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Topical bioequivalence: Experimental and regulatory considerations

Tese no âmbito do Doutoramento em Ciências Farmacêuticas, ramo de Tecnologia Farmacêutica, orientada pela Professora Doutora Carla Sofia Pinheiro Vitorino e pela Dra. Catarina Joana Dias Neto Pratas Cardoso, e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

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à vovó Mila que partiu,
e à Margarida que chegou.

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Forget mistakes. Forget failures. Forget everything except what you are going to do right now, and do it. Today is your lucky day.

Will Durant

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ABSTRACT

Hitherto, the regulatory authorities required that the evaluation of therapeutic equivalence of a topical generic product (TGP) be documented primarily through comparative clinical endpoint studies. Although waivers could sometimes be equated, for most formulations these circumstances did not apply. The variability inherent to topical drug delivery makes comparative clinical studies insensitive, time-consuming, and costly. Their economic burden, coupled with the typically low market price of the reference product (RP) has limited the development of TGPs, as their chance of economic return is lower. These limitations have sparked considerable attention of academia, industry and regulators, having resulted in new regulatory recommendations. The key documents reflecting this paradigm shift are the FDA non-binding product-specific draft guidances, and the EMA draft guideline on quality and equivalence of topical products. Both guidelines advise a modular framework for assessing bioequivalence (BE), wherein qualitative (Q1), quantitative (Q2), microstructure (Q3), product performance (Q4), and local availability sameness should be presented. Against this background, the main objective of this thesis was to develop a methodology to support BE of TGP by comprehensively addressing the requirements of the regulatory guidelines. Since there are myriad specifics ranging from Q1 to local availability, this work specifically focuses on rheological methods to support Q3, *in vitro* release testing (IVRT) and *in vitro* permeation testing (IVPT), to support Q4 and local availability, respectively.

Standardization of procedures is an urgent need when it comes to rheological methods, because according to EMA draft guideline, rheology plays an irrefutable role in the characterization of Q3. Guidance on method development and validation is herein proposed. A risk assessment analysis was used to estimate the impact of selected critical method variables (geometry, temperature and application mode) on a wide range of critical analytical attributes. The proposed validation approach included qualification of the rheometer,

followed by validation of numerous critical operating parameters. The thixotropic relative area, oscillatory yield point, flow point and viscosity related endpoints were found to be highly sensitive and discriminatory parameters.

Considering the regulatory role of IVRT methods, a novel framework based on aQbD principles is described aiming at their development. By defining the analytical target profile, a risk assessment analysis was performed to identify the critical analytical attributes (*in vitro* release rate, cumulative amount released at an initial/final point, and dose depletion) and critical method variables (medium, membrane, and dosage regimen). Based on this information, a 3x2x3 full factorial design was conducted. Statistical modeling and evaluation of system desirability enabled the selection of the most appropriate IVRT parameters, which were fully validated according to EMA requirements.

Although the EMA draft guideline represents a remarkable regulatory advance, there are several restrictive statistical criteria that could undermine its implementation. An attempt was made to critically discuss their plausibility by selecting eight blockbuster RP and considering three batches for each product. Extended pharmaceutical equivalence was evaluated in terms of globule size, pH, rheological properties and IVRT profile. Significant differences were found within the RPs. Statistical analysis showed that when the EMA criteria were applied, none of the same product batches could be considered equivalent. However, when the FDA criteria were used instead, overall equivalence could be inferred.

Finally, in an attempt to provide guidance for the evaluation of topical BE, a decision flow diagram is presented to address distinct scenarios. Three case studies were considered – dimethindene maleate 1 mg/g gel, embodying a simple formulation, bifonazole 10 mg/g cream and diclofenac 20 mg/g emulgel, representing increasingly complex formulations. The RPs for these formulations were compared with commercially available generic products or, alternatively, test products.

For the dimethindene gel, although the rheological tests revealed minor batch-to-batch differences, these were not perceptible in the IVRT results. Therefore, for low complexity formulations, if equivalence of Q1 and Q2 is assured, an adequate and comprehensive characterization of Q4 may be sufficient to demonstrate BE, even if some microstructure requirements fail to be documented.

High rheological variability was observed in the RP of bifonazole cream. Nevertheless, the impact of these differences on Q4 appeared to be negligible. IVPT studies were then conducted and the resulting data were evaluated according to EMA and FDA standards.

Although equivalence could be inferred when applying the FDA scaled average BE assessment criteria, this was not possible when the EMA criteria were considered. For the diclofenac formulation, equivalence pertaining to Q3 was not established. In terms of Q4, equivalence was only found for some batch combinations and when applying the FDA criterion. The IVPT studies also failed to demonstrate equivalence. Nevertheless, since the generic product used in the present study had a pharmacokinetic profile equivalent to that of RP, the observed differences in Q3, Q4 and local availability parameters are not expected to translate into clinically significant differences. Such findings render the one-size-fits-all approach to evaluating topical BE unfeasible and rather point to a case-by-case analysis based on formulation complexity, drug physicochemical properties and site of action. In general, this thesis contributes to a broader understanding of the regulatory constraints to be considered when establishing BE of TGP.

Keywords: Bioequivalence | Topical generic products | Regulatory science | Rheology | IVRT | IVPT

RESUMO

As autoridades regulamentares exigiam, até muito recentemente, que a avaliação da equivalência terapêutica de um medicamento genérico de aplicação tópica (MGT) fosse comprovada através de ensaios clínicos comparativos. Apenas em circunstâncias muito específicas, e para um reduzidíssimo leque de produtos, se poderiam aplicar métodos alternativos. A variabilidade na absorção cutânea torna os ensaios clínicos pouco sensíveis, morosos e dispendiosos. Os elevados custos desta abordagem, associados ao baixo preço que tipicamente caracteriza os medicamentos de referência (MR), conduzem a uma baixa rentabilidade, limitando o desenvolvimento dos MGT. Estes desafios despertaram a atenção de diversos *stakeholders*, nomeadamente universidades, indústria farmacêutica e autoridades regulamentares. Com base em inúmeros estudos científicos, foram emitidas novas *guidelines* europeias e norte-americanas relativas a esta temática. Apesar de existirem diferenças entre as duas agências, ambas recomendam uma estratégia modular no processo de comprovação da bioequivalência (BE). Nesse sentido, o primeiro passo a ser equacionado deve ser o de mimetizar a composição qualitativa do MR (Q1), seguida da similaridade quantitativa (Q2), da microestrutura (Q3), performance do produto, suportada por métodos de libertação *in vitro* (IVRT) (Q4) e, finalmente, a de eficácia.

Tendo por base este enquadramento regulamentar, o presente trabalho teve como principal objetivo detalhar e aprofundar as implicações regulamentares de ambas as agências e, ao mesmo tempo, desenvolver uma metodologia que suportasse a comprovação da BE sob os presentes moldes regulamentares. Dado existir uma vasta panóplia de aspetos a considerar na documentação de Q1 até à do perfil de eficácia, neste trabalho, foi dado especial ênfase aos métodos reológicos na comprovação de Q3 e aos ensaios *in vitro* de libertação e de permeação, usados respetivamente na documentação de Q4 e do perfil de eficácia.

Em face da relevância dada aos métodos reológicos na caracterização de Q3, é necessário proceder-se à sua padronização. Por conseguinte, é proposto um tutorial que engloba tanto o desenvolvimento como a validação desta metodologia. No desenvolvimento dos métodos, foi feita uma análise de risco, na qual se avaliou o impacto das variáveis críticas (geometria, temperatura e modo de aplicação da amostra) num conjunto alargado de atributos críticos analíticos. Na proposta de validação dos métodos incluiu-se a qualificação do equipamento, seguida da validação de vários parâmetros operacionais. Observou-se que a área tixotrópica, o *yield point* obtido através de métodos oscilatórios e todos os parâmetros diretamente relacionados com a viscosidade, mostraram ser indicadores reológicos sensíveis e discriminatórios.

Devido ao enquadramento regulamentar dos métodos IVRT, foi estabelecida neste trabalho uma abordagem para o seu desenvolvimento baseada nos princípios do QbD analítico. Através da definição do perfil alvo analítico, efetuou-se uma análise de risco na qual o efeito das variáveis críticas do método (meio de libertação, membrana e regime de aplicação) foi monitorizado nas variáveis analíticas críticas (velocidade de libertação, quantidade de substância ativa libertada no início e no fim do ensaio e percentagem de libertação). Tendo por base esta informação, foi feito um planeamento fatorial completo do tipo 3x2x3. A análise estatística e a avaliação da *system desirability* permitiram a seleção dos parâmetros de IVRT mais adequados, os quais foram validados de acordo com os requisitos da EMA.

Apesar do presente enquadramento regulamentar estimular o desenvolvimento e comercialização de MGT, na *guideline* europeia, são propostos critérios de aceitação bastante exigentes, que podem inviabilizar a transposição desta directriz. Para contextualizar esta potencial limitação, selecionaram-se 8 MR líderes de mercado, tendo sido escolhidos 3 lotes para cada MR. A microestrutura foi avaliada através do pH, tamanho de gotícula e comportamento reológico, sendo a *performance* avaliada por ensaios IVRT. A análise estatística demonstrou que de acordo com o critério da EMA, lotes do mesmo produto não poderiam ser classificados como bioquivalentes; no entanto, se o critério norte-americano fosse considerado, a BE entre lotes do mesmo produto era, de uma forma geral, comprovada.

No último capítulo da tese é proposta uma "árvore decisional" para utilização aquando da submissão de um MGT. De forma a possibilitar um enquadramento de vários produtos e formas farmacêuticas, foram selecionados 3 casos de estudo: (i) um gel de dimetindeno (1 mg/g), que representa uma formulação simples do ponto de vista tecnológico e cuja substância ativa atua na superfície da pele; (ii) um creme de bifonazol (10 mg/g) e (iii) um emulgel de diclofenac (20 mg/g), que representam formulações mais complexas. Os MR

destas formulações foram comparados com produtos genéricos, produtos testes ou, em alguns casos, com produtos comparadores.

No gel de dimetindeno, apesar do comportamento reológico revelar diferenças entre lotes, estas diferenças não se refletiram no perfil de libertação. Deste modo, para formulações pouco complexas, mediante a comprovação de Q1 e Q2, a demonstração exaustiva de Q4 poderá ser suficiente para suportar a BE, mesmo que alguns dos requisitos referentes a Q3 não sejam comprovados. Os lotes selecionados do MR de creme de bifonazol apresentaram uma grande variabilidade reológica, que não se traduziu em diferenças no perfil de libertação, tendo sido comprovada a equivalência entre o produto teste e o MR. Sendo o creme de bifonazol uma formulação complexa, a comprovação da eficácia poderá ser sustentada através de ensaios *in vitro* de permeação. Os resultados demonstraram mais uma vez, que se os critérios da EMA forem aplicados, a BE não pode ser comprovada; no entanto, se os critérios da FDA forem usados, o resultado já suporta a BE. Relativamente ao emulgel de diclofenac a similaridade de Q3, assim como de Q4, não foram verificadas; apenas em algumas combinações de lotes foram observados perfis de libertação equivalentes. Além disso, a equivalência do perfil de eficácia também não foi comprovada. No entanto, o MG usado nestes estudos apresenta o mesmo perfil farmacocinético do MR; portanto, não se prevê que diferenças em Q3, Q4 e no perfil de eficácia, de acordo com o parâmetro estatístico da EMA, afetem a eficácia terapêutica do produto.

Os resultados obtidos permitem concluir que uma abordagem geral para suportar a BE para todos os produtos tópicos não é exequível. Desta forma, deverá ser equacionada uma abordagem específica para cada produto, tendo em conta a complexidade da formulação, o local de ação, assim como as características físico-químicas da substância ativa.

Como nota final, este trabalho contribui, significativamente, para um melhor enquadramento das limitações regulamentares, que necessitam de ser consideradas no que diz respeito à comprovação da BE de MGT.

Palavras-chave: Bioequivalência | Medicamentos genéricos de aplicação tópica | Ciência regulamentar | Reologia | Ensaios de libertação *in vitro* | Ensaios de permeação *in vitro*.

ABBREVIATIONS

ACV	Acyclovir
ANDA	Abbreviated new drug application
API	Active pharmaceutical ingredient
aQbD	Analytical quality by design
A_{TOTAL}	Cumulative amount of drug permeated at the end of the IVPT study
ATP	Analytical target profile
ATR-FTIR	Attenuated total reflectance-Fourier transformed infrared
AUC	Area under the curve
BA	Bioavailability
BCS	Biopharmaceutical classification system
BE	Bioequivalence
BFZ	Bifonazole
C	Compliant
CAA	Critical analytical attributes
CES	Clinical endpoint studies
CI	Confidence intervals
CLB	Clobetasol
CLT	Clotrimazole
C_{MAX}	Maximal concentration

CMV	Critical method variables
Cn	Negative control
Cnm	Negative matrix control
Cp	Positive control
Cpm	Positive matrix control
CPA	Comparator product A
CPB	Comparator product B
CQA	Critical quality attribute
CRS	Confocal Raman spectroscopy
CV	Coefficient of variation
RSD	Relative standard deviation
DD	Dose depletion
DF	Diclofenac
DMK	Dermatopharmacokinetic
DoE	Design of experiments
DSC	Differential scanning calorimetry
EMA	European medicine agency
EPE	Extended pharmaceutical equivalence
ETF	Etofenamate
FDA	US-Food and drug administration
G´	Storage modulus
G´´	Loss modulus
GMR	Geometric mean ratio
GP	Generic product
HC	Hydrocortisone
HCA	Hierarchical cluster analysis
HIE	Heat isolated epidermis
HPLC	High performance liquid chromatography

IVPT	*In vitro* permeation testing
IVRR	*In vitro* release rate
J_{MAX}	Maximal flux (IVPT studies)
LOD	Limit of detection
LoF	Lack of fit
LOQ	Limit of quantification
LVR	Linear viscoelastic region
M	Molar mass
N.D	Not-determined
η_0	Zero-shear viscosity
η_∞	Infinite-shear viscosity
NC	Non-compliant
NSAIDs	Nonsteroidal anti-inflammatory drugs
OECD	Organization for economic cooperation and development
Osc. Tests	Oscillatory tests
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCC	Physicochemical characterization
PE	Pure error
PKE	Pharmacokinetic endpoints
PPG	Propylene glycol
Q1	Quanlitative sameness
Q2	Quantitative sameness
Q3	Microstructure sameness
Q4	Performance sameness
QbD	Quality by design
QC	Quality control
Q_f	Cumulative amount of drug released at the end of the IVRT experiment

Q_i	Cumulative amount amount of drug released at an initial time point of the IVRT experiment
REM	Risk estimation matrix
Rot. Tests	Rotational tests
RP	Reference Product
RP-HPLC	Reversed-phase high performance liquid chromatography
RSD	Relative standard deviation
RSD	Relative standard deviation
SOP	Standard operating procedures
S_R	Relative thixotropic area
SS	Statistical significance
SUPAC-SS	Nonsterile semisolid dosage forms–scale-up and postapproval changes
SWR	Within-subject standard deviation
TCS	Topical classification system
TCZ	Tioconazole
TEWL	Transepidermal water loss
TGA	Thermogravimetric analysis
TGP	Topical generic product
TP	Test product
TS	Tape-stripping
USP	United States Pharmacopeia
VCA	Vasoconstriction assay
VDC	Vertical diffusion cell
$\tau_{0.OSC}$	Yield point obtained through oscillatory methods
$\tau_{0.ROT}$	Yield point obtained through rotational methods

THESIS STRUCTURE

In the beginning of this PhD thesis (March 2017), the methods accepted by regulatory agencies for evaluating the bioequivalence (BE) of topical generic products (TGPs) relied solely on lengthy and expensive clinical endpoint studies. The only alternative consisted on pharmacodynamic trials, which for physiological reasons are limited to corticosteroids. The economic burden of these clinical studies, combined with the typically low market price of the reference product, significantly limited the development and commercialization of TGP, as chances of economic return were considerably lower. In this context, there was a lack of investment in TGP by many companies and also a reluctance of manufacturers to make significant formulation improvements of a pre-approved product, since it required a clinical BE study to be validated. Nevertheless, throughout 2017-2018 considerable efforts, by the academia, pharmaceutical industries alongside with regulatory agencies such as European Medicine Agency (EMA) and US-Food and Drug Administration (FDA), have been channelled towards the development and validation of other methods.

With this in mind, the **first chapter** of this thesis reviews the regulatory accepted methods, as well as alternative approaches for BE documentation of TGP. Particular attention was given to the FDA draft guidances of TGP and to relevant European Public Assessment Reports, in which alternative BE documentation pathways were partially disclosed. Other regulatory initiatives driven primarily by the FDA, such as the Strawman decision tree and the topical drug classification system were also cited as examples of collaborative efforts amongst regulators, pharmaceutical industries and academia.

This is followed by a **second chapter**, where the EMA draft guideline on quality and equivalence of topical products, issued on December 2018, is critically presented. This draft guideline introduced the extended pharmaceutical equivalence concept and by doing so, new

pathways for TGP BE demonstration could be equated within the European regulatory scenario. Nevertheless, the approach presented by the EMA also comes with some relevant constraints that may hinder its successful implementation. In this context, this chapter presents the most controversial regulatory topics within the guideline and attempts to shed light on some possible solutions. Based on this information, the framework, hypothesis and objectives of this thesis are clearly stated.

To avoid the need for clinical endpoint studies, the European Agency, as well as the United States Agency, proposed a modular framework for TGP BE documentation. First of all, the qualitative (Q1) and quantitative (Q2) equivalence of the test product towards the reference product must be sustained. This is followed by the demonstration of microstructure sameness (Q3). Although there are several tests that should be performed within this scope when dealing with semisolid dosage forms, rheological properties play a central role in the product microstructure and are, for this reason, thoughtfully addressed throughout the draft guideline. Nevertheless, despite their undeniable regulatory importance, there is no science-based framework aimed at their development and validation. In this context, the **third chapter** of this thesis intends to provide guidance on rheological methodology specific to topical products. A risk assessment analysis was presented to estimate the impact of selected critical method variables on previously defined rheological critical analytical attributes. This was followed by formal validation of the optimized method conditions, which included rheometer qualification studies and formal validation of the critical operational parameters. For this chapter, a 1% hydrocortisone cream was used as the model formulation. To further illustrate the applicability of the proposed strategy, this tutorial was successfully used to develop and validate a rheological method aimed at comparing a clotrimazole 10 mg/g cream test product (TP) with the corresponding reference product (RP).

The next parameter that should be evaluated as part of an abridged TGP BE demonstration process in accordance with EMA/FDA requirements is product performance equivalence (Q4). Product performance is evaluated through *in vitro* release testing (IVRT), which allows the determination of the release profile. Because the release profile is generally sensitive to physicochemical differences, it is an effective approach for monitoring batch-to-batch consistency, post-approval changes, scale-up and stability studies. Other applications include TGP development studies. Although regulatory recommendations for IVRT method validation are clearly defined in EMA and FDA guidelines, there is no standardized methodology to support IVRT method development. With this in mind, the **fourth chapter** of this thesis presents a framework for the development of a discriminatory IVRT method that follows the principles of analytical quality by design (aQbD). To this end, the analytical target profile of

the IVRT was defined and a risk assessment analysis was carried out to identify the critical analytical attributes and critical method variables. Afterwards, a full factorial design was performed. Statistical modelling and system desirability assessment enabled the selection of the most suitable IVRT parameters, which were then validated according to the new EMA requirements. For this chapter, a commercially available diclofenac emulgel formulation was used as the model product.

As discussed in detail in Chapter 2, a thorough reading of the new EMA guideline revealed several challenges that could hamper its actual applicability. The **fifth chapter** intends to shed light on some of these challenges, in particular those related to the demonstration of the extended pharmaceutical equivalence. The cornerstone of an abridged TGP BE assessment process is an in-depth characterization of the reference product. Although both manufacturers and regulators actively seek to negligible batch-to-batch differences, there are still products for which batch variability is deeply observed. These variations are largely prompted by raw materials / manufacturing process fluctuations. Semisolid dosage forms, which make up the majority of topically applied products, are particularly prone to batch variations, as their microstructure is highly sensitive to the aforementioned sources of variability. In this context, we intended to evaluate the feasibility of the regulatory limits for Q3 and Q4 assessments indicated in the EMA guideline. More specifically, the 90% confidence interval pertaining to all aspects of the microstructure and the 90-111% confidence interval with respect to the release profile were thoroughly inspected. To this end, in Chapter 5, a panel of 8 reference blockbuster semisolid topical products was considered, and for each product, three batches were selected. These were evaluated in terms of globule size, pH, rheological properties and performance. Furthermore, an integrated multivariate analysis was likewise performed to determine which parameters were responsible for the differences between batches.

According to the EMA guideline, BE can be demonstrated for simple formulations by documenting Q1/Q2/Q3 and Q4 equivalence. Nevertheless, when addressing complex semisolid dosage forms, equivalence in terms of product permeation profile (IVPT) should be demonstrated in addition to extended pharmaceutical equivalence. In this context, the **sixth chapter** presents customized rationales for BE documentation according to the formulation complexity and overall pharmaceutical technology features. A dimethindene maleate 1 mg/g gel, a bifonazole 10 mg/g cream and a diclofenac 20 mg/g emulgel were selected as model products. Reference products, test products and, whenever possible, generic products, were cross compared during the rheology, release and permeation experiments. All methods were validated according to the framework previously reported. In line with Chapter five, a critical

evaluation of the regulatory limits (FDA/EMA) is presented, particularly with respect to IVPT parameters.

Finally, on the **seventh chapter**, a discussion where all issues are addressed and the results are unified. On this chapter, the main conclusions and forthcoming work are also disclosed.

SCIENTIFIC PUBLICATIONS AND CONFERENCES

The following publications have so far resulted from the work presented in this thesis:

International peer-reviewed Q1 journals

Miranda, M., Sousa, J.J., Veiga, F., Cardoso, C., Vitorino, C., 2018b. Bioequivalence of topical generic products. Part 1: Where are we now? Eur. J. Pharm. Sci. 123, 260–267. https://doi.org/10.1016/j.ejps.2018.07.050

Miranda, M., Sousa, J.J., Veiga, F., Cardoso, C., Vitorino, C., 2018a. Bioequivalence of topical generic products. Part 2. Paving the way to a tailored regulatory system. Eur. J. Pharm. Sci. 122, 264–272. https://doi.org/10.1016/j.ejps.2018.07.011

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International peer-reviewed Q2 journals

Miranda, M., Cardoso, C., Vitorino, C., 2020a. Fast Screening Methods for the Analysis of Topical Drug Products. Process. 2020, Vol. 8, Page 397 8, 397. https://doi.org/10.3390/PR8040397

Oral Communications

Miranda, M., Pais, A.A.C.C., Cardoso, C., Vitorino, C. aQbD as a Framework for *in vitro* Release Testing Development.

This oral communication has been presented in:

- Control Release Society 46th Annual Meeting, Valencia (Spain) 21-24th July 2019

- Brain delivery workshop, Coimbra (Portugal), 19th June 2019

- 1st Annual Meeting in Pharmaceutical Sciences, Coimbra (Portugal), 15th November 2019

Poster Communications

Miranda, M., Cardoso, C., Vitorino, C. Bioequivalence of topical generic products: Where are we now?

Poster presented at Skin Forum 2018 Annual Metting", Tallinn (Estonia), 20-21st June 2018.

Miranda, M., Pais, A.A.C.C., Cardoso, C., Vitorino, C., 2019b. aQbD as a Framework for *in vitro* Release Testing Development.

Poster presented at Skin & Formulation 5th Symposium & 17th Skin Forum, Reims (France), 23-24th September 2019.

Miranda, M., Cova, T., Augusto, C., Pais, A.A.C.C., Cardoso, C., Vitorino, C. Batch-to-batch variability of semisolid topical drug products

Poster presented at:

- Okinawa Colloids 2019, Okinawa (Japan), 5-8th November 2019

- 1st Annual Meeting in Pharmaceutical Sciences, Coimbra (Portugal), 15th November 2019

Conference Papers

Miranda, M., Veloso, C., Cardoso, C., Vitorino, C., 2021. From lab to upscale – Boosting formulation performance through *in vitro* technologies. p. 8674. https://doi.org/10.3390/iecp2020-08674

Book chapters

Miranda, M., Cardoso, C., Vitorino, C., 2021. A regulatory framework for the development of topical nanomedicines, in: Theory and Applications of Nonparenteral Nanomedicines. Elsevier, pp. 55–78. https://doi.org/10.1016/b978-0-12-820466-5.00003-x

1 INTRODUCTION

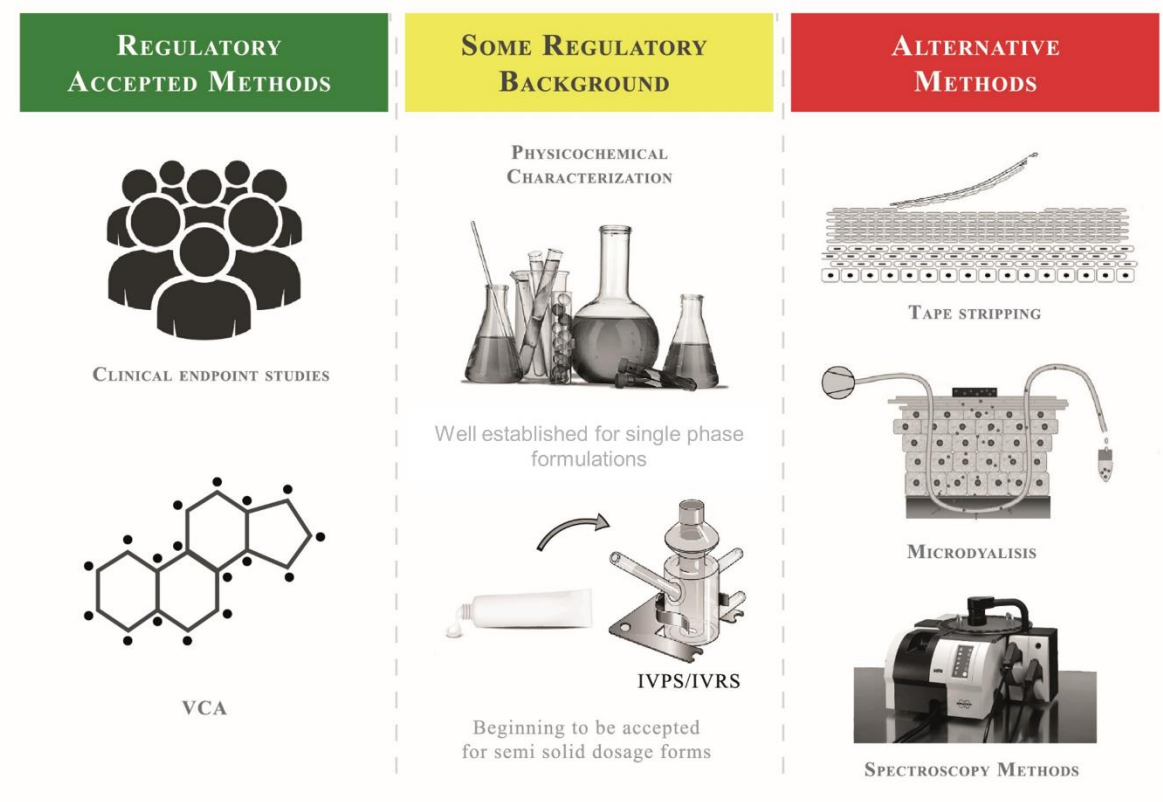


Fig.1.1 – Graphical abstract: Introduction.

This chapter has been adapted from the following publications:

Miranda, M.; Sousa, J.J.; Veiga, F.; Cardoso, C.; Vitorino, C. Bioequivalence of topical generic products. Part 1: Where are we now? Eur. J. Pharm. Sci. 2018, 123, 260–267.

Miranda, M.; Sousa, J.J.; Veiga, F.; Cardoso, C.; Vitorino, C. Bioequivalence of topical generic products. Part 2: Paving the way to a tailored regulatory system. Eur. J. Pharm. Sci. 2018.

M.M and C.V conceived the presented idea and established the research design and implementation. M.M wrote the first draft of the manuscript and all other authors substantially contributed to revisions.

KEY CONCEPTS

- **Bioequivalence:** Absence of significant differences in the rate and extent to which the API, present in pharmaceutical equivalent products, becomes available at the site of action, when administered under similar conditions in an appropriately designed study.
- **The absence of systemic absorption** in the vast majority of topical products intended for a local therapeutic effect, requires that clinical efficacy should be assessed by non pharmacokinetic approaches, such as CES and VCA methods. For the same reason, for TGP, therapeutic equivalence has also to be supported by such methods.
- Due to the disadvantages of these methods, and in an attempt to enrich TGP market portfolio, combined efforts between regulators, academia and industry have targeted the proposal and **development of surrogate methods** for bioequivalence assessment of TGP.

1.1 Overcoming the skin barrier

The skin, the largest organ of the human body, acts as a dynamic barrier between the organism and its environment. Besides protecting internal structures from various external stressors (radiation, chemicals, microorganisms), the skin also acts as a homeostatic platform which regulates several physiological parameters such as body temperature and pressure (Benson and Watkinson, 2012; Menon *et al.*, 2012; Shahzad *et al.*, 2015).

In essence, three functional layers can be identified: hypodermis, dermis and epidermis, being the latter the outermost layer. In the epidermis, a multilayered structure can also be detected. Due to its avascular nature, epidermal cells must source essential nutrients and remove waste through diffusion mechanisms (Wiedersberg *et al.*, 2008). Accordingly, the basal layer possesses metabolic elongated active cells, while the following ones predominantly acquire a highly keratinized structure, with high density and low hydration (Sivaraman and Banga, 2015; Wiedersberg *et al.*, 2008). The *stratum corneum* (SC), the epidermis top layer, is accountable for the barrier function of the skin. The brick and mortar analogy is readily understandable, since this membrane is mainly composed by corneocytes, proteinaceous cellular compartments, interconnected by desmosomes and embedded within a lipid matrix (Chang *et al.*, 2013a; Shahzad *et al.*, 2015).

Locally applied products, commonly developed to exert a local action, have been used throughout history for cosmetic and therapeutic purposes. Their ease of administration and reduced side effects profile reinforce patient compliance (Folzer *et al.*, 2014). Three possible macroroutes of drug permeation can be considered when dealing with dermal absorption: the intercellular, transcellular and the follicular route, see Fig.1.2.

The transcellular pathway comprises the transport through the corneocytes, stimulating the uptake of hydrophilic compounds, due to the hydrated keratin presence. Inversely, the diffusion of lipophilic substances mainly occurs within the lipid matrix, via intercellular transport. Both routes are collectively called the transepidermal route, and they represent the most significant pathways of dermal absorption (Shahzad *et al.*, 2015).

The follicular route is considered to be less significant, since hair follicles only occupy 0.1% of the skin surface. Nevertheless, many studies highlight its importance in dermal/transdermal absorption of large polar compounds (Kattou *et al.*, 2017).

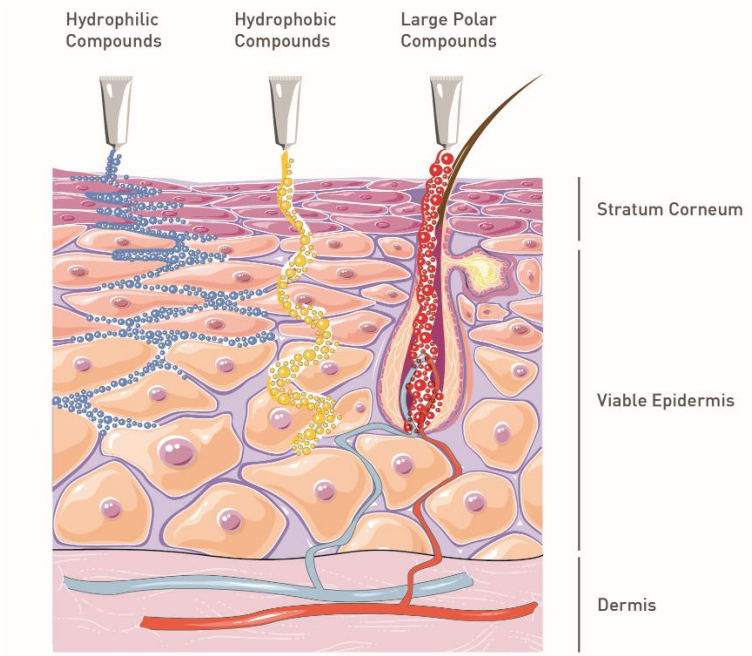


Fig.1.2 – Permeation pathways in topical drug delivery.

To ensure an efficient pharmacological action, the active moieties need to overcome the sinuous and tortuous path provided by the *stratum corneum*. For this to occur, three sequential processes must take place: (i) release of the active substance from the dosage form; (ii) diffusion into and through the SC and; (iii) partitioning to the viable epidermis, where the active substance elicits the desired therapeutic effect (Lauterbach and Müller-Goymann, 2014; Lopes, 2014).

These successive steps rely on the physicochemical characteristics of both active pharmaceutical ingredient (API) and formulation, as well on some physiological factors. A brief summary on each contributor role is following presented:

- The formulation physicochemical characteristics influence the vehicle-SC partition coefficient, which is directly influenced by the particle size, interfacial tension between phases and formulation microstructure. All the above mentioned factors, greatly depend

on the formulation vehicle. This reason sustains the considerable impact of the vehicle in the therapeutic response. A common example that addresses this situation concerns the application of an ointment to the skin. This vehicle prevents transepidermal water loses thus enhancing drug permeation due to its occlusive properties (Chang *et al.*, 2013b). In this context, vehicle deviations may elicit different therapeutic responses (EMA, 1994).

- Similarly, the API physicochemical characteristics also impact the API diffusion/ partition coefficient within the skins lipophilic environment. Factors such as the molecular size (preferably less than 500 Da), lipophilicity (Log P 2-3), melting point, ionization and potential ability to bind to other molecules, have also to be accounted for (Benson and Watkinson, 2012; Shah *et al.*, 2015).
- The physiological factors that influence dermal delivery include age, gender, anatomical site, ethnicity and diseased skin.

These aspects condition the release and permeation profile of a topical product, thus impacting its pharmaceutical performance, as well as therapeutic effect (Yacobi *et al.*, 2014). For this reason, they should be collectively and carefully assessed while developing a topical product.

1.2 Development and production of topical generic products

The constraints regarding the development of new chemical entities and patients need to acquire more affordable drug products have led to an expansion of the generic drug products market (Fernández-Campos *et al.*, 2017).

For a product to be considered as a generic, bioequivalence (BE) has to be ensured. As defined in the Code of Federal Regulations Title 21 (21 CFR 320.24), two products are considered bioequivalent if there are no significant differences in the rate and extent to which the active ingredient, in pharmaceutical equivalent products, becomes available at the site of drug action, when administered under similar conditions in an appropriately designed study. BE investigations are designed to evaluate if the test product has comparable biopharmaceutical properties to a previously approved pharmaceutical equivalent. Taking this into account, a marketing authorization is solely given if the test product fulfils clear and strict regulatory acceptance criteria (Endrenyi *et al.*, 2017).

BE assessment of systemically available drugs is usually performed through pharmacokinetics endpoints, which require no less than 12 subjects (EMA, 2010). Moreover, if a meaningful correlation between the *in vitro* release profile and the *in vivo* bioavailability parameters is

established, the need to conduct such trials can be overpassed (EMA, 2018a, 2014a, 2010). However, the same scenario is generally not applicable for topical drug products aiming at a local action, especially with semisolid dosage forms, since there are several regulatory issues which difficult the marketing authorization approval. These will be carefully debated in the following chapters.

While developing a topical generic product (TGP), both pharmaceutical and therapeutic equivalency towards the reference product must be ensured. A reverse engineering approach, if feasible, is highly recommended, since changes in the vehicle may condition the product pharmaceutical characteristics and therapeutic profile, as previously discussed (Mugglestone *et al.*, 2012; Sivaraman and Banga, 2015). In this context, and as stated by Chang and collaborators, having the same components (Q1), in same concentration (Q2) with the same microstructure (Q3), is the most transparent approach to avoid regulatory approval issues (Chang *et al.*, 2013a).

When the quantitative composition is not known (Q2), a factorial design-based approach is encouraged to ensure that the proposed formulation has a similar quantitative profile when compared to the reference product. Attaining Q3 similarity can be more challenging as this factor is substantially dependent on the manufacturing process, raw material characteristics and on the qualitative and quantitative composition of the product (Osborne, 2016).

Topical semisolid drug products can be classified as complex for multiple aspects. First, dermal delivery is a complex route of administration, since in the majority of the situations the drug needs to overcome the *stratum corneum* in order to reach the viable epidermis, through a process highly dependent on the physicochemical characteristics of both active substance and formulation, as previously reviewed (Lauterbach and Müller-Goymann, 2014). Second, there are several locally acting topical drug products which have complex dosage forms (e.g. a topical patch), or complex drug-device combination (e.g. sprayable foam formulation). Third, topical drug products frequently have complex formulations (e.g. emulgel, complex cream, among others). As the complexity of the product increases, so do the potential failure modes for BE and therapeutic equivalence of TGP towards the reference product (Sinamora, 2017). To minimize this risk, it is essential to acquire relevant product and process understanding, by identifying the critical material attributes, as well as the critical process parameters, and studying their impact on critical quality attributes, so that the quality target product profile defined for the test product (generic) may be accomplished.

To generate product understanding, an adequate characterization of the reference product should be performed. By doing so, several critical questions must be answered: the influence

of the composition and grade of inactive ingredients, if the product dispensing stresses/forces and the inertness of the container closure system (adsorption/absorption issues) affect the product quality and performance, among others. Moreover, these studies should also provide a significant insight on the product *in vivo* performance by assessing factors, such as the metamorphosis of the vehicle on the skin (Sinamora, 2017). This concept, intrinsically connected to dermal bioavailability of drugs, regards the structural and quantitative changes that the majority of dermatological vehicles face when are applied to the skin, either by mechanical agitation during product application (e.g. rubbing), or by ingredient evaporation (phase inversion) (Roberts *et al.*, 2017; Surber and Smith, 2005). On the other hand, a deep process understanding provides a significant insight on the impact of several manufacturing operations, such as the mixing sequence, rates and duration, temperature influence, and also on the impact of orifice diameters, tube lengths, pressures throughout product transfer, holding and packaging (Fernández-Campos *et al.*, 2017; Sinamora, 2017). The contribution of the previously mentioned factors should be carefully assessed, since it may have a direct influence on the formulation microstructural characteristics, and for this reason, an impact on formulation performance and efficacy (Chang *et al.*, 2013a, 2013b; Murthy, 2017). Methods able to discriminate the product microstructure, such as the *in vitro* release and *in vitro* permeation, are for these reasons indispensable tools throughout the entire TGP lifecycle (Roberts *et al.*, 2017).

The development of generic products should be cost and time efficient, since it is usually associated to low profit margins due to the competition with other manufacturers. Against this background, the development of TGP, especially those concerning semisolid dosage forms, can be very challenging, as the demonstration of the therapeutic equivalence is substantially more complex when compared to other products.

1.3 Bioequivalence of Topical Generic Products

1.3.1 Regulatory accepted methods for BE assessment

For the majority of TGP, BE establishment requires the demonstration of both pharmaceutical and therapeutic equivalence. The regulatory authorities frequently request the evaluation of the therapeutic equivalence by one of two options: (i) pharmacodynamic assays, only suitable for corticosteroids or; (ii) comparative clinical endpoint studies (CES) (Chen *et al.*, 2011). Although a waiver from clinical endpoint studies is commonly granted for liquid formulations, such as solutions, for other pharmaceutical dosage forms, convenient for topical administration e.g. creams, ointments, emulsions, this situation mostly does not apply. Since

CES can be applied to any pharmacotherapeutic class, these are often the "go to approach" for establishing BE. There are, however, surrogate methods that can be used to assess topical bioavailability, and by extrapolation, infer on the BE of TGP (Cordery *et al.*, 2017).

The following sections aim to review the regulatory accepted methods for BE assessment of TGP, and also to discuss the suitability of alternative techniques. Regardless of the fact that only semisolid dosage forms are considered in this thesis, please note that solid forms intended for topical application, such as medicated plasters and topical patches have specific requirements concerning BE documentation. An example of this is the recently approved generic lidocaine topical patch, for which adhesion, skin irritation and sensitization, beyond pharmacokinetic studies, are required to support BE (FDA, 2016a).

CES represent the “gold standard” method for TGP BE. Since skin permeation is affected by several factors and some reference products possess a modest therapeutic efficacy, these studies usually require a complex structure – randomized, double-blind, placebo-controlled, crossover and a parallel grouped (FDA, 2016b; PAR, 2007). Moreover, it is anticipated a high number of subjects enrolled (n > 500) (Boix-Montanes, 2011; Chang *et al.*, 2013b; Harris, 2015; Narkar, 2010). Besides these challenges, the selection of the appropriate clinical endpoints is also demanding, as certain diseases have several possible endpoints and the selection of one in detriment of the other, may influence the trial outcome (Braddy *et al.*, 2015).

Additionally, there are several criteria that have to be clearly defined to select the study population, these include:

- Skin integrity, which can be highly compromised in certain diseases;
- Differences in race, gender, age;
- Differences in the immunological state of the individual, especially when considering antiviral topical therapy;
- Differences in the disease state and dosage regimens.

All of the former factors need to be properly addressed while selecting the study population. Moreover, the standardization of the applied dose can be difficult to assure, given the study dimension.

In line with the previously presented data, these studies are simultaneously the most expensive part of the TGP R&D programs and the one with more entailed risks. For these reasons, many authors state that clinical trials are the least accurate, sensitive and reproducible method to demonstrate bioequivalence (Chen *et al.*, 2011; Harris, 2015; Zhang *et al.*, 2013). Moreover, due to the highly complex structure of these studies, these are

undoubtedly more expensive than the pharmacokinetic trials used for BE assessment of generic oral dosage forms.

The economic burden of CES, linked with the typically low market price of the Reference Product, significantly limits the development of these products, as the chances of the TGP economic viability are considerably decreased (Boix-Montanes, 2011; Lionberger, 2008). Therefore, two situations take place: (i) lack of investment in TGP by many companies, reducing the number of generic competitors (Lionberger, 2008) and (ii) reluctance of manufacturers to make significant formulation improvements of a pre-approved product, which require a clinical BE study to be validated (Lionberger, 2008).

Pharmacodynamic assays constitute an alternative to CES when assessing the BE of TGP. This method relies on the vasoconstriction properties of corticosteroids, which are capable of inducing a local skin blanching response. By means of a chromameter the skin blanching effect can be measured, yielding a dose-response curve which can then be used to compare two products (e.g. a reference drug product with a generic product).

The FDA Guidance Topical dermatologic corticosteroids: *in vivo* bioequivalence, is currently accepted by other international regulatory agencies, including Australia, Canada, Chinese Taipei, EMA, New Zealand and Switzerland (Braddy *et al.*, 2015; FDA, 1995). Regardless of the broad acceptance of this guidance, there are several reports that highlight the lack of sensitivity when comparing different dosage forms, and the high inter-subject variability (Franz *et al.*, 2009; Yacobi *et al.*, 2014). To overcome some of these drawbacks, the regulatory authorities demand the conduction of a pilot and pivotal studies, requiring the inclusion of several dozens of subjects, and consequently increasing both time and costs related to BE assessment.

In particular circumstances, a waiver from clinical trials or pharmacodynamic studies may be granted. For this to occur, the generic product has to be a solution, with the same qualitative and quantitative composition towards the reference product and the same functional attributes (pH, particle size and viscosity) (EMA, 2014b; Harris, 2015).

1.4 Alternative methods for BE assessment of Topical Generic Products

Even though a waiver from CES is mostly applicable to topical solutions, a concept paper emitted by EMA for locally acting, locally applied products for cutaneous use, accepts a waiver extension to other pharmaceutical dosage forms (EMA, 2014b). Although the document states that BE assessment of TGP usually is insufficient to predict the product therapeutic

equivalence, the advantages of the establishment of an extended concept of pharmaceutical equivalence are highlighted. In this context, alternative methods for equivalence assessment such as tape stripping, *in vitro* permeation testing (IVPT) and possibly microdialysis would sustain a more precise pharmaceutical characterization, which in turn, could support a claim of therapeutic equivalence. The concepts addressed in this document were further reinforced in the EMA draft guideline on quality and equivalence of topical products (solely released in the end of 2018). This guideline will be carefully detailed throughout this thesis. Besides the concept paper, there are other European initiatives that suggest a paradigm shift in BE assessment of TP. A clear example regards the release of several European Public Assessment Reports, in which *in vitro* methods were used to partially document TGP bioequivalence (PAR, 2016, 2009, 2007). These will be highlighted throughout this chapter.

The development and validation of surrogate methods for BE assessment of TGP has also been a long-standing concern of the U.S. FDA. Several initiatives, such as the Critical Path Opportunities for Generic Drugs, the Evaluation of Topical Drug Products - Current Challenges in Bioequivalence, Quality, and Novel Assessment Technologies workshop in 2013, and, more recently, the Bioequivalence Testing of Topical Drug Products workshop in 2017 have been specifically promoted to address this issue (Lionberger, 2008; Sinamora, 2017; Yacobi *et al.*, 2014). In these meetings, the importance of *in vitro* methods, such as the permeation assay using excised human skin, and *in vivo* tape stripping has been similarly stated. Moreover, in the past few years, several non-binding product-specific guidances for generic product development have presented specific equivalence protocols solely based on *in vitro* methods (FDA, 2016c).

Based on this information, *in vitro* methods are clearly gaining regulatory status as surrogate methods for TGP BE assessment. Moreover, their application as risk assessment tools for establishing appropriate dermal absorption values of pesticides, biocides and cosmetics is already a reality (EPA, 1992; OECD, 2010, 2004; WHO, 2006). Tape stripping, microdialysis and spectroscopy methods constitute other analytical surrogates that may also provide a valuable and complementary contribution within this field.

The next sections aim to summarize the main characteristics of the above mentioned methods, as well as respective advantages and disadvantages.

1.4.1 Microdialysis

Microdialysis is a promising tool in dermal BE research, since it enables the real-time determination of various pharmacokinetic parameters, such as absorption, half-life,

metabolism, elimination, among others. A significant advantage of this technique concerns its ability to reflect a permeability increase in diseased skin, as proven by the works of García Ortiz and collaborators (García Ortiz *et al.*, 2009, 2008). Several sampling sites of the same volunteer can be studied simultaneously, and the detection of specific biomarkers produced in response to the drug stimuli is also possible (Holmgaard *et al.*, 2010; Narkar, 2010). Even though an initial investment for the pump acquisition is required, the method is quite cheap, since the probes can be laboratory manufactured.

Nevertheless, microdialysis has some intrinsic limitations including (i) difficulty to assess low dosage topical formulations (like corticosteroids), (ii) experiments are usually limited to 8-10 hours, since the probe induces tissue inflammation, (iii) difficulty in accurately reproduce the probe insertion and manufacturing, (iv) difficulty in the selection of an adequate flow rate. In addition, monitoring highly lipophilic and protein-bound substances can be challenging. Nevertheless, the use of an open-coil probe can overcome the difficulties addressed. Such probes are used in open-flow microperfusion, a technique that shares the same principles as microdialysis. However, in this method, the interstitial fluid is continuously collected without the need of a membrane (Abd *et al.*, 2016). Even though the analyte chemical composition or size is no longer an obstacle, open flow microperfusion samples are for the same reason more complicated to process than microdialysis samples and require pre-treatment (Yacobi *et al.*, 2014). Schimek and collaborators tried to address this problem by developing a faster ultra-high-performance liquid-high-resolution tandem mass spectrometry analysis method. Their results are quite promising since they were able to obtain a remarkably lower limit of quantification, which in turn provided excellent accuracy and precision results (Schimek *et al.*, 2018).

1.4.2 Optical Methods

Due to the need to develop simpler and faster methods, capable to reflect *in vivo* conditions, non-destructive optical methods are also an attractive alternative to CES (Franzen and Windbergs, 2015). In the dermal research field, vibrational spectroscopy methods, in particular near infrared (NIR) and Confocal Raman have been applied both *in vivo* and *in vitro* with multiple purposes:

- Monitor water, lipid and protein content;
- Assess the role of lipid polymorphism role in permeation;
- Determine permeation enhancers action mechanism;
- Diagnose skin disorders like atopic dermatitis or psoriasis (Dreassi *et al.*, 1997);

- Evaluate the efficacy of topical moisturizers, through skin hydration monitoring (Crowther *et al.*, 2008; Egawa, 2009);
- Assess topical drug delivery (Medendorp *et al.*, 2006, 2007).

These broad range of applications derive from the ability of these methods to provide information on functional groups.

1.4.3 Confocal Raman Spectroscopy

Confocal Raman spectroscopy (CRS) is an optical method based on inelastic light scattering (Caspers *et al.*, 2001; Mohammed *et al.*, 2013). The sample is illuminated by a monochromatic laser and photons are scattered by the molecules in the sample. A small fraction of the scattered light, the Raman spectrum, is found at different wavelengths than that of the incident light. The positions, relative intensities and shapes of this spectrum entail information about the sample molecular composition, structure and interactions (Caspers *et al.*, 2001).

Many authors have been applying this technique to determine *stratum corneum* thickness and composition *in vivo*, since it can provide detailed information on the skin hydration and chemical profile (Crowther *et al.*, 2008; Mateus *et al.*, 2013; Mohammed *et al.*, 2013).

Additionally, as proven by the work of Mateus and co-workers, this method can also be used to monitor in-depth topically applied substances. The authors developed an *in vivo* CRS method to assess the permeation profile of several ibuprofen solutions. Afterwards, the results were successfully compared with previously published data from tape stripping assays (Mateus *et al.*, 2013). Another study performed by Mohammed and co-workers monitored the permeation profile of a known permeation enhancer DMSO. The experiments were also performed *in vivo* using different vehicle solutions. The obtained data were then compared with the results of an *in vitro* permeation study, also with positive correlations (Mohammed *et al.*, 2014). Even though the results of these studies are quite encouraging due to the *in vivo* real-time profiling of the applied substances, there are several disadvantages regarding CRS that should be considered. Since the Raman scattering is unique for particular chemical functional groups, the high variability of the skin composition and structure can undermine the analysis of the acquired Raman spectra (Franzen and Windbergs, 2015). To correctly interpret the results long acquisition times are needed, which can thermally injure the skin and compromise the study (Franzen and Windbergs, 2015). While conducting *in vitro* permeation studies, factors such as ongoing diffusion, microbial growth and changes in the skin hydration can alter Raman spectra. Another drawback of this method reports to the skin auto fluorescence, which conceals most Raman signals (Franzen and Windbergs, 2015). Even

though several research groups have designed strategies to overcome these drawbacks, CRS is a technique that mainly provides relative measurements instead of absolute determinations. Therefore, it is considered a semi-quantitative technique, meaning that an exact determination of the drug concentration in the skin is not attainable (Narkar, 2010). Against this background, during BE establishment of a TGP, the usage of the CRS methods should be complementary to other techniques.

1.4.4 Near-infrared spectroscopy methods

Near infrared spectroscopy (NIR) is broadly applied in process analytical technology procedures, in particular for the determination of physical and chemical properties of a drug product throughout its production (Juan G Rosas *et al.*, 2011). A significant advantage of this technique is the fact that it does not require derivatization, in another words, there is no need to prepare the sample for analysis (Juan G. Rosas *et al.*, 2011).

As previously mentioned, NIR has also been used in dermal research (Caspers *et al.*, 2001). Its application to BE assessment of TGP, deem from the necessity to analyse in a less time-consuming manner the strips obtained from the dermatopharmacokinetic studies. Tape stripping, which will be carefully debated in the next section, is not ideal for volatile chemicals, since the drug tends to evaporate faster than the time of analysis. So, instead of processing the strips through drug extraction followed by HPLC quantification, infrared imaging methods were selected as a backup (Medendorp *et al.*, 2006; Pirot *et al.*, 1997). However, NIR waves can penetrate the skin up to several cm, depending on the wavelength, therefore when combined with linear multivariate statistics, NIR spectroscopy can be used to quantify a substance in the skin, representing a considerable advantage over CRS (Narkar, 2010).

Medendorp and collaborators made a promising contribution in the field of NIR application to TGP bioequivalence assessment. In their work, NIR spectrophotometry was used to monitor the concentration of several drugs in human and pig skin. Firstly, the authors assessed if the selected active substances possessed NIR chromophores. Afterwards, the permeation profile of different products (solutions and semi-solid formulations with different strengths) were determined. The results were quite promising and showed that NIR can be a viable option to establish BE of TGP, since it is a flexible, non-destructive, non-invasive and rapid technique, requiring minimal sample preparation (Medendorp *et al.*, 2006, 2007). Nevertheless, this method also shares some disadvantages with Confocal Raman Spectroscopy, namely the complexity of the obtained spectra prompted by the skin heterogeneity. To correctly interpret

this information, the development of sophisticated chemometric calibration models is required (EMA, 2014c).

Despite these drawbacks, optical methods applied to skin research are not only academically appealing, but they have also proved to be industrially attractive. In fact, there is a high number of patents and utility models that encourage the applicability of these imaging methods to the dermal field. Just in the last five years, more than 220 patents have been published on this subject, encompassing biometric and monitoring devices, cosmetic, diagnostic and therapeutic applications. The reasons that sustain this enhanced industrial interest are related with the NIR radiation ability to permeate the skin.

Patented devices that use NIR tissue spectroscopy usually comprise the application of a NIR laser onto a specific area. The light propagates accordingly with the scattering and absorption properties of the skin tissue. Since each skin layer contains a unique heterogeneous environment, light absorbance changes through scattering. As previously mentioned, if proper chemometric models are developed, the quantification of parameters such as water, proteins, lipids and drugs, is possible (Wenzel *et al.*, 2002). Additionally, the obtained spectral information can be converted into an image, useful in many surgical interventions as well as a non-invasive diagnosis tool. For all the above-mentioned reasons, NIR provides appealing research opportunities, and it is expected to continue generating important data related to skin physiology and pathology (Narkar, 2010; Juan G Rosas *et al.*, 2011).

1.4.5 Dermatopharmacokinetic Methods

The dermatopharmacokinetic method, commonly referred to as tape stripping, is a minimally invasive *in vivo* procedure in which tape strips are sequentially applied and removed from the skin surface (Benson and Watkinson, 2012). Through this technique, *stratum corneum* layers are collected in each tape strip, being their content processed by suitable analytical methods (Russell and Guy, 2012; Selzer *et al.*, 2013). The basic assumption of this method is that the amount of drug collected in the strips throughout time, represents the rate and extent of the drug in the skin. In other words, this method enables the determination of the drug dermatopharmacokinetic profile, which can be further used to compare the test product (generic) with the reference product (Benson and Watkinson, 2012; Cordery *et al.*, 2017; Yacobi *et al.*, 2014).

In 1998, the FDA issued an interim tape stripping guidance to assess topical drug products BE. The suggested protocol required that skin tape stripping should be performed in no less than eight sites, being the first four used to characterize the drug uptake phase, and the remaining

sites to depict the clearance phase. The guidance suggested that the tape stripping procedure should be made at different time points, previously defined in accordance with the drug steady state period (FDA, 1998). Nevertheless, due to conflicting results with the same commercially available tretinoin gel, the FDA withdrawn this guidance in 2002 (Au *et al.*, 2010; Yacobi *et al.*, 2014).

Several reports addressed the main problems of the technique, which dictated its lack of reproducibility. First, the results may be affected if the *stratum corneum* is not the action site, if the follicular route is a relevant permeation pathway for the tested product and if the barrier function is compromised (N'Dri-Stempfer *et al.*, 2009; Navidi *et al.*, 2008; Praça *et al.*, 2018). Second, the guidance did not provide a robust insight on the amount/depth of the *stratum corneum* that should be collected in the tape strips. Third, and perhaps the most important, the guidance demanded complex validation procedures requiring a large number of subjects and, consequently, a considerable amount of application sites, since both skin stripping technique and analytical method should be validated. In these terms, a dermatopharmacokinetic approach would have no tangible economic benefits, when compared to the gold standard method - clinical endpoint studies (FDA, 1998; N'Dri-Stempfer *et al.*, 2009). Even though the method is currently not included in an US guidance, nowadays EMA in the draft guideline on quality and equivalence of topical products already accepts the use of the dermatopharmacokinetic approach as a BE tool. Furthermore, other agencies, such as the Japanese, South African and Brazilian, also accept tape stripping with this purpose. Therefore, a certain regulatory "opening" regarding the acceptance of this technique is being registered (EMA, 2014b; Yacobi *et al.*, 2014). There are in fact, several pertinent advantages of skin tape stripping method:

- It is non-invasive, since corneocytes are denucleated, flattened and non-living cells, a noteworthy advantage when compared to open flow microperfusion or microdialysis, which are semi-invasive (Abd *et al.*, 2016);
- Since it is an *in vivo* technique, the topical drug delivery assessment is conducted with a fully cutaneous microcirculation;
- It is a faster method when compared to *in vitro* permeation studies (Cordery *et al.*, 2017). Additionally, the information provided by this method can be useful to complement the results of an *in vitro* permeation study through the establishment of an *in vivo-in vitro* correlation (Bunge, 2017). Moreover, the method is not solely applicable to biopharmaceutical investigations, but also in other research fields e.g. in the evaluation of the skin barrier function, gene expression monitoring and dermatological skin pathologies diagnosis.

Motivated by the methods range, several research groups, as well as regulatory authorities, continued to develop new protocols able to minimize the procedure complexity, and at the same time, increase its reproducibility (Benson and Watkinson, 2012; Bunge, 2017; Cordery *et al.*, 2017; Leal *et al.*, 2017). A particularly relevant optimization strategy of the tape stripping technique regards the "two-time" approach, developed by Professor Richard H. Guy and Professor Annete Bunge. The main changes of this new procedure are the following: (i) solely one uptake and one clearance time are analysed; (ii) the authors developed strict cleaning procedures, which assure that the formulation excess is properly removed prior to tape stripping; (iii) the removal of nearly all *stratum corneum* during the experiment (and therefore most, if not all of the drug); and finally, (iv) the method does not discard the first tape strips (Benson and Watkinson, 2012; N'Dri-Stempfer *et al.*, 2008). Due to the simplicity of this updated procedure, the method is less prone to interlaboratory differences. Moreover, less subjects are required since each volunteer is able to provide more replicate measurements, thus enhancing the analysis statistical power (Cordery *et al.*, 2017; N'Dri-Stempfer *et al.*, 2009).

An interesting work by Cordery and collaborators compared the results of the tape stripping method (using the previously described methodology) with *in vitro* permeation studies to assess the bioavailability of three marketed diclofenac topical formulations with different strengths (1%, 2% and 3% w/w) and different dosage forms (solution and gels) (Cordery *et al.*, 2017). The obtained tape stripping results were able to correlate the superior performance of one formulation (a 2% w/w diclofenac solution), which had a known permeation enhancer (DMSO) over the other two formulations that did not possess DMSO. Both methods – *in vitro* permeation and tape stripping – revealed similar results, reinforcing the potential of these techniques to generate complementary information, useful while assessing bioavailability of topical drugs (Cordery *et al.*, 2017).

1.4.6 *In vitro* methods

In vitro dissolution methods are a valuable tool throughout the pharmaceutical product lifecycle. They are broadly used during the product development phase, and their importance as a quality control tool is undeniable. Through these methods, the product dissolution profile can be traced, thus yielding valuable information on its pharmaceutical performance. These reasons sustain their possible applicability as an extended pharmaceutical equivalence assessment tool (Braddy *et al.*, 2015). For solid dosage forms, these methodologies are adequately addressed in pharmacopoeias and are widely approved by the regulatory authorities in the validation of batch-to-batch uniformity, generic drug approval and so on

(Braddy *et al.*, 2015; Petró *et al.*, 2013). However, for topical formulations, the same scenario is still to be registered.

In vitro permeation testing (IVPT) and *in vitro* release studies (IVRS), specifically tailored for topical products, share the same scientific principles of the dissolution methods of solid dosage forms (Flynn *et al.*, 1999). Nevertheless, until very recently, these methods were not commonly regarded as BE tools by the regulatory authorities. This view has been subject to several changes, as both IVPT/IVRT are becoming more and more established. Regulatory agencies, such as ANVISA, FDA and EMA are gradually accepting them as clinical trials surrogates in BE assessment of TGP (Braddy *et al.*, 2015).

Both methods require a diffusion cell with the following structure: (i) a donor compartment where the topical formulation is applied; (ii) a receptor compartment that entails the receptor solution, and (iii) a membrane that separates both chambers. If the membrane is from biological origin (human or pig), the permeation profile of a substance is traced, see Fig.1.3. If a synthetic membrane is used, the release profile is obtained (OECD, 2010). Throughout the analysis, several samples from the receptor compartment are collected at pre-defined times, followed by replenishment with fresh medium (Sivaraman and Banga, 2015). The rate of drug release from the donor to the receptor compartment is traced throughout time, thus yielding the permeation or release profile (Chang *et al.*, 2013a).

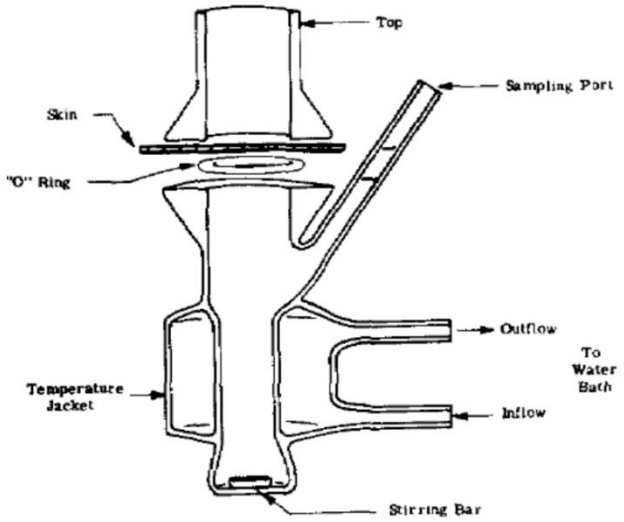


Fig.1.3 – Schematic representation of the diffusion cell developed by Thomas J. Franz (Franz, 1975).

1.4.6.1 *In vitro* release testing

The determination of the *in vitro* release profile is useful in many scenarios. During product development, it can provide essential data on the product's microstructure, valuable to optimise both formulation and production process. On the other hand, during late-stage development, as well as post-marketing phase, these assays function as a quality control tool to monitor batch-to-batch consistency. The main advantages of this method rely on its high sensitivity and discriminatory power, which are often able to reflect physicochemical differences of topical semisolid drug products (Dandamudi, 2017). Moreover, in specific conditions, IVRT can be used to document the product pharmaceutical equivalence when compared to a pre-approved product (FDA, 1997). These specific conditions will be extensively debated in Chapters 4, 5 and 6. Nevertheless, since synthetic membranes are used, IVRT is not expected to correlate or be predictive of *in vivo* performance (Dandamudi, 2017).

1.4.6.2 *In vitro* permeation testing

IVPT is a method that has been successfully used to determine the permeation rate of active substances from semisolid vehicles (Flaten, G. E *et al.*, 2015; Klein *et al.*, 2002; Narkar, 2010). The permeation profiles obtained from IVPT can be used to assess topical pharmacokinetics (Abd *et al.*, 2016; Chang *et al.*, 2013a; Leal *et al.*, 2017). The usage of biological membranes, especially human, closely mimic *in vivo* conditions, as the *stratum corneum* is the primary limiting barrier to dermal absorption (Franz, 1975).

In vitro drug permeation/penetration studies are quite numerous in the literature. A work that frequently stands out, reports to study by Franz *et al.*, where a systematic comparison between the clinical performance of seven approved TGP and the corresponding reference products is presented (Franz *et al.*, 2009). Several corticosteroid products were used, as well as two tretinoin formulations. Their results showed that for the majority of the formulations, permeation studies were able to replicate human absorption, previously assessed by CES (tretinoin) and by *in vivo* pharmacodynamic assays (Franz *et al.*, 2009). A similar study was performed by the same group with different dosage forms of clobetasol propionate (Lehman and Franz, 2014). The authors were also able to prove that IVPT was not only able to assess topical drug bioavailability, but also to provide a more sensitive and discriminatory analysis than the one provided by the pharmacodynamic assay, especially when comparing different dosage forms (Lehman and Franz, 2014).

The same rationale was presented in a publication by Trotter *et al*, where 139 acyclovir generic creams from 37 different countries were analysed. A first screening of the formulations was

able to document considerable quantitative discrepancies in the excipients, such as the propylene glycol, a known permeation enhancer, between some generics and the reference product. In this context, the authors performed a pilot IVPT study, using human skin, to compare the reference product with the generic products, that registered the lowest propylene glycol content. The obtained permeation profiles were able to reflect the quantitative differences of the formulations (Trottet *et al.*, 2005).

IVPT are an integral part of the quality control of transdermal drug systems, as addressed in the EMA guideline on quality of transdermal patches. A recent publication by Shin *et al.*, used IVPT to evaluate and/or compare the effects of heat on various transdermal products. The authors were able to document a strong *in vivo-in vitro* correlation between the IVPT (using human skin) of a nicotine transdermal product, with the plasmatic concentrations of the drug, when subjected to increased temperatures (Shin *et al.*, 2018b, 2017). As previously discussed, in the EMA concept paper on quality and equivalence of topical products and in the guideline of transdermal patches, IVPT methods are also explored as surrogate methods of CES.

Moreover, in the EMA draft guideline on quality and equivalence of topical products, experimental considerations addressing method development and validation procedures are thoughtfully addressed. These aspects will be further detailed in Chapter 6 of the present thesis. Moreover, other guidances also recommend the use of IVPT methods to predict local bioavailability (EMA, 2014d, 2014b; EPA, 1992; WHO, 2006). These can be further divided into three major categories: risk assessment of agrochemicals (EFSA, 2012), bioavailability evaluation of dermal products (OECD, 2010) and those encompassing a broad field of application (OECD, 2004). One must take into account that in this context, “skin absorption” regards local bioavailability and not absorption through the skin, designed to achieve a systemic therapeutic action.

The OECD guidance document on the conduct of skin absorption studies and the SCCS/1358/10 document emitted by European Council entitled “basic criteria for *in vitro* assessment of dermal absorption of cosmetic ingredients”, provides detailed guidelines regarding *in vitro* permeation testing (EC, 2010; OECD, 2004). In fact, dermal absorption of personal care/cosmetic, biocide and agrochemical products, is mostly assessed through these methodologies (Almeida and Costa, 2016; Davies *et al.*, 2017).

There are, however, important limitations of IVPT that should be addressed whenever considering this approach. These mainly regard the inter and intra variabilities of the human skin, which pose a challenge to method standardization and reproducibility compliance (Praça *et al.*, 2018).

In spite of these disadvantages, combined efforts of the regulatory agencies, pharmaceutical industry and the academia have been established to validate this method further. The main objective is to reinforce the usage of IVPT with the same criteria as CES (Shah *et al.*, 2003).

As extensively discussed in the previously presented sections, several alternative methods to CES can be used to establish the bioequivalence of TGP. Amongst them, IVPT are the most commonly used, relying on sound scientific principles, analogous to the dissolution methods used to establish the bioequivalence of solid dosage forms.

Other alternative approaches, such as microdialysis, tape stripping and spectroscopic methods, have also sparked considerable interest. Newer combinational techniques, amongst these analytical surrogates, seem to be of great usefulness, since bioequivalence could be assessed by a more straight-forward, cost-effective and robust approach.

In Table 1.1, the main disadvantages and advantages of each method for bioequivalence assessment of TGP are briefly summarized.

Table 1.1 – Main advantages and limitations of BE assessment approaches of topical generic drug products.

BE assessment methods	Advantages	Limitations
Clinical studies	Regulatory accepted method	Inter-subject variability (disease state, race, gender, age, difficulties in dose standardization) Large number of patients (Increases trial times and cost)
VCA studies	Regulatory accepted method	Only applicable to corticosteroids Doubtful in certain dosage forms Inter-subject variability
MD methods	Real-time determination of pharmacokinetic parameters Suitable to study diseased skin Allows testing of both RP and GP at the same time, in the same individual Can detect drug stimuli specific biomarkers Inexpensive	Difficult analysis of highly lipophilic and protein bound Difficult analysis of formulations with low drug content Semi-invasive technique → tissue inflammation to 8-10 hours' assay duration Specialized personnel Difficult selection of an adequate flow rate
NIR	No sample derivatization All optical approach NIR radiation permeates the skin, thus enabling the determination of the formulation permeation profile	Especially relevant with NIR active molecules Requires the development of chemometric models Inter individual variability must be considered
PKD methods	"Switch-status" regulatory methods Determination of pharmacokinetic parameters Non-invasive technique	Only applicable for SC acting drugs Poor model to assess the BE in diseased skin Reproducibility issues Lack of "tape equivalence" Does not distinguishes crystallised drug from the drug in solution
IVPT	"Switch-status" regulatory methods Based on sound scientific principles Simple and effective method Experimental conditions can be easily controlled Can predict the absorption profile Quantitative Sensitive to changes solubility, particle size and viscosity Regulatory accepted method: SUPAC-SS + BE tool for level 2 changes (see next chapter)	Ethical consent can be difficult to obtain Inter individual variability must be considered

Key: VCA – Vasoconstrictor assay; NIR – Near Infrared studies; PKD – Dermatopharmacokinetic studies; IVPT – *in vitro* permeation testing.

1.5 A new paradigm in the pharmaceutical equivalence concept

As previously explored in section 1.4, the development and validation of surrogate methods has been a long-standing concern of several regulatory agencies, especially EMA and the US-FDA. In this context, the following sections intend to present an updated analysis on the most significant regulatory developments addressing alternative approaches for BE assessment of

TGP. Particular attention was given to the FDA draft guidances of complex generic topical products. Some of these non-binding recommendations already accept a biowaiver of clinical trials if a proper physicochemical characterization is provided. Furthermore, some relevant European Public Assessment Reports in which *in vitro* methods played a significant part of the product bioequivalence assessment process, are also mentioned.

The Strawman decision tree and the topical drug classification system were also referred as examples of the combined efforts among the regulators, pharmaceutical industries and academia. These fruitful strategies intend to outline the principles that would substantiate an extension of the pharmaceutical equivalence concept, and at the same time, promote harmonisation on the regulatory approaches used for establishing the therapeutic equivalence of these dosage forms.

1.5.1 *In vitro* methods regulatory background

BE assessment of semisolid dosage forms for topical administration has to be performed not only for abbreviated new drug applications (ANDA), but also for pre-approved products that have been changed (e.g. formulation, manufacturing process, scale-up/scale-down of manufacturing and site of manufacturing). In such situations, the performance of complex clinical endpoint studies as the only resource to prove bioequivalence is not feasible.

Motivated by these constraints, the US-FDA emitted a guideline to overcome the regulatory restrictions in the above-mentioned situations – the Nonsterile Semisolid Dosage Forms – Scale-Up and Post approval Changes (SUPAC-SS): Chemistry, Manufacturing and Controls (FDA, 1997). This guidance regulates the use of IVRT methods as a tool to generate information on the equivalence between the pre-change (approved product) and post-change (test product) (Shah *et al.*, 2015, 2003)

As a consequence, IVRT have been used for years by the pharmaceutical industry during formulation development and also during quality control analysis (Chang *et al.*, 2013b; Sivaraman and Banga, 2015). This extensive use has produced both knowledge and comprehensive experience in all marketed complex semi-solid dosage forms.

The SUPAC-SS applies to pharmaceutical sponsors of new drug applications (NDA), and ANDAs, as well as abbreviated antibiotic drug applications (Sivaraman and Banga, 2015). The guidance is applicable in the following circumstances: (i) changes in components and composition; (ii) changes in the manufacturing equipment; (iii) changes in the manufacturing process; (iv) changes in batch size and; (v) manufacturing site changes. For each field, three levels of

change are established. In most cases, the test products which were solely exposed to level two changes, are able to document product sameness through IVRT. Level two changes include, but are not limited to:

- Changes in the component or composition, between 5 and 10% of excipients, with the total additive effect of all excipients changes being no more than 10%;
- Changes in the equipment (different design or principle);
- Changes in the manufacturing process, such as rate of mixing, rate of cooling, operating speeds and holding time;
- Changes in the scale-up/scale-down of manufacture, more specifically changes in batch size beyond a factor of 10.

In these circumstances, if the ratio between the median release rate for the post-change product over the median release rate for the pre-change product falls within 75% to 133.3%, IVRT can be used to requalify the product (FDA, 1997) The application of this guidance significantly reduces the regulatory complexity of post-approval changes of a topical drug product (Shah *et al.*, 2015; Sivaraman and Banga, 2015).

The scientific relevance of IVRT has been exhaustively documented, since the release profile of a given formulation is highly dependent of its qualitative, quantitative and microstructure attributes, being a useful tool to assess product sameness (Shah *et al.*, 2003). However, permeation studies using human skin provide a more significant insight on the products *in vivo* performance. For this reason, there are several regulatory authorities which recognize the importance of developing a discriminative IVPT method. A clear and updated example on the regulatory background of IVPT methods regards the Public Assessment Report of Tactuo, an adapalene/benzoyl peroxide gel formulation emitted by the Swedish regulatory agency. This was considered an innovative product, since only the isolated drug substances had marketing authorisations, therefore CES, as well as pharmacokinetic studies were performed. Nonetheless, three *in vitro* studies were conducted in order to support the absence of significant differences between the dermal absorption of the active components whether administered in combination or as single components.

In SUPAC-SS, the US-FDA only considers artificial membranes which are less representative of *in vivo* topical permeation since they do not offer the same degree of resistance as *stratum corneum.* This guidance explicitly states that IVRT, alone, cannot be considered a surrogate test for *in vivo* BE of topical semi-solid products, since there was no convincing *in vitro-in vivo* correlation (FDA, 1997). This opinion was also stated in the AAPS/FDA workshop report held in 1997 (Flynn *et al.*, 1999). In this workshop, IVRT was found very useful in what regards

formulation design and optimisation, as well as in assessing product performance when dealing with slightly different formulations / production process changes (Flynn *et al.*, 1999). Nevertheless, the workshop highlighted that IVRT continue to be non-predictive of i*n vivo* performance since there was still to be registered a systematic investigation, capable of comparing the influences of raw materials and process variables, with the release profile of topical semi-solid formulations (Flynn *et al.*, 1999). Under this "regulatory perspective", IVRT could not be used to assess TGP bioequivalence, but could instead be used to document product pharmaceutical performance equivalence.

Nowadays, many jurisdictions such as Australia, the EMA, South Africa, Switzerland, ANVISA, and the USA-FDA are more and more permeable to IVPT methods as complementary tools to *in vivo* studies, but also as a biowaiver methods. This is a direct result of the combined effort between industry, regulators and academia that through many initiatives such as the Strawman Decision Tree and the Topical Classification System, are defining a new concept of pharmaceutical equivalence. In the following sections, these multidisciplinary approaches will be highlighted.

1.5.2 The Strawman Decision Tree

The workshop on "Current challenges in bioequivalence, quality and novel assessment technologies for topical products", greatly contributed for the development of a science-based approach towards the simplification of the regulatory requirements for TGP (Harris, 2015; Shah *et al.*, 2015; Yacobi *et al.*, 2014).

In this meeting, pharmaceutical scientists and dermatologists from academia, industry and regulatory agencies discussed the currently available methods for therapeutic equivalence assessment of TGP, detailing their advantages and limitations (Harris, 2015; Yacobi *et al.*, 2014). This multidisciplinary meeting concluded that a "whole toolkit" approach to BE assessment would benefit TGP development. Hence, a "Strawman" decision tree was proposed. A schematic adaptation of this model is presented in Fig.1.4. The proposed approach underlines the importance of accounting product specificities, namely the API properties, the disease and the pharmaceutical dosage form, in order to conduct a more rigorous investigation on TGP bioequivalence assessment (Yacobi *et al.*, 2014).

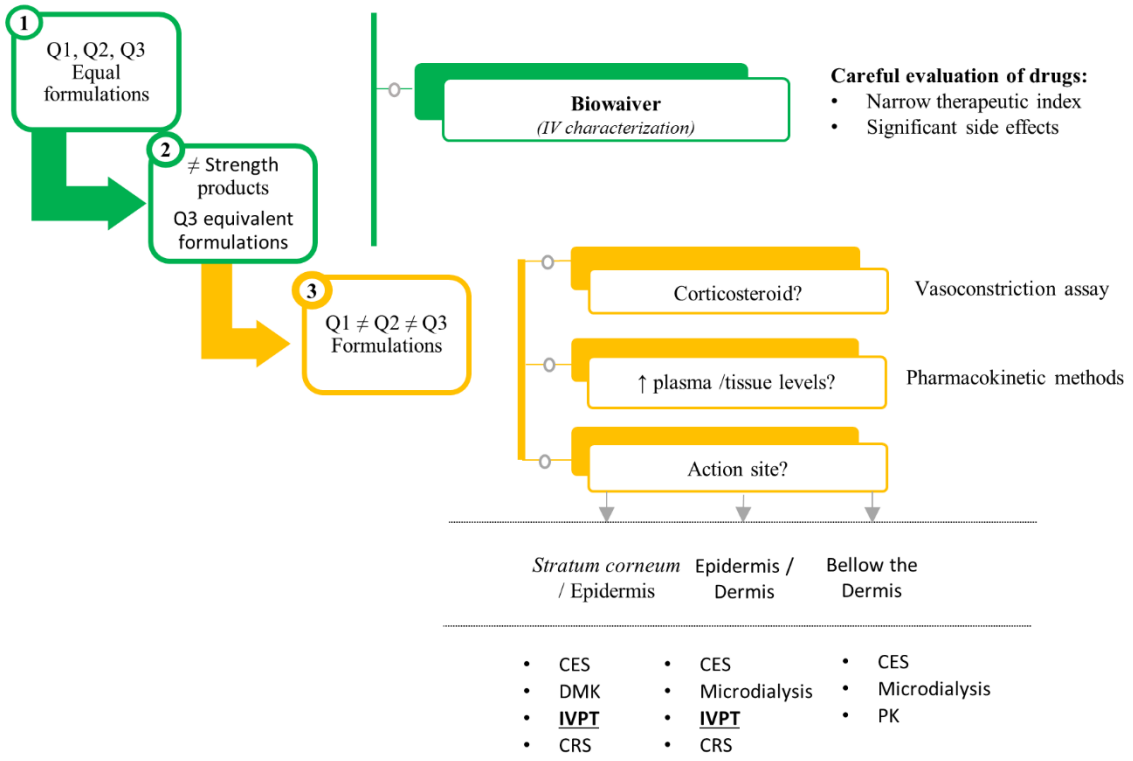


Fig.1.4 – Schematics of bioequivalence assessment methods for TGP. Adapted from (Yacobi *et al.*, 2014).

Key: CES – Clinical Endpoint Studies; DMK – Dermatopharmacokinetic methods; IVPT – *In vitro* permeation testing; CRS – Confocal Raman Spectroscopy; PK – Pharmacokinetic endpoints; Q1 – Qualitative sameness; Q2 – Quantitative sameness; Q3 – Microstructure sameness.

As displayed in Fig.1.4, the first step that should be evaluated is the qualitative sameness, quantitative sameness and microstructure sameness of the test product (generic) and innovator product (Chang *et al.*, 2013a).

If both products are qualitatively and quantitatively equal, microstructure sameness can be assessed by *in vitro* tests, such as IVPT and IVRT. In this scenario, the need for clinical evaluation could be waived. However, as mentioned in the EMA concept paper, a critical evaluation has to be considered in the following circumstances: drugs with narrow therapeutic index, drugs with significant side effects and with drugs that oblige additional safety requirements (EMA, 2014b; Yacobi *et al.*, 2014).

In the case of formulations that do not meet the same Q1 and Q2 criteria, additional tests must be conducted to properly document the generic product therapeutic equivalence. In this case, the Strawman decision tree considers three main scenarios:

- If the drug product is a corticosteroid, the *in vivo* vasoconstrictor assay (VCA) can be used;

- If the drug induces significant plasma/tissue levels, a pharmacokinetic analysis can be performed, similar to BE assessment of systemically available drugs. Nevertheless, this method in the vast majority of topical products is not applicable, since these dosage forms are primarily developed to exert a local effect. Rare examples of pharmacokinetic application include lidocaine patches, lidocaine-prilocaine creams, diclofenac emulgels and etofenamate formulations (Chang *et al.*, 2013b; Drossapharm, 2019; Efe *et al.*, 2014).
- If the test product is not eligible neither for pharmacodynamic or pharmacokinetic evaluations, other BE assessment methods have to be considered. These should be decided accordingly with the product action site. CES can be applied in all circumstances (*stratum corneum*, epidermis or dermis). However, there are alternative methods that can be used to supplement clinical data. Examples of such methods include IVPT, CRS, MD and TS, whose application is reinforced, thus unveiling their use in dermal research (Yacobi *et al.*, 2014).

There are, however, products that do not meet Q2 sameness criteria, but where a biowaiver could also be safely and scientifically justified – Different strength products. The scientific reasons that support this extension are quite simple to identify. Different strength products only differ in the amount of API, being the basic formulation, equipment and manufacturing process the same for all strengths. Therefore, if there is proper documentation of the proportional or pseudo-proportional release/permeation profiles, obtained by methods such as IVRT or IVPT respectively, a biowaiver could be also equated (Shah *et al.*, 2015, 2003).

1.5.3 The Topical Drug Classification System

Besides the Strawman decision tree, where different BE assessment methods are investigated bearing in mind the specificities of the evaluated product, there are some authors that further explore the biowaiver concept for TGP (the first stage of the decision tree).

This complementary approach to the Strawman decision tree is based on the proposal of a topical classification system, analogue to the Biopharmaceutics Classification System (BCS), established for oral solid dosage forms (Lu *et al.*, 2016; Shah *et al.*, 2015).

The BCS institutes four classes of API based on their solubility and permeability characteristics. For generic immediate release dosage forms of the BCS class 1 (high solubility and permeability) and class 3 (high solubility and low permeability) API, a biowaiver can be applied, if proper documentation of the dissolution profiles similarity is presented (Shah *et al.*, 2015).

The TCS differentiates the generic formulations from the reference product, based on Q1, Q2 and Q3 profile. This system was purposed, mainly focusing the rationale behind the SUPAC-SS guidance. A schematic diagram of TCS is presented in Fig.1.5.

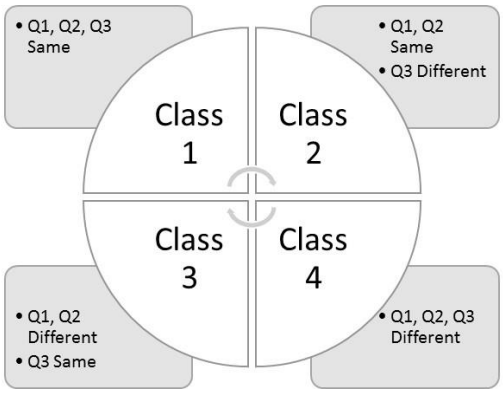


Fig.1.5 – Topical Drug Classification system. Modified from (Lu *et al.*, 2016; Shah *et al.*, 2015).

Similarly to the Strawman decision tree, if the generic product is Q1, Q2 and Q3 equivalent (class 1), the need to prove therapeutic equivalence by CES is not scientifically demanded.

Accordingly, with this model, when Q3 criteria are met, for products that are not Q1 and Q2 equivalent (class 3), a biowaiver could also be considered. For instance, API with specific physicochemical properties that strongly influence the formulation main critical attributes, i.e. extreme lipophilicity, the excipients impact on the permeation kinetics is not significant. Additionally, if product performance, evaluated through IVRT, is not altered by Q1/Q2 changes, there is no scientific reason to believe that the product will not be therapeutically equivalent (Shah *et al.*, 2015). As previously referred, the TCS is based on the SUPAC-SS guidance, so only a maximum of 10% change in the quantity of any or collectively, all excipients would be acceptable.

For class II and IV topical drug products, the evaluation is not that simple, since there are formulation differences that, in the majority of the cases, impact the product performance. For these classes, therapeutic equivalence must be proved by other methods. In spite of this attempt of standardization, the present approach has been continuously evolving to better fit the purpose of bioequivalence establishment without the need of clinical endpoint studies.

Using these scientific principles, both approaches – the Strawman decision tree and the TCS – would contribute to faster development and approval of TGP, maintaining at the same time all the efficacy and safety requirements (Shah *et al.*, 2015). Both approaches are based on the fact that Q3 characteristics highly influence topical dosage forms bioavailability. Under this

perspective, *in vitro* diffusion tests, would be key decision tools for BE assessment (Shah *et al.*, 2015).

1.5.4 FDA draft guidances

Since 2007, the FDA has been publishing non-binding product-specific guidances for generic product development. These intend to identify the tests that should be presented while submitting a new ANDA. In these guidances, the FDA reaffirms the need to prove the TGP pharmaceutical equivalence (same active ingredient, dosage form, strength and route of administration). Due to the complexity underlying CES, in the past 2-3 years, the US-FDA has been accepting, for some products, alternative BE assessment methods (Braddy *et al.*, 2015; FDA, 2017).

To proper address this situation, the draft guidances regarding TGP were reviewed and according to the results, these can be further assigned into two groups: (i) products in which the agency recommends a fixed BE assessment method and; (ii) products in which the applicant may select, within the suggested alternatives, which BE study to present.

The majority of the products falls within the 1st group. Clinical endpoint studies are appointed as the main method to support BE. Nevertheless, there are guidances, such as the micronized 0.1 and 0.04% tretinoin gel, which explicitly address the importance of performing IVPT studies, alongside with CES, to confirm the products equivalence. If the product is a corticosteroid, vasoconstriction studies are also appointed. If the product is a liquid, the applicant may submit a biowaiver. Pharmacokinetic studies are also referred to when the product is systemically absorbed. Nevertheless, these methods solely cover a negligible percentage of products aiming at a local action. Despite this scenario, there are a growing number of products for which the approval may be solely supported by an adequate physicochemical characterization, where *in vitro* techniques partake a predominant role, see Table 1.2. Other tests that need to be presented to provide conclusive evidence of the TGP pharmaceutical sameness include comparative:

- Visual and microscopy appearance;
- Rheological properties at multiple shear rates;
- Drug particle/globule size and size distribution;
- API polymorphic form;
- Vehicle metamorphosis;
- API distribution;

- pH, water activity and other potentially relevant physical and structure similarity characterisation.

To properly document the physicochemical and structural similarity, these tests have to be performed in a minimum of three lots of the GP and three lots of the RP.

For the second group of products, where BE can be portrayed by hither two options, a biowaiver from CES can be requested if the product is a solution. Whenever a pharmacokinetic evaluation is possible, it should be performed in conjunction with IVRT/IVPT. The alternative hypothesis to document BE for these products, frequently falls within clinical endpoint studies, or, if suitable, pharmacodynamic assays.

Table 1.2 – FDA non-binding product-specific guidances for generic drug development where BE can be documented through *in vitro* methods.

API	Dosage Form	1st Option	2nd Option	Date
Acyclovir	Ointment	PCC + IVRT	CES	2016
	Cream	PCC + IVRT + IVPT	CES	2016
Benzyl alcohol	Lotion	PCC + IVRT	CES	2017
Bexarotene	Gel	PCC + IVRT + IVPT		2019
Crisaborole	Ointment	PCC + IVPT/IVRT + PKE		2019
Crotamiton	Lotion	PCC		2017
	Cream	PCC		2017
Cyprofloxacine hydrochloride	Ointment	PCC + IVRT		2018
Clindamycine phosphate	Gel	PCC + IVRT		2020
Clindamycine phosphate and tretinoin	Gel	PCC + IVRT		2020
Dapsone	Gel	PCC + IVRT + IVPT + PKE	CES	2019
Doconazol	Cream	PCC + IVRT	CES	2017
Doxepin hydrochloride	Cream	PCC + IVRT + IVPT	PKE	2019
Erythromycin	Gel	PCC		2017
Gentamicin sulfate	Cream	PCC		2017
	Ointment	PCC		2017
Hydrocortisone	Cream	PCC		2017
Ivermectin	Cream	PCC + IVRT + IVPT	CES	2019
Lidocaine	Ointment	PCC		2016

API	Dosage Form	1st Option	2nd Option	Date
Luliconazole	Cream	PCC + IVRT + IVPT		2018
Metronidazole	Gel	PCC + IVRT + IVPT		2019
Nystatin and triamcinolone acetonide	Cream	PCC		2017
	Ointment	PCC		2012
Ozenoxacin	Cream	PCC + IVRT + IVPT		2019
Oxymetazoline hydrochloride	Cream	PCC + IVRT + IVPT		2019
Penciclovir	Cream	PCC + IVRT + IVPT		2018
Pimecrolimus	Cream	PCC + IVRT + IVPT		2019
Silver sulfadiazine	Cream	PCC + IVRT		2017
Tacrolimus	Ointment	PCC + IVRT + IVPT		2018
Tretinoin	Gel	PCC + IVRT		2020
	Cream	PCC + IVRT	CES	2020
Triamcinolone acetonide	Lotion	PCC		2014
	Ointment	PCC		2017
	Ointment	PCC		2017
	Cream	PCC		2017
	Ointment	PCC		2017

Key: PCC – Physicochemical Characterization; CES – Clinical Endpoint Studies; IVPT – *In vitro* permeation testing; IVRT – *In vitro* release testing; PKE – Pharmacokinetic Endpoints; Group A – fixed studies products; Group B – alternative methods products (Ilić *et al.*, 2021).

As can be seen in Table 1.2, with exception of lotions, all dosage forms are semi solid and some products are corticosteroids.

A similar situation is being registered with locally acting topical drug products intended for ophthalmic administration, such as cyclosporine emulsion or bacitracin ointment. These products share the same complex dosage forms than TGP, and consequently the same BE challenges. Some draft guidances of these products include IVRT as bioequivalence tools (Al-Ghabeish *et al.*, 2015; Krishnaiah *et al.*, 2014).

The addressed examples, clearly reinforce the broader acceptance of *in vitro* approaches as integral part of the overall quality assessment of semi-solid products (Al-Ghabeish *et al.*, 2015).

1.5.5 European appraisal

In Europe, TGP generally do not follow a centralized procedure, instead they are often submitted through mutual recognition/decentralized procedures via a bibliographic application, according to Article 10a of Directive 2001/83/EC. This is applicable to products with a well-established medicinal use within the Community for at least ten years, and recognized efficacy as well as acceptable level of safety. In such conditions, the applicant is

able to demonstrate the product efficacy and safety in the proposed indications, based on previously published preclinical and clinical data (European Council, 2001; PAR, 2016, 2015a, 2014).

However, hybrid products, usually benefit from this submission strategy. A product can be registered as a hybrid product whenever: (i) the strict definition of a 'generic medicinal product' is not met; (ii) when bioavailability studies cannot be used to demonstrate bioequivalence, which is commonly applicable to topically applied and locally acting drug products and when; (iii) there are changes in the active substance(s), therapeutic indications, strength, pharmaceutical form or route of administration of the generic product compared to the reference product. In such circumstances, the applicant may use the pre-clinical and clinical data pertaining to the "reference product"; nevertheless, new studies should be conducted to support the marketing authorization (European Council, 2001). There are however, publicly available European assessment reports of hybrid applications, where the regulatory authorities granted a marketing authorization without new clinical data presentation. A clear example regards Grinvix, a dimetindene maleate aqueous gel, where only information regarding the pharmaceutical equivalence of the test products towards the reference product was provided (PAR, 2015b).

Another example of a hybrid application regards Solaraze 3%, a diclofenac sodium 3% gel. Even though, for this product, CES were performed, the submission process also included *in vitro* studies. These were presented in the pharmaceutical equivalence section, as well as in the clinical aspects. This occurrence is highly indicative of the ongoing importance of these methods in Europe (PAR, 2018).

A further example regarding a mixed bioequivalence assessment process using *in vitro* and CES concerns a lidocaine 25 g/g and prilocaine 25 mg/g cream. This generic product was registered via a mutual recognition procedure. In the clinical aspects, the applicant presented an *in vitro* permeation study on human skin. Additionally, data concerning the amount of active drug (prilocaine and lidocaine) retained in each layer of the skin – *stratum corneum*, epidermis and dermis – was presented. Other interesting IVPT study addressed in this PAR regards the evaluation of the stability of the test product. For this, the principal metabolite of prilocaine, ortho-toluidine, was added to the formulation in known concentrations. The applicant was able to prove that the test formulation did not induce a significant accumulation of the degradation product in the skin, similarly to what was registered for the reference product (PAR, 2009). *In vitro* tests in this application represented the main clinical studies presented; nevertheless, a pivotal comparative clinical study was also performed.

Similar skin retention studies are described in another PAR regarding a Diclofenac Sodium 4% Spray Gel, a product also submitted under a mutual recognition procedure. Bioequivalence was demonstrated by a pharmacokinetic study, which was possible since diclofenac concentrations can be monitored in plasma even if the product is topically applied, by clinical studies to assess both product safety and efficacy and by IVPT methods. The applicant presented data on the distribution profile of diclofenac in the skin, when compared to a different strength reference product, Voltaren® Emulgel (1.16% diclofenac diethylammonium). In this case, the applicant was able to prove that the *in vitro* percutaneous absorption was higher than the one registered in the reference product (PAR, 2002).

The use of in *vitro* methods for bioequivalence assessment in European countries, when compared to the USA example, is still far from harmonization, since as already mentioned in the majority of situations, TGP are not submitted via a centralized procedure. As such, it is often dependent on the differing assessments of the Member States involved. Nevertheless, although to lesser extent than in the US, IVPT is increasingly recognized as a bioequivalence tool, as evidenced by the numerous cases where these methods have been used to partially document BE and, more recently, by the release of the EMA draft guideline on quality and equivalence of topical products.

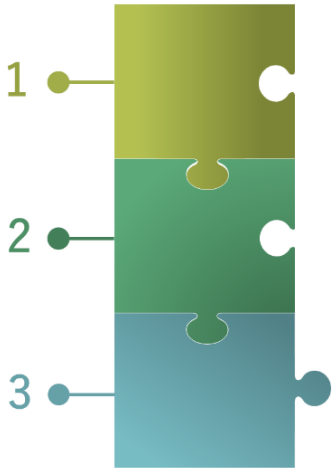
The key scientific principles that allow the use of *in vitro* methods have been carefully investigated by several research groups. One of the most frequently cited examples in the literature is the acyclovir ophthalmic ointment.

Xu *et al.* assessed the IVRT profile of oleaginous and water-soluble acyclovir ointment formulations using Hanson vertical diffusion cells (Xu *et al.*, 2015a). It was observed that the *in vitro* release profile was highly dependent on the type of ointment bases and that for oleaginous bases, drug release followed a particular logarithmic-time dependent profile. The authors further characterize this specific kinetic mechanism and call it the “transient-boundary hypothesis”. According to this model, the API diffusion is firstly conditioned by the limited miscibility between petrolatum and water. However, in a second stage, the drug diffusion profile changes due to the presence of an aqueous transient layer that expands over time. This expansion is closely related with the absence of water soluble components in an oleaginous ointment base. In absorption or water-soluble base ointments, the water is able to diffuse into the matrix, which leads to a “inward moving boundary”. However, in oleaginous ointment bases the transient layer, and consequently drug diffusion into the bulk aqueous medium, moves outwards (Xu *et al.*, 2015a). A second study carried out by the same group, performed a design of experiments (DoE) with an acyclovir ointment composed of an oleaginous base as a model dosage form (Xu *et al.*, 2015b). Outputs such as particle size,

content uniformity and assay, and rheological studies were further analysed. By performing a principle component analysis, the authors were able to identify drug loading, as the most influential parameter on the product quality and performance (Xu *et al.*, 2015b). Through *in vitro* release and *in vitro* permeation testing across rabbit cornea, the authors were able to discriminate the product and process variability, and at the same time confirm product sameness.

A recent study by Tiffner *et al.* presented an integrated approach to qualify and validate an IVRT method for acyclovir cream 5% (Tiffner *et al.*, 2018). Even though the authors based their research on previous works, such as the one presented by Thakkar and Chern, their approach was essentially methodological, with focus on individual parameters of IVRT, but also on other critical components of the test system, including: apparatus and laboratory qualification, HPLC method validation and also IVRT method validation (Thakker and Chern, 2003; Tiffner *et al.*, 2018). For each critical component, specific acceptance criteria were presented. Some of the procedures used for the IVRT validation presented in this paper are also considered in the FDA draft guidance on acyclovir cream. This comprehensive and standardized approach to validate an IVRT method clearly represents a valuable tool to assess the pharmaceutical equivalence not only for acyclovir creams, but also for other semisolid TGP (Tiffner *et al.*, 2018).

1.6 Highlights



Clinical endpoint studies are the gold method for therapeutic assessment of TGP. However, due to the intricacies linked with dermal absorption, these studies are simultaneously the most expensive part of the TGP R&D programs and the one with more entailed risks.

Microdialysis, tape striping and spectroscopic methods constitute **alternative methods for assessing TGP therapeutic equivalence**. However, from a regulatory perspective, *in vitro* methods such as IVRT and IVPT, have been increasingly used as CES biowaivers.

Multidisplinary initiatives such as the Strawman decision tree and the topical drug classification system, have been proposing tailored frameworks for bioequivalence assessment, reliant on the products extended pharmaceutical equivalence.

2 FRAMEWORK, HYPOTHESIS AND OBJECTIVES

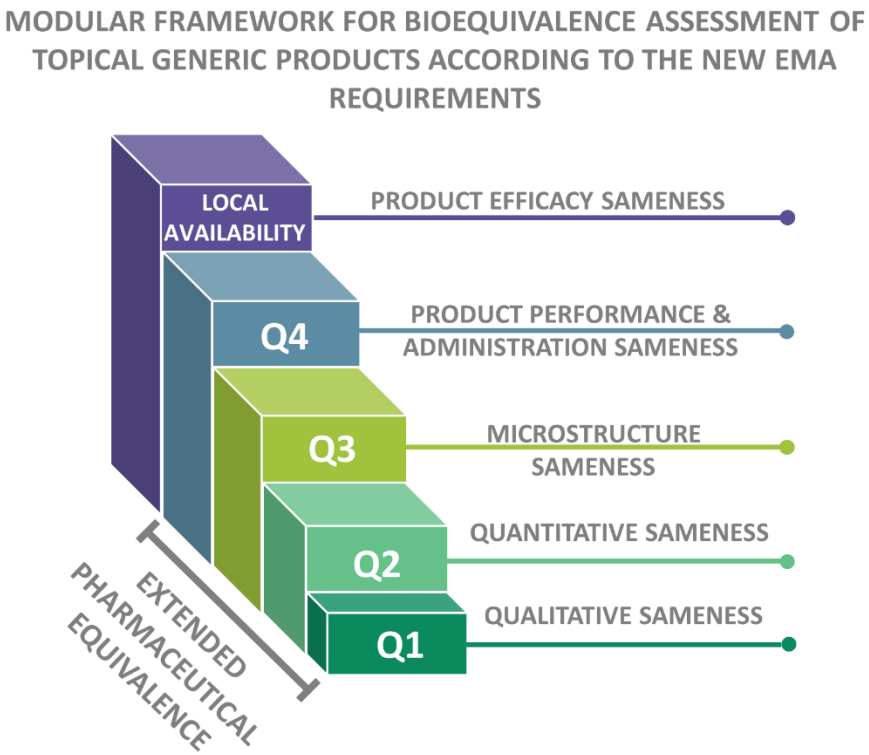


Fig.2.1 – Graphical abstract: Quality and equivalence of topical products: A critical appraisal.

This chapter has been adapted from the following publication:

Miranda, M., Cardoso, C., Vitorino, C., 2019. Quality and Equivalence of Topical Products: A Critical Appraisal. Eur. J. Pharm. Sci. 105082. https://doi.org/10.1016/j.ejps.2019.105082

M.M and C.V conceived the presented idea and established the research program and implementation. M.M wrote the first draft of the manuscript and all the other authors substantially contributed to revisions.

KEY CONCEPTS

- **EMA draft guideline on quality and equivalence of topical products** was released for consultation on October 2018. In line with the previously published acyclovir draft guidance by the US-FDA, this document purposes a modular framework for equivalence demonstration of topical generic products.
- In spite of the noteworthy regulatory advance concerning the approval of topical generic semisolid dosage forms, this document entails **some critical aspects, which may undermine its successful translation** into practice.

2.1 Introducing the new guideline – What does it change?

As already outlined in the 1st chapter, the regulatory authorities have been advancing relevant regulation concerning topical bioequivalence assessment.

Significant on-going and emerging focus areas have been frequently addressed: *in vitro* release testing, i*n vitro* permeation testing, dermatopharmacokinetic methods, rheology and skin metamorphosis. These were mainly purposed by the FDA. In fact, this agency has proven to be more dynamic than the EMA, not only through the release of the FDA Product-Specific Guidances for Generic Drug Development (2015-2018), but also through its continuous efforts to sponsor several scientific workshops addressing this issue. Nevertheless, in October 2018, EMA released a draft guideline on quality and equivalence of topical products. This long awaited document presents a modular framework for equivalence demonstration of topical products, where the use of surrogate methods to clinical endpoint studies is accepted. Despite of its immense applicability, there are critical aspects that should be further highlighted and discussed in order to enable a more concrete applicability of the guideline. In this context, this chapter pertains to call attention to significant parameters addressed in the guideline.

Several issues are raised after a thorough reading of the new EMA guideline, since this document imposes several challenges, which might hamper the real applicability of the alternative bioequivalence methodologies. These have been extensively discussed in the recent EUFEPS Open Forum Discussion on the Draft Guideline on Quality and Equivalence of Topical Products (Bonn, June 2019).

In this context, this section attempts to discuss some of the difficulties regarding the extended pharmaceutical equivalence characterization, where IVRT and product metamorphosis will be specially highlighted, as well as efficacy equivalence studies.

The key alterations imposed by this document can be summarized as follows:

- It clearly defines the extended concept of pharmaceutical equivalence – Qualitative, quantitative, microstructure aspects, product performance and administration equivalence.

Furthermore, it introduces specific protocols for evaluating product efficacy equivalence through *in vitro* permeation studies and *in vivo* tape stripping.

- It significantly limits the conditions for approval of topical generic products submitted via a bibliographic application, in particular when the extended concept of pharmaceutical equivalence is not properly documented.
- It defines the *in vitro* release rate (IVRR) as a critical quality attribute (CQA) of the formulation. The determination of such a parameter should be assessed at several stages in the development of a generic topical product (scale-up, equivalence of excipient homologues, post-authorization changes, definition of acceptance limits for both degradation products and impurities, batch-to-batch consistency, life cycle management).
- It calls for the need to develop topical product specific equivalence protocols.
- It enables the acceptance of a biowaiver for different strength products, if specific requirements are met.
- It introduces the importance of documenting mass balance studies while performing *in vitro* skin permeation testing.
- It accepts dermatopharmacokinetic studies as a reliable method for bioequivalence assessment of topical drug products.

2.2 Guideline background

The main principle behind the redaction of the draft guideline clearly relies on a patient focus and patient driven pharmaceutical development of topical generic products.

The applicant should previously establish the quality target product profile, addressing efficacy, safety and quality aspects. Afterwards, an extensive and comprehensive characterization should be performed both for test product and the reference product.

According to the EMA draft guideline, a modular framework for equivalence demonstration for TGP can be set based on the product dosage form, see Fig.2.1. If the test product is a simple formulation, wherein the API is included within a single-phase base, such as a solution, suspension, gel or ointment, product equivalence can be justified through an extensive pharmaceutical characterization. However, qualitative, quantitative and microstructure sameness (Q1, Q2, Q3, respectively) towards the reference product should be established. Moreover, product performance (which in our perspective could be termed Q4, since it also regards a quality aspect of the formulation), given by the *in vitro* release profile, should also be documented.

Alternatively, if the test product is a complex dosage form (e.g. multiple phase, with permeation enhancers), besides the extended pharmaceutical characterization, the applicant should provide information on the product efficacy equivalence.

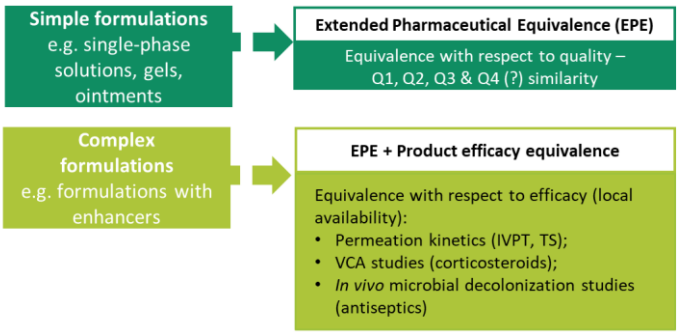


Fig.2.2 – Modular framework for bioequivalence assessment according to the EMA draft guideline on quality and equivalence of topical products.

Key: Q1 – Qualitative sameness; Q2 – Quantitative sameness; Q3 – Microstructure sameness; Q4 – Product performance sameness; IVPT – *In vitro* Permeation Testing; TS –Tape stripping; VCA – Vasoconstrictor assay.

2.2.1 Q1, Q2, Q3 similarity

For both simple and complex formulations, EMA requires the documentation of both qualitative and quantitative equivalence between the RP and the TP. Despite warrant qualitative composition may not be an issue, the same does not happen to ensure quantitative sameness, for which reverse engineering procedures are demanded. Moreover, as the draft guideline clearly states, special attention needs to be directed towards the grade of the excipient, since this aspect may compromise drug product quality. As mentioned by Chang *et al.* a low-melting-grade material may melt under accelerated stability conditions, while a high melting-grade excipient can withstand higher storage temperatures. On the other hand, an excipient with high viscosity will have a distinct impact on the product rheological profile, when comparing to a low viscosity excipient (Chang *et al.*, 2013b, 2013a). In such circumstances, and as reported in the EMA draft guideline, when the grade of the excipient may compromise active substance bioavailability, product manufacturability or product quality, the grade of excipient should be the same as the RP.

Even though there are sound scientific reasons that state the importance of attaining Q1 and Q2 sameness in a semisolid product quality profile, from a practical perspective, attaining such sameness, within the defined criteria (±5%) may prove to be extremely challenging, due to patent protection issues, undesirable RP characteristics, among other factors (Chang *et al.*, 2013b). Moreover, information regarding the excipient grade is solely accessible to the agency, making it difficult for generic manufacturers to assure such conditions.

The topical drug classification system proposed by Shah and collaborators highlights the importance of microstructure sameness when evaluating topical semisolid products. For BE assessment of a class III product (different Q1, Q2 and same Q3), the authors support a waiver for clinical trials, if the excipients proved to be inert and the *in vitro* release testing (IVRT) profile meets the confidence interval criteria comparing to the RP (Shah *et al.*, 2015).

In addition to IVRT, there are multiple methods useful to characterize product microstructure, such as:

- Rheology behaviour – Viscosity of the product directly impacts topical delivery, since it affects the drug diffusion rate. High viscosity formulations exhibit high diffusion rates when compared to low viscosity ones (Bao and Burgess, 2018). The draft guideline mentions specific requirements that should be presented while documenting the products rheological profile. These include a complete flow curve of shear stress (or viscosity) versus shear rate; Linear viscoelastic response (storage and loss moduli *vs.* frequency); Classification of the product's behaviour according to shear and time effects (EMA, 2018b). Equivalent requirements have been previously presented by the FDA in the acyclovir draft guidance (FDA, 2016c). There is, however, one drawback in the EMA criteria that concerns the restriction of the confidence interval (CI) between the RP and generic product (GP) to 90-110%. This CI does not take into account the intrinsic variability of semisolid products, which may impose added challenges in the documentation of rheology profile similarity.
- Careful assessment of the physical state and crystal habit of the drug in the semisolid system – drug polymorphism.
- Analysis of particle size distribution with representative microscopic images at multiple magnifications.
- Analytical centrifugation, a powerful tool which helps derisk product development since it predicts potential destabilization processes, such as sedimentation, creaming, flocculation, coagulation, coalescence, or phase inversion phenomena's, under accelerated conditions.

Besides these methods, an overgrowing importance is being given to analytical strategies used to evaluate the formulation thermodynamic activity (Chang *et al.*, 2013a). In addition to thermal methods, such as differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA), metamorphosis of the topical product upon administration is being frequently addressed when establishing the pharmaceutical equivalence of a topical generic product.

Drug metamorphosis/transformation refers to the changes that most dermatological vehicles undergo after being removed from the primary container and applied to the skin. The mechanical shear associated with the application of the product and/or evaporation of

ingredients, besides promoting a rearrangement of the initial structural matrix, also changes the quantitative composition of the product (Surber and Smith, 2005). These processes may induce skin drug crystallisation, by other words, the formation of a drug reservoir (Goh *et al.*, 2017). Since crystallization phenomenon bears a direct impact in drug permeation, consequently affecting bioavailability, the same process may condition bioequivalence between products (Hadgraft and Lane, 2016). Monitoring excipients in the skin using methods, such as ATR-FTIR spectroscopy, localized nanothermal analysis and photothermal microspectroscopy, combined with multivariate data analysis, are referred in the literature as strategies to assess product transformation (Goh *et al.*, 2018, 2017). However, such methods are still being outlined and the draft guideline does not provide a significant insight on the appropriate methodologies to evaluate product transformation. Therefore, this requirement may prove to be challenging to demonstrate.

2.2.2 Q4 similarity – Product performance IVRT

IVRT has been extensively used during formulation development and quality control analysis. Its wide spread applicability has produced both knowledge and comprehensive experience in all marketed complex semi-solid dosage forms, as reviewed in the 1st chapter. In this context, the acceptance of IVRT methods by EMA as a product qualifying tool in what regards the establishment of an extended pharmaceutical equivalence, comes with no surprise. Similar efforts have been previously carried out by the FDA in some product specific guidances, as well as by other agencies. The European agency requires the performance of extensive IVRT validation procedures which include: membrane inertness, linearity, precision, sensitivity, discriminatory power and robustness evaluation of the method. Moreover, the diffusional system should also be qualified in what concerns diffusional area and volume of the cells, dispensed sampling volume and temperature at the membrane surface (EMA, 2018b). There are, however, some aspects mentioned in annex 1 of the EMA draft guideline (IVRT methods) that may impose significant challenges. The next paragraphs highlight some of these difficulties.

The guideline refers that at least 70% of the active substance applied should be released. However, the majority of topical products do not attain a 70% release of drug substance throughout drug exposition time. In many cases, it will require a prolonged assay duration that does not mimic *in vivo* conditions. Moreover, in topical drug products the active substance is usually in excess, in order to account for drug loss due to skin turnover, among other reasons. Therefore, monitoring the amount of drug released per (exposed) unit area is a far better indicator for assessing individual's drug exposure, when compared to the percentage of drug

released which may greatly underestimate the impact of drug release (Al-Ghabeish *et al.*, 2015; Bao *et al.*, 2017; Fernández-Campos *et al.*, 2017; Goebel *et al.*, 2013; Khanolkar *et al.*, 2017; Krishnaiah *et al.*, 2014; Kriwet and Müller-Goymann, 1995; Lauterbach and Müller-Goymann, 2014; Nallagundla *et al.*, 2014; Petró *et al.*, 2013; Xu *et al.*, 2015a, 2015b).

Another significant restriction that the draft guideline purposes is the reduction of the relative standard deviation (RSD) in IVRT to 10%, as well as the restriction of the CI to 90-111%. Both requirements may compromise the acceptance of many TGP already market approved, as well as many topical reference products due to their intrinsic variability.

Even though the FDA presents broader criteria (RSD 15% and CI 75-133%), attaining these, especially RSD, may also prove to be challenging, especially when dealing with complex formulations such as emulsions or creams (Goebel *et al.*, 2013; Nallagundla *et al.*, 2014).

There should be predefined CI according to the dosage form instead. In this context, EMA defends the publication of Product-Specific Guidances. The redaction of such documents should take into account the nature and complexity of the drug product and active substance, and might be an excellent opportunity to establish more realistic and adapted criteria.

One additional challenge that the guideline purposes is the inclusion of the *in vitro* release rate (IVRR) as a critical quality attribute (CQA) of the topical product. In light of this requirement there would be the necessity to conduct release experiments on a daily routine basis, which may prove to be too demanding for generic manufactures.

2.2.3 Local availability similarity – Equivalence with respect to efficacy

2.2.3.1 IVPT

As the *stratum corneum* is the main barrier for percutaneous absorption, the usage of human skin as a membrane in topical product diffusion tests, closely mimics *in vivo* drug permeation. The appointed reason is the basis of the regulatory overgrowing importance of IVPT as a method to establish product equivalence with respect to efficacy, as previously referred in the 1st chapter.

When submitting IVPT to regulatory agencies, the applicants should perform, similarly to pharmacodynamic endpoint studies, both pilot and pivotal studies. Method development reports, method validation reports and standard operating procedures (SOP) should be likewise presented (Katragadda, 2018).

Validation procedures include membrane qualification, receptor solution qualification and quantification, among other parameters. Membrane qualification studies are especially important due to human skin variability. Accordingly, skin donor inclusion/exclusion criteria should be defined, as well as methods to carefully assess skin integrity. Moreover, several donors should be included, as well as multiple sections per donor.

The OECD guidelines on dermal absorption as well as the guideline on quality of transdermal patches emitted by EMA, provide specific and detailed information concerning this aspect. The variability of the human membranes should be confirmed at least in 2 donors, for EMA, while for the OECD the usage of 3 donors is required. The FDA does not define a minimum number of donors required to adequately power the IVPT pivotal study, however, it clearly states that a minimum of 4 dosed replicates per donor per treatment group should be used (EMA, 2014d; FDA, 2016c; OECD, 2010). In fact, inter- and intra-individual variability of human skin, poses a challenge to method standardization and verification of reproducibility (Praça *et al.*, 2018). This reason may be the basis of the new EMA criteria to use in each IVPT 12 donors with at least 2 sections per donor. This significant increase when compared to pre-existent guidelines, may pose an additional challenge to the performance of IVPT. Human skin for these tests is usually retrieved from plastic surgery, with ethical consent being required, therefore to have 12 simultaneous donors would be extremely difficult.

EMA also requires the conduction of mass balance studies, where an overall recovery 90-110% should be obtained. Once again, these limits may be too restrictive.

2.2.3.2 Tape stripping

The acceptance of dermatopharmacokinetic methods to establish BE of topical drug products is one of the most noteworthy changes of the EMA draft guideline. This method had already been approved as a surrogate method for BE assessment by the FDA, however, as reviewed in the 1st chapter, the agency withdrawn the guidance in 2002 (Au *et al.*, 2010; Yacobi *et al.*, 2014). Due to the method wide range of applicability, especially in what concerns *stratum corneum* acting products, several groups continued to work in the optimization and validation of tape stripping method. The EMA guideline in section III – "*stratum corneum* sampling" presents a specific protocol, based on the work of Professor Richard Guy and Professor Annete Bunge, whose main contributions were already reviewed in Chapter 1.

Fig.2.3 summarizes the main challenges in the transposition of the updated EMA criteria in the establishment of topical generic products bioequivalence.

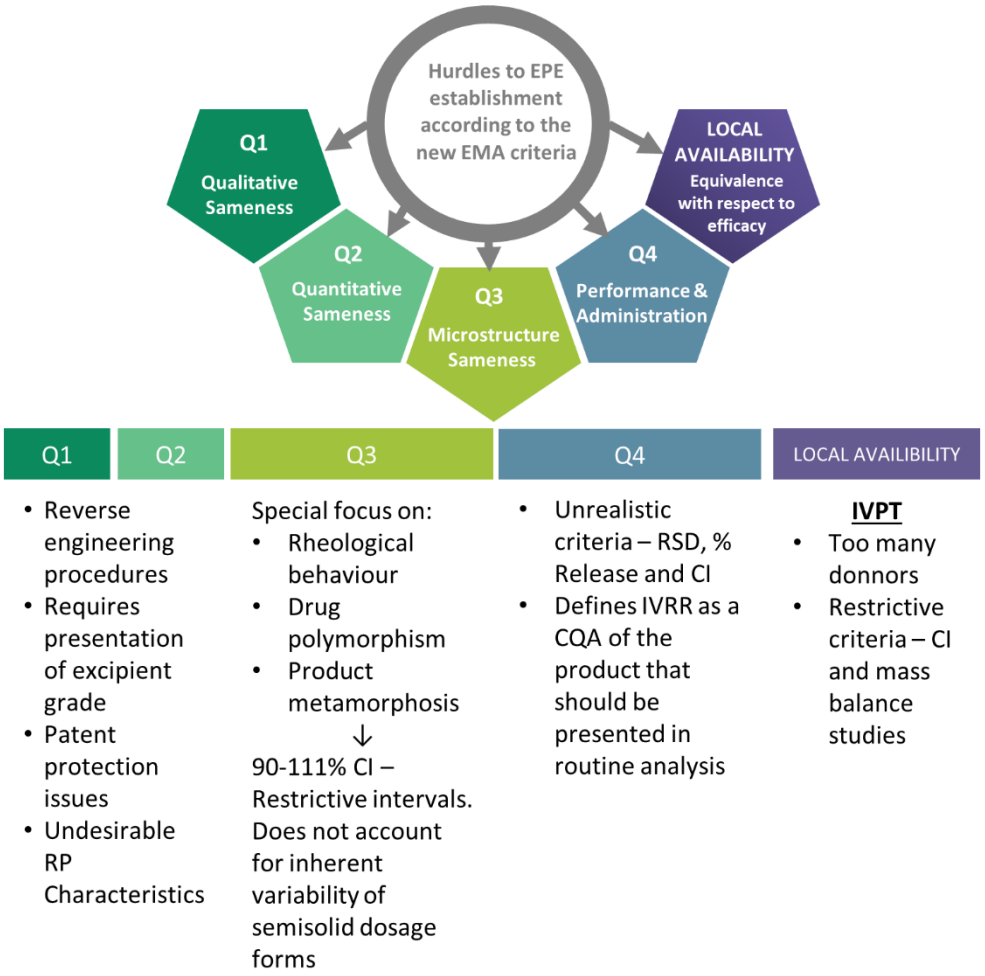


Fig.2.3 – Main hurdles of the new criteria of the EMA draft guideline on the quality and equivalence of topical products.

Key: Q1 – Qualitative sameness; Q2 – Quantitative sameness; Q3 – Microstructure sameness; Q4 – Product performance sameness; IVRR – *In vitro* release testing; IVPT – *In vitro* Permeation Testing; CI – Confidence interval; EPE – Extended Pharmaceutical Equivalence; CQA – Critical Quality Attribute; RP – Reference Product; RSD – Relative Standard Deviation.

2.2.4 SWOT analysis

To properly address all the above changes a strength, weakness, opportunity and threats, (SWOT) analysis was made considering the EMA guideline. Please see Fig.2.4.

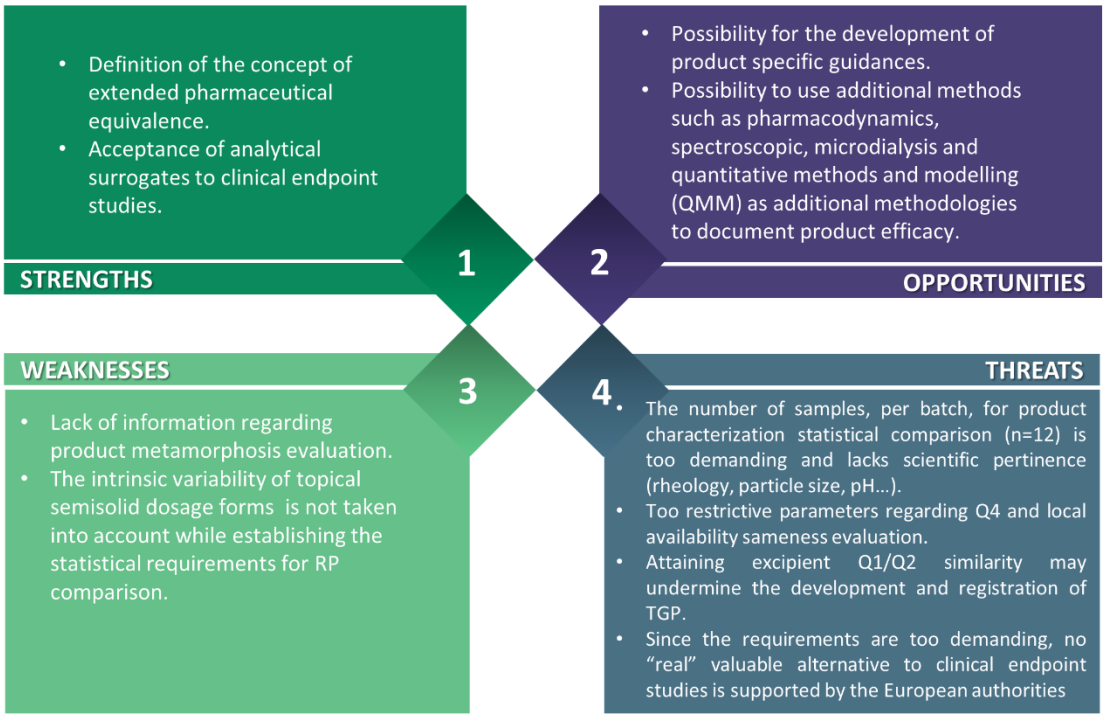


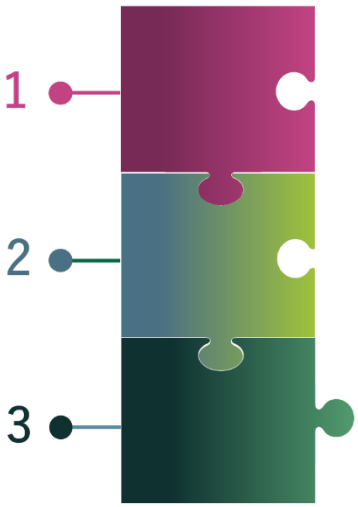
Fig.2.4 – SWOT analysis of the draft guideline on quality and equivalence of topical drug products.

Envisioning possible solutions to the above mentioned threats and weaknesses we timely propose some decision making strategies:

- Taking into account that there are no clear or regulatory established methods for assessing metamorphosis of topical products so far, this evaluation, should be regarded as a complementary analysis.
- Maintain the SUPAC criteria regarding both IVRT and IVPT confidence interval limits. The 90% confidence interval for the ratio of means of the test and comparator products should be contained within the acceptance interval of 75-133%.
- Clarification of the statistical treatment used to compare reference with test products in both release and permeation studies. The EMA draft guideline suggest that the principles stated in the guideline on the investigation of bioequivalence should be used. However, the terms in which ANOVA is performed (subject within sequence, period and formulation) do not apply to IVRT or IVPT. We believe, that a non-parametric analysis, as described in USP (adapting the matrix to n=12) would better fit this purpose.
- For IVRT validation studies, adopt the FDA threshold for the assessment of the method intermediate precision (CV < 15%). The same applies to the comparison of permeation parameters between test and reference products.

- A threshold regarding the % of release in IVRT should not be imposed. As compared to the 70% of drug release, the monitoring of the amount of drug released per (exposed) unit area is by far a better indicator for assessing individual's drug exposure.
- Reduce the donors involved in IVPT to a more reasonable and ethical number.

2.3 Highlights



Extended Pharmaceutical Equivalence (EPE) Concept
For extended pharmaceutical equivalence to register, Q1, Q2, Q3, Q4 and administration sameness must be observed.

EMA draft guideline on quality and equivalence of topical products
A valuable input on the development, validation and implementation of the extended pharmaceutical equivalence concept, supported by *in vitro* technologies, is carefully presented in this draft guideline. By doing so, a safe and scientific driven assessment of TGP BE is stimulated.

Translating the draft guideline into practise
Several issues may hinder the draft guideline applicability: (i) lack of information regarding some methods to establish EPC equivalence; (ii) imposition of strict statistical criteria; (iii) the high number of samples/donors required for IVRT and IVPT analysis, respectively. All these aspects may prove to be challenging to assure.

2.4 Problem, hypothesis and objectives

Assessing the therapeutic profile of a TGP by its local action and comparing it towards the RP can be quite challenging. As such, a pharmacokinetic evaluation is generally unfeasible. Even though pharmacodynamic studies can be used for corticoids, clinical endpoint studies (still) represent the "gold standard" method for establishing bioequivalence.

As previously mentioned, skin permeation and subsequent topical product efficacy is highly dependent on skin integrity, race, gender, age, overall immunological state, disease state and dosage regimens. To surpass these limitations, clinical endpoint studies (CES) usually require a complex structure (randomized, double-blind, placebo-controlled, crossover and parallel grouped studies) and also possess a high number of enrolled subjects. Under these conditions, the evaluation of therapeutic equivalence is the most expensive part of TGP R&D programs and the one with more entailed risks, as some reference products possess modest efficacy. The economic burden of CES, combined with the typically low market price of the reference product, significantly limits the development of TGPs, as their chances of economic viability are considerably decreased. On the other hand, innovation in topical drug formulations for skin diseased has been lagged behind other pharmaceutical product classes. This makes topical products more vulnerable to price increases (Kwa *et al.*, 2020). A recent study by Kwa *et al.* has revealed that in the US, from 2011–2015, total spending on topical drugs has increased by 61%, whilst the number of units increased by only 18% (Kwa *et al.*, 2020). The authors examined the relationship between net changes in the number of therapeutic options and spending on prescription topical drugs. For this purpose, topical drugs were divided into several categories according to their therapeutic profile: analgesic/antipruritic, antibacterial, antifungal, antineoplastic, antiparasitic, antiseptic, antiviral, corticosteroids, retinoids, and other. For each drug class, the log of the ratio of total spending per unit in 2015 to total spending per unit in 2011 was modelled as linear function of the net number of topical therapeutic options over that time period (Kwa *et al.*, 2020). Interestingly, the classes with higher price disparities (topical corticosteroids and antifungals) involved categories with few therapeutic options. This study indicated that if the number of therapeutic options, especially generics, were to increase, an effective cost control strategy could be implemented and benefit the patients (Kwa *et al.*, 2020). Despite being a different economic perspectives, it clearly highlights a potential market opportunity.

Given the limitations associated with CES, surrogate methodologies have been explored. As discussed in the introductory chapter, these mainly rely on *in vitro* methodologies that allow a more sensitive and reproducible permeation profile assessment. Since the *stratum corneum*

is the primary limiting barrier to dermal absorption, the determination of the permeation profile closely mimics *in vivo* topical drug delivery. By doing so, these methods can be used as BE assessment tools for TGP. The development and validation of these techniques is a strategic area for generic drugs research, since by enabling faster submission and approval processes, an expansion of topical drug products with a generic version is more attainable.

Taking this background into account, several multidisciplinary initiatives, such as the Strawman Decision Tree, the Topical Drug Classification System and the FDA product-Specific Guidances for Generic Drug Development, aim to outline the principles that would substantiate the use of these *in vitro* surrogates. Even though these meetings and guidances provide insight on innovative regulatory approaches to be used for establishing therapeutic equivalence, they were only urged by the US-FDA. Moreover, their focus mainly involves semisolid ophthalmic preparations and topical acyclovir. In what concerns the European Medicine Agency, CES continues to be the accepted approach, even though some exceptions could be pointed out. These mainly regard bibliographic applications, which are not submitted through a centralized procedure and are increasingly becoming rarer, and some case studies, as reviewed on Chapter 1.

Motivated by these constraints in their daily practice, Laboratórios Basi® aim to generate more knowledge on the plausibility as well as on the validation procedures of these *in vitro* surrogate methods. To do so, Basi enrolled in a PhD company based program – Drug R&D – in partnership with the Faculty of Pharmacy of the University of Coimbra. Please note that on the beginning of this PhD, March 2017, EMA draft guideline had not been released yet, so the main goals of the investigation were:

- To develop by means of an analytical quality by design strategy, *in vitro* permeation and near infrared spectroscopy methods to assess topical drug permeation, using human skin as a biological subtract;
- To validate the proposed methods. These should provide enough sensitivity, robustness and discriminatory power in order to be able to (i) discriminate the permeation profiles of different topical semisolid dosage forms (gels, creams, ointments) and to (ii) assess the permeation impact of Q1/Q2/Q3 differences towards a specific reference product;
- To compare the obtained results with the regulatory approved approaches – pharmacodynamic and clinical endpoint studies;
- To implement the cited methods, on a routine basis, as a quality control and as a bioequivalence tool during R&D programs of TGP.

These goals had to be partially changed after the release of the EMA draft guideline. Even though this document represents a long-awaited advance on regulation concerning TGP development and approval, a careful analysis of the guideline clearly revealed some aspects that might undermine its successful translation into practice. These could be summarized into three key points: (i) The development of the methods used to support the extended pharmaceutical equivalence was not carefully detailed; (ii) Imposition of strict statistical criteria and (iii) Additional requirements for IVRT and IVPT studies, including a higher number of samples/donors, respectively.

Bearing in mind the constraints introduced by this guideline, some questions have been raised:

Do *in vitro* methodologies entail sufficient regulatory power to support *per se* BE assessment? Can these methodologies be regarded as a "stand-alone" surrogate method? If so, how can they be tailored and which premises are required for their development and validation?

Taking into account the updated regulatory background, the specific objectives of this thesis were outlined as follows:

- **To provide scientifically sound guidance for the development and validation of a rheology method.**

  A detailed rheological characterization of a semisolid dosage form provides information on product aesthetics properties, therapeutic effectiveness, patient compliance and overall quality / stability profile. Even though comprehensive rheological tests are thoughtfully addressed in the guideline, general development and validation procedures are not pointed out. In an attempt to surpass this limitation, specific rotational and oscillatory measurements, alongside with data analysis were carried out considering all the relevant components of a conventional analytical validation, including precision, discriminatory power and robustness. A 1% w/w hydrocortisone cream was used as a model product. Additional recommendations pointed out in the draft guideline were also addressed. To further highlight the applicability of the purposed strategy, the same rationale herein proposed, was transposed to a distinct case study – a clotrimazole 10 mg/g cream.

- **To Apply aQbD principles to the development of a IVRT test.**

  Even though regulatory recommendations addressing IVRT method validation are clearly defined, there is not a standardized methodology to support overall method development. According to a workshop promoted by the FDA, with the overgrowing regulatory importance of IVRT, the absence of method development protocols/reports is often appointed as one of the aspects that might impair submission and approval processes

related to TGP applications. Taking this information into account, this chapter aims to offer a robust and regulatory oriented platform for IVRT method development, based on aQbD principles. To do so, the IVRT analytical target profile was firstly traced, followed by a risk assessment analysis, which enabled the selection of both critical analytical attributes, as well as critical method variables. Several IVRT experiments were then performed, according to a full factorial design, in order to select the most suitable IVRT parameters. These were then fully validated according with EMA requirements. Similar to the present chapter, a critical and reflective assessment is presented here, focusing on the requirements of the new EMA draft guideline, especially those concerning IVRT method. In this chapter, a commercially available diclofenac emulgel formulation was used as a model product.

- **To discuss the statistical parameters presented in the EMA guideline regarding the extended pharmaceutical equivalence documentation.**

  According to the EMA guideline, a modular framework can be used to support BE of TGP. For a test product to be registered, the qualitative (Q1), quantitative (Q2), microstructure (Q3) and product performance (Q4) sameness must be supported. Strict regulatory limits are defined in the guideline, although their suitability has been subject of intense debate. In this context, this chapter aims to address these issues by characterizing a panel of 8 reference blockbuster semisolid topical products. For each product, three batches were selected and, whenever possible, batches retrieved from different manufacturing sites were considered. Product microstructure was evaluated in terms of globule size, pH, rheological attributes and, if required, the thermal behaviour was also assessed. Performance was evaluated through *in vitro* release testing (IVRT). Finally, an integrated multivariate analysis model was created to highlight the features that most contribute for product variability.

- **To present customized rationales according to formulation technological features, as well as therapeutic action site for BE establishment of TGP.**

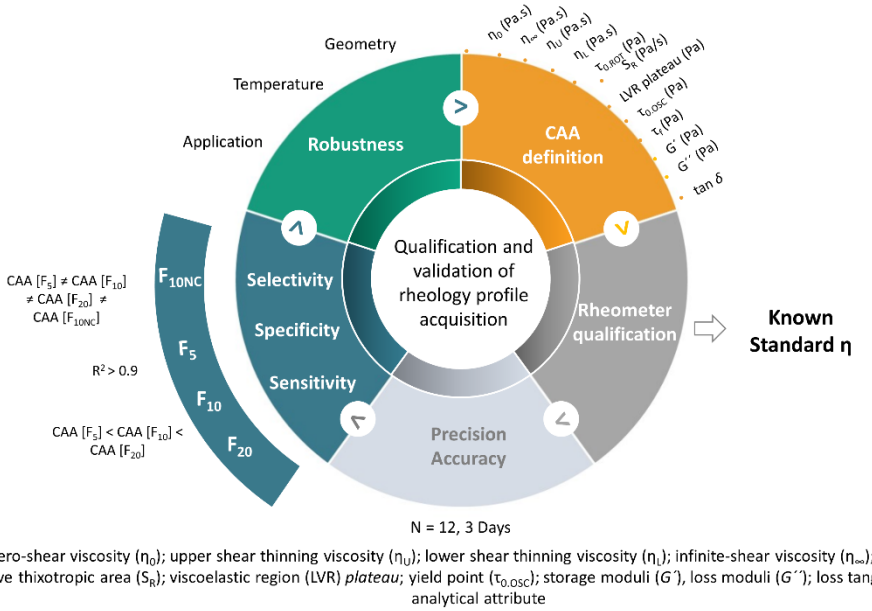
  Considering all the regulatory constraints referred in the previous chapters, it is essential to have a transposable strategy for BE documentation. Even though there are several literature reports addressing TP BE evaluation following the updated regulatory requirements, these mainly focus on acyclovir cream formulations, as this is a complex product where bioavailability is highly dependent on formulation characteristics. To broad the studied products panel, but also to address different challenges that might rise through a BE documentation process, three different case studies were considered: a dimethindene maleate 1 mg/g gel, a bifonazole 10 mg/g cream and a diclofenac 20 mg/g emulgel.

  According to the guideline, for simple formulations such as gels, BE may be demonstrated by documenting Q1-Q4 sameness. This scenario, however, tends to differ when addressing

more complex dosage forms, such as creams or emulgels. The structure arrangement, which directly influences product performance, becomes increasingly dependent on critical quality attribute features. In this context, equivalence regarding product local availability should also be demonstrated, alongside with the extended pharmaceutical equivalence. Reference products, test products and whenever possible generic products, were cross compared during rheology, release and permeation experiments. All methods were validated following the previously reported frameworks. In line with the preceding chapters, a critical evaluation of the regulatory limits (FDA/EMA), especially those pertaining to IVPT parameters, is presented.

- **To implement rheology, IVRT and IVPT methods on a routine basis, as a quality control and as a bioequivalence tool during R&D programs of TGP at Laboratórios Basi®.**

3 RHEOLOGY BY DESIGN: A REGULATORY TUTORIAL FOR ANALYTICAL METHOD VALIDATION



Key: zero-shear viscosity (η_0); upper shear thinning viscosity (η_U); lower shear thinning viscosity (η_L); infinite-shear viscosity (η_∞); viscosity (η); yield point ($\tau_{0.ROT}$); relative thixotropic area (S_R); viscoelastic region (LVR) *plateau*; yield point ($\tau_{0.OSC}$); storage moduli (G'), loss moduli (G''); loss tangent (*tan δ*); CAA: Critical analytical attribute

Fig.3.1 – Graphical abstract: Rheology by design: A regulatory tutorial for analytical method validation.

This chapter has been adapted from the following publication:

Simões, A.*, Miranda, M.*, Cardoso, C., Veiga, F., Vitorino, C., 2020. Rheology by Design: A Regulatory Tutorial for Analytical Method Validation. Pharmaceutics 12, 820. https://doi.org/10.3390/pharmaceutics12090820
*These authors contributed equally to this work and should be regarded as co-first authors.

A.S and M.M conceived the presented idea and established the research program and implementation. A.S and M.M performed the experimental parts of the work and wrote the first draft of the manuscript. Supervision, resources, data curation, review and editing was provided by C.V. Funding resources were provided by C.C, F.V. and C.V.

KEY CONCEPTS

- A **detailed rheological characterization** of a semisolid dosage form provides information on product aesthetics properties, therapeutic effectiveness, patient compliance and overall quality / stability profile.
- **Rheology attributes** play a central role on TP microstructure characterization. Therefore, according to EMA draft guideline, comparable rheological data should be presented to support Q3 equivalence within an abridged TGP BE referral.
- Despite the undeniable regulatory importance of rheology methods, there is not a **scientific driven framework** aiming the development and validation of such techniques.

3.1 Introduction

Topical semisolid dosage forms include complex multiphase systems demanding a detailed rheological characterization, since these properties may meaningfully affect quality and performance (Krishnaiah *et al.*, 2014; Lucia *et al.*, 2015). Rheology regards the study of the material flow and deformation behaviour and may be measured by applying an external force (shear-induced deformation) to a sample (Kelly *et al.*, 2018). Allied to formulation viscosity, elasticity and plasticity, rheological behaviour may impact product manufacturing, appearance, packaging, long-term stability, dispensing, sensory properties and the *in vivo* performance (Ethier *et al.*, 2019; van Heugten *et al.*, 2017). Therefore, rheology assessment proves to be an useful quality and stability indicator, revealing predictive information concerning batch variability, product aesthetic properties, therapeutic effectiveness and patient compliance (Ghica and Hîrj, 2016; Lucia *et al.*, 2015; Mangas-Sanjuán *et al.*, 2019). An enhanced understanding and control of rheology parameters is the basis for the sustainable development of new or abbreviated drug applications, meeting stakeholders' expectations (Lauterbach and Müller-Goymann, 2014).

Topical semisolid dosage forms predominantly exhibit a non-Newtonian behaviour, since a higher shear rate induces a viscosity decrease, which enables an easier skin application (Marto *et al.*, 2015). As such, a given critical stress value (yield stress) is required for the formulation to start to flow. Below this point, the products majorly present elastic properties; on the contrary, above this endpoint, the material predominantly displays a plastic flow (Namjoshi *et al.*, 2020).

Likewise, there are a plethora of rheology attributes which directly influence topical product microstructure and, consequently, impact several aspects. For instance, the formulation spreadability and bioadhesion to the skin are highly affected by viscoelastic properties. As patients directly apply topical formulations on their skin, these sensorial attributes are of

outmost importance to assure patient acceptability and, therefore, treatment compliance (Binder *et al.*, 2019; Namjoshi *et al.*, 2020; Simões *et al.*, 2019; Sivaraman *et al.*, 2017).

Stability and physical appearance are also dependent on rheological features. A detailed rheological characterization provides valuable insight on why products may settle or separate over shelf life. Furthermore, this tool can determine if there is a significant impact on product microstructure whenever dispensed from a packaging tube/dosing pump (Namjoshi *et al.*, 2020). Biopharmaceutical characteristics, such as drug release and permeation are also reliant on the formulation rheological profile (Sivaraman *et al.*, 2017; Soriano-Ruiz *et al.*, 2019). For all the above reasons, rheology behaviour is a key quality attribute within a target product profile of semisolid formulations (Ethier *et al.*, 2019; Hamed *et al.*, 2016; Namjoshi *et al.*, 2020).

Rheological characteristics are highly dependent on critical material attributes (CMAs) and critical process parameters (CPPs). Therefore, a close rheological monitoring can be a useful tool to guide and shorten product development, as well as to assure product quality and reduce batch variations during manufacturing (Ghica and Hîrj, 2016). This is in line with the pharmaceutical industry growing need to gain process understanding and improve product quality. These are the underlying principles of Quality by Design (QbD). This pharmaceutical regulatory concept is based on a systematic and risk-based approach, where the desired product quality profile is modulated through a detailed understanding of both raw materials and process parameters (ICH, 2009; Simões *et al.*, 2019, 2018a).

QbD methodology firstly involves the definition of the quality target product profile (QTPP) and critical quality attributes (CQAs), in which rheological characteristics should be a primary concern. Afterwards, through a detailed risk analysis, both CMAs and CPPs should be clearly identified. With these parameters well established, design of experiments (DoEs) should be performed in order to finally establish the design space, as well as a viable control strategy (ICH, 2009; Simões *et al.*, 2019, 2018a). This final step, is of outmost importance, since it warrants that the process is controlled and kept within the established design space (Henriques *et al.*, 2019). Measurements during manufacturing with process analytical technologies (PAT) can be integrated as a part of a control strategy. Even though PAT is increasingly applied in solid dosage forms, its application to semisolid systems manufacture is not yet seen as a common solution (Qwist *et al.*, 2019). Nevertheless, several authors have been exploring the potential of rheology as a PAT tool. Qwist *et al.* have developed a pressure difference apparatus, which can retrieve samples from the bulk intermediate/product stream in order to determine the storage moduli (G´) and the loss moduli (G’’), attained through the frequency sweep test, as well as the flow curve (Qwist *et al.*, 2019). Van Heugten and

colleagues have evaluated the filling temperature influence on an ointment yield stress. Based on the results, the authors were able to establish an optimal filling viscosity range, which, in turn, enabled a successful filling operation with minimal weight variation, and consequently, a product with the desired yield stress (van Heugten and Vromans, 2018).

By applying QbD principles, system variability can be understood and by doing so regulatory pathways concerning product development/manufacture can be more flexible. As part of this strategy, the application of rheology as a PAT tool can be helpful to improve formulation and manufacturing capabilities, by reducing product variability and batch rejection (Simões *et al.*, 2019, 2018a).

As outlined in the draft guideline on the quality and equivalence of topical products, a patient-focused approach should be envisioned while developing a product (EMA, 2018b). Therefore, as previously mentioned, aspects such as patient acceptability, highly influenced by rheological attributes, should be primary concerns when developing a product. This is valid for an innovator product, but it is also highly relevant when addressing a generic product.

As previously reviewed in the 2nd chapter, EMA draft guideline proposes, as an alternative to clinical endpoint studies, a modular framework for equivalence demonstration in topical generic products. Accordingly, for a product to apply, extended pharmaceutical equivalence criteria must be fulfilled: (i) qualitative, quantitative and microstructure sameness (Q1, Q2, Q3, respectively) towards the reference product; (ii) product performance (Q4) mainly supported by *in vitro* release testing; and, finally, (iii) if the test product regards a complex dosage form, equivalence regarding the efficacy profile should be supported through *in vitro* permeation or dermatopharmacokinetic studies (Pleguezuelos-Villa *et al.*, 2019). In this context, microstructure equivalence demonstration is a cornerstone for bioequivalence assessment of topical generic products.

There are multiple factors, broadly described in the literature, that influence microstructure, and wherein rheology attributes play an irrefutable role (Benson and Watkinson, 2012; Chang *et al.*, 2013b; Ethier *et al.*, 2019; Mangas-Sanjuán *et al.*, 2019; Pleguezuelos-Villa *et al.*, 2019). For this reason, EMA presents specific requirements concerning the rheological parameters that should be accomplished while describing the rheological behaviour of a given formulation (EMA, 2018b). These include: (i) a complete flow curve of shear stress (or viscosity) *vs.* shear rate; (ii) yield point values; (iii) linear viscoelastic response, (iv) storage and loss moduli *vs.* frequency/stress; and (v) thixotropic relative area. Even though FDA also requires the presentation of rheological endpoints, the list is not as exhaustive when comparing to the European agency.

However, and despite the existence of several literature reports concerning the applicability and overall importance of rheology, there is a lack of understanding and standardization regarding formal validation procedures of such technique. Neither the parameters that define semisolid rheology profile, nor their acceptance limits have so far been defined in the literature. Furthermore, crucial rheology parameters are not included as routine analysis when releasing new batches (Mangas-Sanjuán *et al.*, 2019). In this context, a widespread validation applied to all semisolid dosage forms should be provided, safeguarding that the developed rheology measurement methods have suitable discriminatory abilities to determine formulation "sameness" and also to detect formulation differences, which may affect clinical performance (Rath and Kanfer, 2020). Moreover, a detailed rheology profile, with mandatory quality parameters, should also be available.

Aiming to standardize the rheological methodology, whether for assisting quality control or even a potential PAT tool, a comprehensive characterization of the rheometer operational parameters that could impact the rheology profile was carried out. To this end, the assumptions of the analytical quality by design (aQbD), including risk assessment applied to rank the impact of critical method variables (CMV) over critical analytical attributes (CAA), were considered to systematically validate the operational ranges of the rheometer, the experimental setup and the rheology measurement methods for the acquisition of a suitable rheology profile.

Specific rotational and oscillatory measurements, alongside with data analysis were carried out considering all the relevant components of a conventional analytical validation, including precision, discriminatory power and robustness (CPMP/ICH/381/95, 2005). A 1% w/w hydrocortisone cream was used as a model product. Additional recommendations pointed out in the draft guideline on quality and equivalence of topical products were likewise addressed (EMA, 2018b).

3.2 Materials and Methods

3.2.1 Materials

Micronized hydrocortisone (HC) was kindly provided by Laboratórios Basi Indústria Farmacêutica S.A. (Mortágua, Portugal). Methyl parahydroxybenzoate and propyl parahydroxybenzoate were purchased from Alfa Aesar (Kandel, Germany). Kolliwax® GMS II (glycerol monostearate), Kolliwax® CA (cetyl alcohol), Kollicream® IPM (isopropyl myristate), and Dexpanthenol Ph. Eur. were kindly provided by BASF SE (Ludwigshafen, Germany). Stearic

acid was provided by Acorfarma distribuicion S.A. (Madrid, Spain). Triethanolamine was purchased from Panreac AppliChem (Darmstadt, Germany). Liquid paraffin was provided by LabChem Inc. (Zelienople, PA). Glycerol was purchased from VWR Chemicals (Leuven, Belgium). Water was purified (Millipore®) and filtered through a 0.22 mm nylon filter before use. A viscosity reference standard RT5000 (Fungilab, Spain) was used for rheometer qualification studies.

3.2.2 Methods

3.2.2.1 Preparation of HC Cream Formulations

HC o/w cream formulations were conventionally prepared resorting to an Ultra-Turrax X 10/25 (Ystral GmbH, Dottingen, Germany) equipment. Both continuous and dispersed phases were separately prepared and heated to 70°C (Simões *et al.*, 2020b, 2019). Afterwards, the active pharmaceutical ingredient was solubilized in the dispersed phase. Previous studies established the optimal experimental settings relating to rate, duration and temperature of the manufacturing process. After production, cream formulations were cooled down to room temperature. Batches of 0.5 Kg were considered. All samples were stored at 20-25°C.

In order to document the discriminatory power of the proposed rheological analysis, three formulations were prepared considering different concentration of glycerol monostearate: 5% (F_5), 10% (F_{10}) and 20% (F_{20}). This excipient bears a significant impact on product microstructure due to its thickening properties (Simões *et al.*, 2020b, 2019). Please note that F_{10} was considered as the reference formulation. Moreover, a forth formulation was prepared with 10% of glycerol monostearate, but considering a different homogenization rate during manufacture. This formulation will be further addressed as a F_{10} negative control (F_{10NC}).

3.2.2.2 Rheological characterization

The rheological profile of all products was investigated using a HAAKE MARS 60 6000 (Thermo Scientific, Karlsruhe, Germany) equipped with a peltier system as temperature control unit. The data was evaluated using the Haake RheoWin Data Manager software (Thermo Scientific, Karlsruhe, Germany). For every analysis, a sample hood was used to minimize temperature fluctuations.

Considering EMA recently published draft guideline on quality and equivalence of topical products, a complete rheological profile should include both rotational and oscillatory measurements (EMA, 2018b).

Rotational tests are sample destructive. The information retrieved from these measurements enables the assessment of small periodic deformations, which affect structural breakdown and/or rearrangement. Moreover, with these tests the ability of a material to recover can also be studied.

Oscillatory tests regard amplitude and frequency sweep tests. Generally, these measurements, due to the decreased shear stress applied, can be considered as non-destructive. Nevertheless, it should be pointed out that minor system perturbations can still occur during amplitude sweep tests. Oscillatory measurements aim to assess the material viscoelastic properties, while exposed to small-amplitude deformation forces (Simões *et al.*, 2020b). The following sections detail the main outputs of both methodologies.

Rotational measurements

Rotational tests were performed with a C35/2°/Ti cone geometry at 32°C. Approximately 0.3 g of formulation were placed on a lower plate TMP35 using a positive displacement syringe. A pre-set gap of 0.1 mm was considered.

A linear CS step test from 0.01 to 250 Pa was measured for 800 s, in order to trace the flow curve [$\eta = f(\tau)$]. To characterize the flow behaviour, the following responses, or critical analytical attributes (CAA), were determined: zero-shear viscosity (η_0), upper-shear thinning viscosity (η_U), lower-shear thinning viscosity (η_L), infinite-shear viscosity (η_∞) and yield point ($\tau_{0.ROT}$).

To evaluate the thixotropic behaviour, a CR ramp test was performed with a shear rate from 0.01 to 300 s^{-1} and down again to 0.01, during 300 s [$\tau = f(\dot{\gamma})$]. From this analysis, the thixotropic relative area (S_R) was calculated.

Oscillatory measurements

The viscoelastic properties were investigated using a P35/Ti plate geometry at 32°C. Approximately 1 g of the formulation was applied on a lower plate TMP35, using a syringe. An amplitude sweep test between 0.01 and 600 Pa at 1 Hz was firstly conducted to estimate the linear viscoelastic region (LVR) plateau, yield point ($\tau_{0.OSC}$) and flow point (τ_f). Afterwards, a frequency sweep analysis was conducted within the LVR plateau. The storage moduli (G'), loss moduli (G'') and loss tangent (tan δ) were calculated at 1 Hz.

3.2.2.3 Rheological method validation

The present chapter aimed to establish a practical and straightforward approach concerning the validation of a rheological analysis. In this context, following a traditional validation procedure, precision and robustness, alongside with sensitivity, specificity, selectivity (discriminatory power) were determined (EMA, 2018b, 2009). Please note that linearity was not considered a relevant parameter for the rheological method validation, since there is no inherent linearity within the acquisition of a rheological profile.

Risk assessment

According to prior knowledge, it was possible to extensively identify the analytical settings – CMV – which may pose a direct repercussion on rheological endpoints. To determine which of these parameters need to be further studied and controlled, an Ishikawa diagram was constructed, see Fig.3.2. In addition, a risk estimation matrix (REM) was carried out to rank the previously identified analytical conditions, see Table 3.1 (ICH, 2009; Simões *et al.*, 2018b).

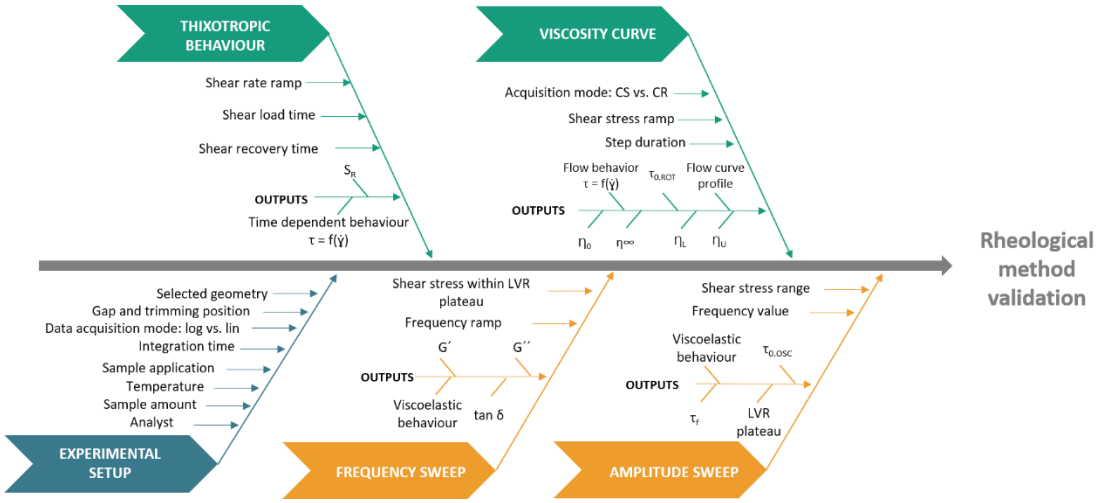


Fig.3.2 – Hypothetical Ishikawa diagram applied to the acquisition and validation of a rheology profile.

Table 3.1 – Initial risk estimation matrix (REM) for rheology method validation.

Test	Viscosity curve					Thixotropic behaviour	Amplitude Sweep			Frequency Sweep		
CAA	η_0	η_∞	η_U	η_L	$\tau_{0.ROT}$	S_R	LVR plateau	$\tau_{0.OSC}$	τ_f	G'	G''	Tan δ
CMV												
Geometry	M	M	M	M	M	M	M	M	M	M	M	L
Temperature	M	M	M	M	M	M	M	M	M	M	M	L
Sample application	M	M	M	M	M	M	M	M	M	M	M	L
Gap and trimming	L	L	L	L	L	L	L	L	L	L	L	L
Data acquisition mode	L	L	L	L	L	L	L	L	L	L	L	L
Integration time	M	M	M	M	M	M	M	M	M	M	M	L
Sample amount	M	M	M	M	M	M	M	M	M	M	M	L
Analyst	L	L	L	L	L	L	L	L	L	L	L	L
Shear stress ramp	H	H	H	H	H		H	H	H			
Step duration	M	M	M	M	M	M	M	M	M	M	M	L
Shear rate ramp						H						
Shear load time						H						
Shear recovery time						H						
Frequency value							M	M	M			
Shear stress within LVR plateau										H	H	L
Frequency ramp										M	M	L

Key: Low risk variable – Low; Medium risk variable – Medium; High risk variable – High.

Equipment qualification

Rheometer qualification was performed by determining the viscosity profile of a reference standard. Two temperatures were considered, 25°C and 32°C. The first one reported to the standard manufacturer specifications, whilst the second aimed to reproduce the previously reported method conditions.

Triplicate measurements were performed, on three different days, in order to evaluate method precision.

Precision

To test precision twelve rheological measurements for each test, were conducted, on three different days in order to comply with the updated EMA requirements. A RSD less than 15% was considered acceptable to validate the previously defined endpoints (FDA, 2016c; Mangas-Sanjuán *et al.*, 2019).

Discriminatory power

One of the most relevant steps during a validation procedure is the evaluation of the method discriminatory ability, i.e., the capacity of the method to discriminate between different formulations. To address so, the method sensitivity, specificity and selectivity, should be proven (EMA, 2018b, 2014a; FDA, 2016c).

The rheological profile of F_5, F_{10} and F_{20} was cross-compared. Furthermore, F_{10NC} rheological profile was determined as an additional discriminatory element. By tracing the rheological profile of such formulations, the discriminatory ability of the method can be sustained, since microstructure differences are highly sensitive to changes in excipient concentration and manufacturing process (EMA, 2018b; Ethier *et al.*, 2019; Ili and Daniels, 2017; Mezger, 2010; Pleguezuelos-Villa *et al.*, 2019). In this context, the sensitivity of the rheological methods was validated by evaluating the CAA response to changes in the concentration of glycerol monostearate. If the CAA obtained with F_5 were lower than F_{10}, and if the F_{20} CAA mean was superior when compared to F_{10}, the rheological methods are considered sensitive.

On the other hand, the specificity of the method was evaluated by assessing whether the considered CAA of F_5, F_{10} and F_{20} were able to successfully reflect the different glycerol monostearate content. A linear regression model of the CAA as dependent variable by the thickener concentration was used to estimate correlation coefficient (R^2). The method was considered to be specific if the R^2 was larger than 0.9 (FDA, 2016c; Tiffner *et al.*, 2018). The method selectivity was documented statistically. Pairwise comparisons between the reference formulation (F_{10}), and the specifically manufactured formulations F_5, F_{20} and F_{10NC} were conducted. The differences between the means were considered to be significant for values of $p<0.05$. If the CAA of each formulation showed significant differences, the method was considered to be selective. For the discriminatory capacity studies, six replicates per formulation, were set forth for each rheological measurement.

Robustness

To evaluate the method robustness, the impact of three different experimental setups was assessed. These included temperature fluctuations (+2ºC and -2ºC), sample application (positive displacement syringe *vs.* spatula) and finally, geometry impact. For rotational studies, the performance of a C35/2º/Ti cone - TMP35 plate (C35-P35) configuration was compared to a P35/Ti plate - TMP35 plate (P35-P35) configuration. For oscillatory measurements, the impact of P35/Ti plate - TMP35 plate (P35-P35) configuration was compared to P20/Ti plate -

TMP20 plate (P20-P20) configuration. The method was considered to be robust, if the CAA did not deviate by more than 15% from the mean CAA, at nominal method parameter settings.

3.2.2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software (San Diego, CA) by applying a one-way ANOVA with Tukey multiple comparison test. Differences among mean values were considered statistically significant when $p<0.05$.

3.3 Results and Discussion

3.3.1 Rheological method validation

In the quest of a standardized procedure to assess the rheology profile of topical dosage forms, and underlying the aQbD principles, CMV and CAA were previously identified and their impact crosswise assessed, based on the pillars of method validation. Results are discussed in the sections that follow.

3.3.1.1 HC cream rheological characterization

As displayed in Fig.3.3, all formulations exhibited a non-Newtonian, and shear thinning behaviour with a consistent decrease in apparent viscosity while increasing the shear stress. The acquired rheograms clearly show three distinct regions: (A) 1st Newtonian range with a plateau value corresponding to the zero-shear viscosity (η_0); (B) shear-thinning range with shear stress-dependent viscosity function $\eta=f(\tau)$ and (C) 2nd Newtonian range with the plateau value corresponding to the infinite-shear viscosity (η_∞). The η_0 depicts a formulation viscosity towards an infinitely low-shear rate, close to zero, whereas η_∞ represents a formulation viscosity towards an infinitely high-shear rate (Kim *et al.*, 2003; Mezger, 2010). The upper (η_U) and lower (η_L) shear-thinning viscosities were also considered. These CAAs encompass the initial and final borderline viscosity values of the shear-thinning range.

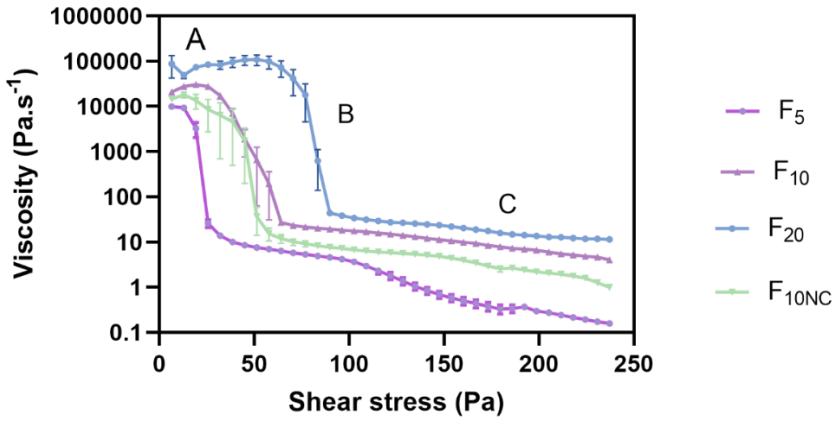


Fig.3.3 – Effect of glycerol monostearate content (F_5, F_{10} and F_{20}) and homogenization rate (F_{10NC}) on formulation viscosity curve. A: 1st Newtonian plateau; B: Shear-thinning range; C: 2nd Newtonian plateau. Results report to a 6<n<12.

Formulation viscosity provides a useful information on the release of the active substance from the vehicle. In highly viscous systems, drug release is hampered, affecting its bioavailability and inherent therapeutic effectiveness (Lucia *et al.*, 2015). Moreover, viscosity results can also shed light on formulation resistance to structure breakdown (Ribeiro *et al.*, 2004). Besides the impact on product performance and stability, this CAA also determines formulation appearance, spreadability and retention at the application site, fundamental aspects for patient compliance (Clares-Naveros *et al.*, 2019).

The viscosity curves of all formulations displayed a specific yield point ($\tau_{0.ROT}$). This CAA was estimated from rotational measurements via tangent crossover method. τ_0 is known as the minimum shear stress required to initiate material flow or the stress below which a material exhibit gel-like and elastic behaviour. Beyond τ_0, cream microstructure changes, causing elasticity loss and the flowing of the sample. Formulations with raised τ_0 consisted on more structured network systems and with increased viscosity, offering higher resistance to external deformation forces (Ribeiro *et al.*, 2004). These reasons reinforce the suitability of yield point as a stability indicator CAA. Furthermore, τ_0 values of pharmaceutical products should be high enough to avoid material flow out of a container when the container is placed in an upside-down position. However, it should not be so large that it offers resistance to flow when spread over the skin (de Souza Mendes, 2009; Mahdi *et al.*, 2011). Spreadability is a critical sensory property highly dependent on formulations τ_0 (Ili and Daniels, 2017). Thereby, this CAA is likewise an essential element for patient acceptance.

The flow curves (Fig.3.4) enabled the classification of all formulations as thixotropic systems, since hysteresis loop areas were promptly observed with the rising curves located above the

return curves. Thixotropy is a reversible phenomenon exhibited by non-Newtonian materials, characterized by a reduction in the apparent viscosity when the material is subjected to a constant shear rate (deformation), which returns to its viscosity and initial structure, when the shear rate is ceased (recovery) (Głowińska and Datta, 2014).

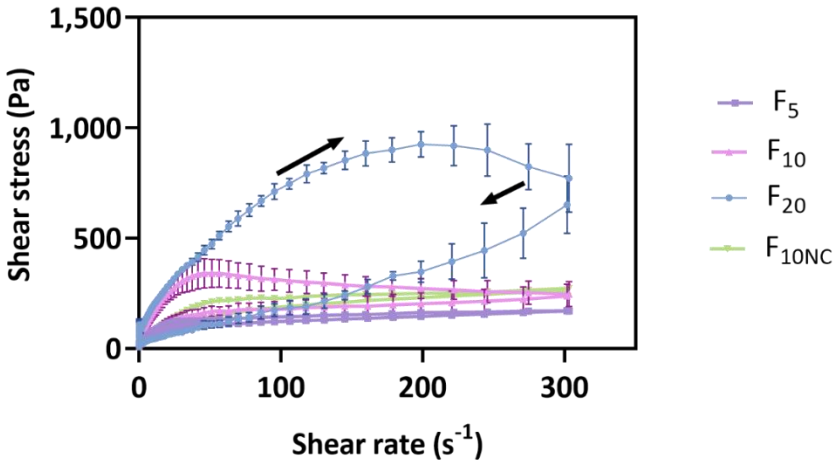


Fig.3.4 – Effect of glycerol monostearate content (F_5, F_{10} and F_{20}) and homogenization rate (F_{10NC}) on formulation thixotropic relative area.

From data analysis, it is possible to observe that the glycerol monostearate content significantly affects cream microstructure, with flow curves displaying different thixotropic relative areas (S_R). More structured systems required more time to rebuild he damaged bonds. Such changes are attributed to structure rearrangements that involve rupture and subsequent reformation of weak bonds (Mewis and Wagner, 2009). Besides the impact on product performance, thixotropy also contributes to an easy formulation spreadability at the application site, fundamental for patient acceptance and compliance (Ghica and Hîrj, 2016; Tadros, 2010). Moreover, during shelf-life, cream formulations undergo repeated shear forces when extruded from the container. Hence, to guarantee physical stability against breakdown, microstructure recovery must be ensured through a thixotropic behaviour (Mezger, 2010). For that reason, this CAA is also a good stability indicator.

Regarding the formulation amplitude sweep behaviour (Fig.3.5), a linear viscoelastic region (LVR) was likewise observed. The LVR is a constant plateau where storage moduli (G') or loss moduli (G'') values are independent of the strain and only correlated with molecular structure. Within LVR, microstructure is maintained intact and any disruption will be instantaneously recovered (Hamed *et al.*, 2016; Pisal *et al.*, 2013). All formulations exhibited a well-established yield point ($\tau_{0.OSC}$) and flow point (τ_f) values. Similar to $\tau_{0.ROT}$, $\tau_{0.OSC}$ is defined as the minimum shear stress that must be applied to induce material flow. However, this CAA corresponds to

the shear stress value detected at the end of LVR plateau, obtained through oscillatory measurements.

Beyond $\tau_{0.OSC}$, a deviation from LVR is observed with G' decreasing while G'' simultaneously increasing until τ_f (Li *et al.*, 2011; Nguyen *et al.*, 2015). τ_f is an important rheology property which corresponds to the shear stress value where the moduli crossover (G'= G'') occurs. τ_f can be considered as the borderline between the (gel = solid like) and the fluid = liquid-like state. Before τ_f, G´ is higher than G´´, suggesting that the system predominantly exhibits elastic properties. Nevertheless, if surpassing this point, the prevalence of G´´ over G´ suggests a more viscous system. Any microstructure disturbance after τ_f will then produce irreversible deformations in the gel network structure (Mezger, 2010).

Rheological data suggested that more structured and viscous formulations offer more resistance to deformation forces, which is denoted by higher LVR plateau, τ_0 and τ_f results (Nguyen *et al.*, 2017). Similar to the τ_0 response, LVR plateau and τ_f are also important stability references.

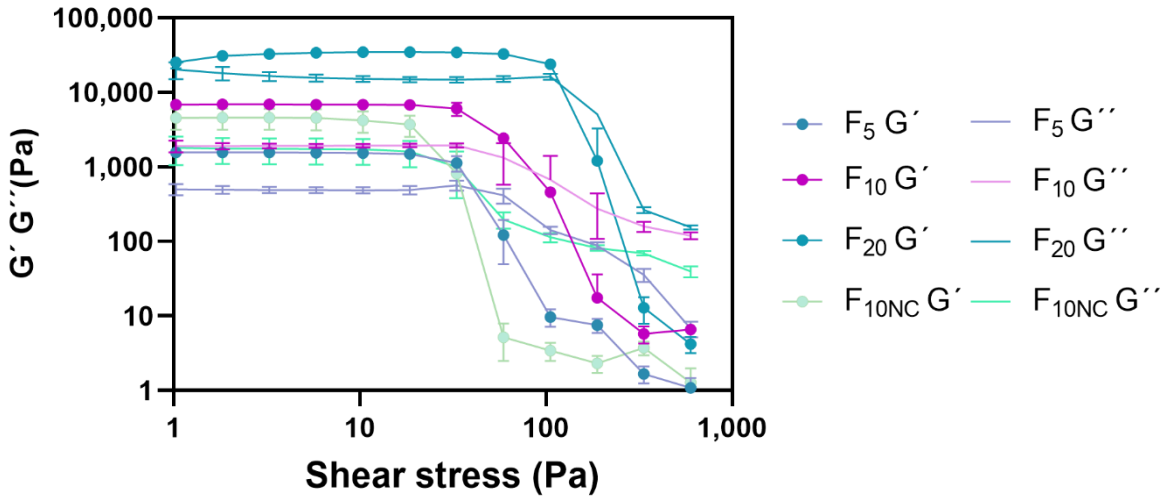


Fig.3.5 – Effect of glycerol monostearate content (F_5, F_{10} and F_{20}) and homogenization rate (F_{10NC}) on the formulation amplitude sweep.

Regarding frequency sweep profile (Fig.3.6), the four formulations exhibited a dominance of the storage moduli (G') over the loss moduli (G''). When the material displays a more viscous behaviour, a G'<G'' tendency is observed; conversely, when the elastic properties of a material prevail, G' > G'' (Li *et al.*, 2020). Accordingly, the HC cream herein under evaluation can be considered as an essentially viscoelastic system, being its microstructure dominated by a gel network structure (Li *et al.*, 2011). Viscoelastic materials combine two different characteristics: the viscous and the elastic. The first one, implies that they deform slowly when

exposed to external forces (G´ < G´´). The term "elastic" implies that once a deforming force has been removed the material will return to its original structure (G´> G´´) (Mezger, 2010). By other words, during the deformation process, the prevalence of elastic properties also determines a more stable microstructure, since reversible deformations (G') overcome the irreversible ones (G'') (Mezger, 2010). Besides physical stability, formulation spreadability, drug release and skin bioadhesion, can be impacted by viscoelastic properties (Batheja *et al.*, 2011; Jones *et al.*, 2009).

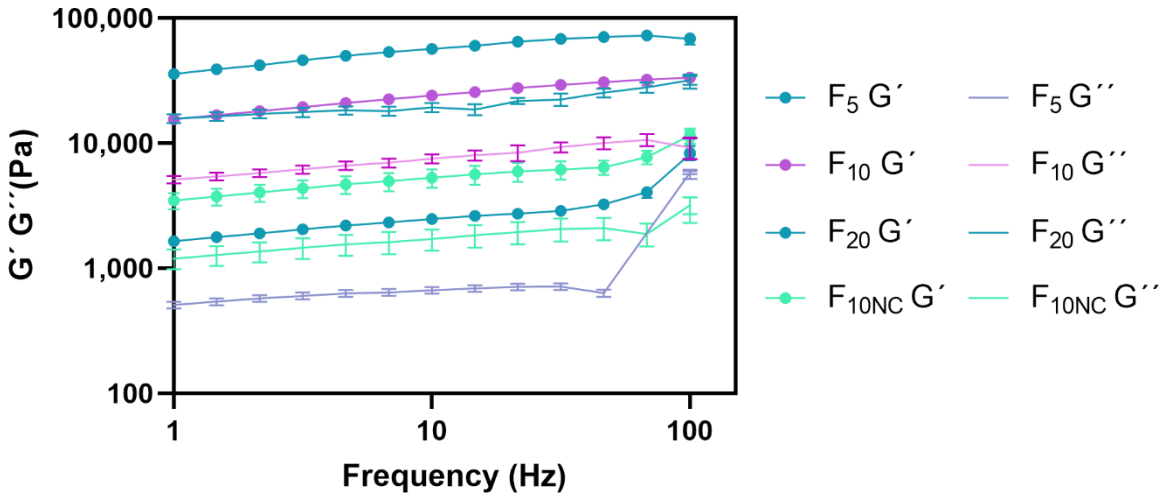


Fig.3.6 – Effect of glycerol monostearate content (F_5, F_{10} and F_{20}) and homogenization rate (F_{10NC}) on the formulation frequency sweep.

The loss tangent (tan δ) was also considered. Tan δ is a dimensionless term that describes the ratio between G'' and G'. This endpoint is useful to elicit information regarding system structure. When tan δ<1 (G''<G'), elastic properties and gel-like or solid state dominate; when tan δ>1 (G''>G'), viscous properties and a liquid-like or fluid state prevail; when tan δ=1 (G''=G'), τ_f is achieved (Jones *et al.*, 2001). For all the formulations, a tan(δ) close to zero was observed, confirming the gel-like state and elastic properties prevalence. Besides the effect on product performance, those CAAs are important stability indicators with meaningful impact on patient compliance.

3.3.1.2 Equipment qualification

Equipment qualification studies were firstly performed to investigate and compare a Newtonian standard flow curve profile to its manufacturer specifications. Viscosity values were provided for two different temperatures – 20°C and 25°C.

To determine the standard viscosity at 32°C, Andrade equation can be employed, see Equation 3.1.

$$\eta = De^{\frac{B}{T}} \quad (3.1)$$

where D and B correspond to empirical constants and T to the absolute temperature.

Afterwards, the resulting equation provides a close approximation of viscosity as a function of temperature (Goodrum *et al.*, 2002; Wong *et al.*, 2016). Through this model, it was possible to determine the theoretical viscosity of the standard sample at 32ºC – the selected temperature for this study.

In order to provide a reliable strategy for qualification studies, the standard sample viscosity was determined at two different temperatures. Firstly, at 25ºC to directly compare with the manufacturer specifications, and then at 32ºC. The obtained viscosity at 32ºC was then cross-compared with the theoretical value calculated through Eq. 3.1.

Acceptance criteria and interday results of equipment qualification studies are summarized in Table 3.2.

Table 3.2. – Predefined acceptance criteria and results for equipment qualification tests. Interday results report to an n=3 performed on three consecutive days. A Newtonian standard with known viscosity was used as reference.

	CAA	Acceptance criteria	Results	Status
Standard 25ºC	η (Pa.s)	4.984 Precision (RSD) < 15% Accuracy (Bias) < 15%	Mean = 5.27 ± 0.14 RSD = 2.67% Bias = 5.74%	C
Standard 32ºC		4.360 Precision (RSD) < 15% Accuracy (Bias) < 15%	Mean = 4.8 ± 0.2 RSD = 4.17% Bias = 10.09%	C

Key: Shear viscosity (η); Compliant (C); Non-compliant (NC).

Since the standard presents a Newtonian behaviour, some of the rheological endpoints previously reported for hydrocortisone cream are not applicable. The viscosity of a Newtonian sample is independent of both degree and duration of the applied shear stress, therefore infinite-shear viscosity, lower and upper-shear viscosity all share the same value. Furthermore, according to the same rationale, no yield point is verified (Mezger, 2010; Soriano-Ruiz *et al.*, 2019).

Viscosity results at 25ºC comply with the manufacturer specifications. Likewise, the theoretical viscosity at 32ºC, estimated through the Andrade equation, is also compliant with the experimentally determined values. As expected, higher temperatures led to a decrease in

viscosity. This is mainly related with an increase of the molecular kinetic energy alongside with the attenuation of weak intermolecular attractions (London dispersion forces). Both occurrences stimulate a molecular realignment in the direction of shear, thus decreasing viscosity (Wong *et al.*, 2016). Viscosity results for both temperatures meet the inter-day specification, therefore the equipment proved to be compliant.

3.3.1.3 Precision

To address the method precision, an n=12 was considered during three independent days to evaluate both intraday and interday variability. Results are displayed in Table 3.3.

Table 3.3 – Acceptance criteria and results of precision evaluation. Results report to an n=12.

CAA	Results					
	Acceptance criteria	Intraday variability		Interday variability		Status
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	
η_0 (Pa.s)	[CAA±15%]	26293 ± 6538	24.87	26338 ± 7474	28.38	NC
η_∞ (Pa.s)		17.3 ± 1.5	8.85	17.3 ± 1.6	9.28	C
η_U (Pa.s)		23277 ± 7231	31.06	23277 ± 8168	35.09	NC
η_L (Pa.s)		40.8 ± 5.8	14.14	40.8 ± 6.1	14.83	C
$\tau_{0.ROT}$ (Pa)		27.2 ± 1.7	6.36	27.2 ± 1.8	6.71	C
S_R (Pa/s)		25041 ± 2548	10.17	24576 ± 3238	13.17	C
LVR *plateau* (Pa)		6649 ± 454	6.83	6659 ± 492	7.38	C
$\tau_{0.OSC}$ (Pa)		34.6 ± 4.5	13.00	34.7 ± 4.6	13.38	C
τ_f (Pa)		76.6 ± 5.3	6.88	76.9 ± 6.4	8.30	C
G' (Pa)		6867 ± 484	7.05	6853 ±634	9.25	C
G'' (Pa)		1942 ± 148	7.63	1941 ± 184	9.49	C
Tan δ		0.28 ± 0.02	5.57	0.28 ± 0.02	6.87	C

Key: zero-shear viscosity – η_0; upper shear thinning viscosity – η_U; lower shear thinning viscosity – η_L; infinite-shear viscosity –η_∞; yield point – $\tau_{0.ROT}$; relative thixotropic area – S_R; viscoelastic region – LVR plateau; yield point – $\tau_{0.OSC}$; storage moduli – G´; loss moduli – G''; loss tangent – Tan δ; Compliant – C; Non-compliant – NC.

The majority of the CAA displayed compliant results concerning both intra and interday evaluations, thus reinforcing the suitability of the proposed methods. Nevertheless, two variables presented high and non-compliant precision results: η_0 and η_U.

The main reason that supports this occurrence mainly relates with the non-Newtonian behaviour of the hydrocortisone cream. As previously explained in 3.3.1.1, to warrant a detailed characterization of the flow curve, the acquisition of 3 different segments, 1st Newtonian plateau, shear thinning region and 2nd Newtonian plateau, were actively pursuit. Both η_0 and η_U are retrieved from the first segment of the flow curve. The first endpoint concerns viscosity values at an "infinitely low" shear rate, whilst the second one concerns the

viscosity registered prior to the shear thinning behaviour, which occurs at medium shear rates (Mezger, 2010).

During the 1st Newtonian plateau, at low shear rates, some sample macromolecules start to lean into a given shear direction. For some of them, this induces partial disentanglements. Consequently, a viscosity decrease is denoted in these parts of the sample. Nevertheless, due to the sample intrinsic viscoelastic behaviour, some other macromolecules, which were already oriented and disentangled, start to recoil and re-entangle all over again, thus inducing a viscosity increase. As a result, during this segment of the viscosity curve, the sum of the partial orientations and re-coilings with the sum of all disentanglements and re-entanglements, results in no significant changes in viscosity (Mezger, 2010). However, these interactions are difficult to replicate, thus explaining the high RSD values, which are not registered in the other rotational endpoints, such as infinite-shear viscosity, lower-shear thinning viscosity, rotational yield point and relative thixotropic area. Regarding oscillatory measurements, all the selected CAA demonstrated to be precise in both intraday and interday levels.

Even though the majority of the CAA proved compliance with the previously established criteria (RSD<15%), which are in agreement with FDA guidelines, a critical evaluation should be made (FDA, 2016c). If the updated EMA criteria (RSD<10%) was to be followed, three CAA would display non-compliant results (η_L, S_R and $\tau_{0.OSC}$) (EMA, 2018b).

Similar results were attained by Victor Mangas-Sanjuán and collaborators (Mangas-Sanjuán *et al.*, 2019). The authors performed a comprehensive rheological analysis of 10 different batches of a reference ointment containing calcipotriol and betamethasone. The selected endpoints were: relative thixotropic area, rotational yield stress, zero-shear viscosity, viscosity at 100 s^{-1}, loss tangent, elastic and viscous moduli at 1 Hz, and finally m' and m" which regard fit and spreadability parameters. The authors evidenced high intra-batch variability in relative thixotropic area and zero-shear viscosity, which were also registered in the present work. Moreover, variability in both elastic and viscous moduli at 1 Hz was also presented. Through different batches comparison, the authors were able to draw several conclusions: (i) some endpoints do not follow a normal distribution and, therefore, do not qualify for comparison according to the EMA criteria; (ii) if a parametric evaluation is performed for low inter-batch variability endpoints EMA criteria can be successfully applied. Nevertheless, endpoints which display high inter-batch variability, equivalence cannot be supported. In conclusion, this work was able to support that a CV of 10% is too strict to conclude equivalence regarding the rheology profile of topical semisolid drug products. In order to promote a practical applicability of the extended pharmaceutical equivalence concept, as desired in the European

draft guideline, it is imperative to establish wider criteria based on the variability of the product being studied.

3.3.1.4 Discriminatory power

A solid documentation of the method discriminatory ability is progressively being demanded by the regulatory authorities, in order to prove that the methods are able to assure a critical distinction among samples.

Even though comprehensive reports addressing the evaluation of this validation component for *in vitro* release (IVRT) and *in vitro* permeation methods (IVPT) can be found in the literature, the scenario is slightly different when considering rheology methods (EMA, 2018b; Mudyahoto *et al.*, 2020; Tiffner *et al.*, 2018). However, since these methods play a central role during semisolid microstructure characterization, the development of a scientific driven platform able to sustain their discriminatory capacity, could be beneficial in regulatory terms. This fact has been extensively discussed in Skin forum (Reims, September 2019) and in EUFEPS Open Forum Discussion on the Draft Guideline on Quality and Equivalence of Topical Products (Bonn, June 2019).

To document the discriminatory power of a method, three concepts should be addressed: sensitivity, specificity and selectivity (FDA, 2016c). For IVRT and IVPT, different strength formulations can be tested to evaluate these concepts. If the methods are able to reflect distinct and proportional *in vitro* release rate (IVRT) or alternatively, maximal rate of absorption (IVPT), the discriminatory power of both methods is adequately supported. The same rationale can be transposed to rheology methods.

An in-depth formulation knowledge is required to design appropriate and complete validation procedures able to assess the sensitivity, specificity and selectivity. As previously mentioned, two contributions should be mainly accounted for: the impact of the quantitative profile (CMA) and also the influence of critical production parameters (CPP). According to prior knowledge from our group, formulation impact was assessed by varying glycerol monostearate content, since due to its thickening properties, this excipient highly impacts hydrocortisone cream microstructure. Regarding CPP, the homogenization rate proved to be a highly influent CPP and was for this reason selected (Simões *et al.*, 2019, 2018b).

Discriminatory power results are summarized in Table 3.4.

Table 3.4 – Acceptance criteria and results of discriminatory power evaluation. Results report to mean ± SD. A An $6 \leq n \leq 36$ was used.

	Sensitivity						Specificity			Selectivity	
CAA	Results				Acceptance criteria	Status	Results	Acceptance criteria	Status	Acceptance criteria	Status
	F_5	F_{10}	F_{20}	F_{10NC}							
	Mean ± SD						R^2				
η_0 (Pa.s)	8600 ± 2409	26338 ± 7474	62870 ± 6630	19785 ± 6121	CAA [F_5] < CAA [F_{10}] < CAA [F_{20}]	C	1.000	R^2>0.9	C	CAA [F_5] ≠ CAA [F_{10}] ≠ CAA [F_{20}] ≠ CAA [F_{10NC}]	C
η_∞ (Pa.s)	3.57 ± 0.56	17.3 ± 1.6	17.3 ± 0.4	6.96 ± 0.55		C	0.972		C		C
η_U (Pa.s)	6422 ± 553	23278 ± 8168	69250 ± 5260	12815 ± 5969		C	0.995		C		C
η_L (Pa.s)	18.4 ± 2.3	40.8 ± 6.1	56.2 ± 3.4	32.2 ± 4.9		C	0.914		C		C
$\tau_{0.ROT}$ (Pa)	10.8 ± 0.1	27.2 ± 1.8	55.2 ± 7.4	10.7 ± 0.05		C	0.998		C		C
S_R (Pa/s)	5006 ± 325	24576 ± 3228	136625 ± 9419	9062 ± 1195		C	0.962		C		C
LVR plateau (Pa)	1636 ± 106	665 ± 491	33721 ± 2446	4081 ± 900		C	0.965		C		C
$\tau_{0.OSC}$ (Pa)	16 ± 2.2	34.7 ± 4.6	67.9 ± 5.4	10.9 ± 0.2		C	0.999		C		C
τ_f (Pa)	44.4 ± 1.9	76.9 ± 6.4	124 ± 10	26.4 ± 3.5		C	0.993		C		C
G' (Pa)	1649 ± 97	6853 ± 634	35787 ± 634	3419 ± 487		C	0.964		C		C
G'' (Pa)	509 ± 35	1941 ± 184	15739 ± 184	1169 ± 202		C	0.940		C		C
Tan δ	0.23 ± 0.13	0.28 ± 0.02	0.44 ± 0.02	0.28 ± 0.12		C	0.991		C		C

Key: Compliant – C; Non-compliant – NC.

Sensitivity evaluation showed that the four rheological methods – CS step test, thixotropy, amplitude and frequency sweep were able to distinguish the three formulation with different glycerol monostearate content. The reference formulation (F_{10}) CAA presented higher values whenever compared with F_5 CAA, and as expected, with increasing thickener concentrations (F_{20}), all CAA displayed a higher response, see Table 3.4. For this reason, sensitivity was established.

Rotational and oscillatory methods were also able to successfully establish a linear relationship between thickener concentration and all CAA, thus documenting the method specificity. The determination coefficients for all endpoints were mostly in the range of (0.914 - 1), indicating a good fitting, see Table 3.4 and Fig.3.7.

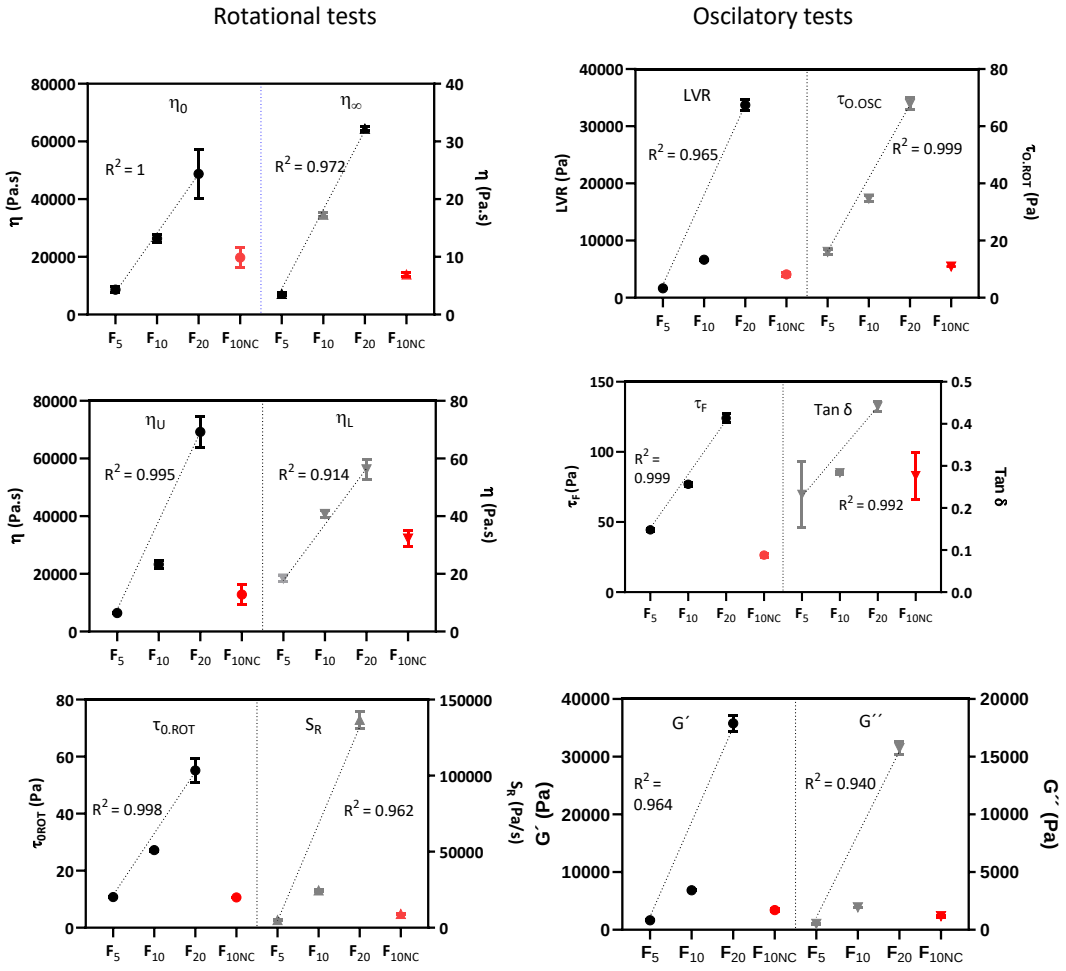


Fig.3.7 – Specificity results. Results are expressed as mean ± SEM. A 6≤n≤36 was used.

To evaluate selectivity, the ability of the methods to accurately identify distinct formulations, three pairwise statistical comparisons were performed: (i) F_{10} *vs.* F_5; (ii) F_{10} *vs.* F_{20} and; (iii) F_{10} *vs.* F_{10NC}. The results, summarized in Table 3.5, demonstrate that for most comparisons

low *p*-values are attained, suggesting that there are significant differences among the formulations.

There were, however, non-compliant results observed for tan δ comparison between F_{10} and F_5 (*p* value = 0.4165). Notwithstanding, since this parameter regards the ratio between G'' and G', and these two CAA display significant differences between F_{10} - F_5. Therefore, this punctual lack of compliance does not undermine overall selectivity results.

Table 3.5 – Selectivity results. A one-way analysis of variance (ANOVA) with a Tukey multiple comparison test was performed. The differences between the means were considered to be significant for values of $p<0.05$.

CAA	F_{10} *vs.* F_5	F_{10} *vs.* F_{20}	F_{10} *vs.* $F_{10.NC}$
η_0 (Pa.s)	Normal distribution? Yes. CI: [-27445 – -8031] p value: < 0.0001	Normal distribution? Yes. CI: [-34029 – -10854] p value: < 0.0001	Normal distribution? Yes. CI: [-5034 – 18141] p value: 0.4403
η_∞ (Pa.s)	Normal distribution? Yes. CI: [-15.49 – -12.03] p value: < 0.0001	Normal distribution? Yes. CI: [-17.23 – -12.52] p value: < 0.0001	Normal distribution? Yes. CI: [8.311 – 12.44] p value: < 0.0001
η_U (Pa.s)	Normal distribution? Yes. CI: [-26774 – -6936] p value: 0.0003	Normal distribution? Yes. CI: [-61072 – - 30873] p value: < 0.0001	Normal distribution? Yes. CI: [-492 – 21417] p value: 0.0659
η_L (Pa.s)	Normal distribution? Yes. CI: [-29.36 – -15.52] p value: < 0.0001	Normal distribution? Yes. CI: [-26.67 – 4.147] p value: 0.0040	Normal distribution? Yes. CI: [0.3611 – 16.81] p value: 0.0380
$\tau_{0.ROT}$ (Pa)	Normal distribution? Yes. CI: [-21.04 – -11.90] p value: < 0.0001	Normal distribution? Yes. CI: [-31.92 – -23.91] p value: < 0.0001	Normal distribution? Yes. CI: [12.01 – 21.15] p value: < 0.0001
S_R (Pa/s)	Normal distribution? Yes. CI: [-25597 – -13543] p value: < 0.0001	Normal distribution? Yes. CI: [-118645 – -105452] p value: < 0.0001	Normal distribution? Yes. CI: [9487 – 21541] p value: < 0.0001
LVR plateau (Pa)	Normal distribution? Yes. CI: [-6224 – -3821] p value: < 0.0001	Normal distribution? Yes. CI: [-28200 – -25925] p value: < 0.0001	Normal distribution? Yes. CI: [1376 – 3779] p value: < 0.0001
$\tau_{0.OSC}$ (Pa)	Normal distribution? Yes. CI: [-23.67 – -13.6] p value: < 0.0001	Normal distribution? Yes. CI: [-37.97 – -28.41] p value: < 0.0001	Normal distribution? Yes. CI: [18.41 – 29.11] p value:< 0.0001
τ_f (Pa)	Normal distribution? No. CI: [-40.5 – -24.55] p value: < 0.0001	Normal distribution? No. CI: [-54.46 – -39.88] p value: < 0.0001	Normal distribution? No. CI: [42.52 – 58.49] p value: < 0.0001
G' (Pa)	Normal distribution? Yes. CI: [-6958 – -3451] p value: < 0.0001	Normal distribution? Yes. CI: [-30402 – -27466] p value: < 0.0001	Normal distribution? Yes. CI: [1966 – 4902] p value: < 0.0001
G'' (Pa)	Normal distribution? Yes. CI: [-2120 – -745.8] p value: < 0.0001	Normal distribution? Yes. CI: [-14373 – -13223] p value: < 0.0001	Normal distribution? Yes. CI: [197.6 – 1348] p value: < 0.0044
Tan δ	Normal distribution? Yes. CI: [-0.1384 – 0.03657] p value: 0.4165	Normal distribution? Yes. CI: [-0.2315 – -0.08499] p value: < 0.0001	Normal distribution? Yes. CI: [-0.06649 – 0.08001] p value: 0.9947

As previously mentioned, to further challenge the method selectivity, a fourth formulation (F_{10NC}) was manufactured to serve as a negative control for the reference formulation. The sole purpose was to assess if the methods were able to distinguish formulations obtained considering a different CPP. As displayed in Table 3.4 and Fig.3.7, the mean CAA values obtained for F_{10NC} are smaller when compared to the reference formulation, reinforcing the predominant impact of the homogenization rate in hydrocortisone cream rheology behaviour. Generally, all CAA displayed statistically different results when comparing F_{10} with F_{10NC}, therefore selectivity is overall adequately demonstrated. Nevertheless, η_0, η_U and tan δ lack to report a statistical difference since high *p*-values were reported, see Table 3.5.

3.3.1.5 Robustness

Method robustness was evaluated by assessing the impact of minor fluctuations in temperature, geometry and sample application. An important outcome of the robustness analysis is to establish appropriate analytical parameters to ensure method validity (ICH, 2005).

Table 3.6 – Acceptance criteria and results of robustness evaluation for the optimal formulation. Results report to mean ± SD. An n=12 was used.

	TEMPERATURE					GEOMETRY					APPLICATION				
CAA	**Acceptance criteria**	**Condition**	**Mean ± SD**	**RSD (%)**	**Status**	**Acceptance criteria**	**Condition**	**Mean ± SD**	**RSD (%)**	**Status**	**Acceptance criteria**	**Condition**	**Mean ± SD**	**RSD (%)**	**Status**
η_0 (Pa.s)	[CAA±15%]	32±2˚C	25953 ± 6810	26.24	NC	[CAA±15%]	C35-P35 *vs.* P35-P35	22981 ± 10560	45.95	NC	[CAA±15%]	Syringe *vs.* Spatula	26363 ± 7070	26.82	NC
η_∞ (Pa.s)			17.2 ± 1.6	9.17	C			15.7 ± 4.4	27.93	NC			17.7± 2.0	11.38	C
η_U (Pa.s)			22194 ± 7370	33.21	NC			20702 ± 10313	49.82	NC			22203 ± 8126	36.60	NC
η_L (Pa.s)			41 ± 6	14.06	C			37 ± 11	30.17	NC			44 ± 10	22.46	NC
$\tau_{0.ROT}$ (Pa)			27.0 ± 2.1	7.74	C			28.4 ± 5.8	20.49	NC			27.3 ± 1.7	6.36	C
S_R (Pa/s)			27602 ± 6397	23.18	NC			30014 ± 5245	60.53	NC			27349 ± 8034	29.38	NC
LVR plateau (Pa)			6539 ± 453	8.61	C		P35-P35 *vs.* P20-P20	6879 ± 996	14.48	C			6704 ± 503	7.51	C
$\tau_{0.OSC}$ (Pa)			39 ± 9	25.43	NC			36 ± 7	19.92	NC			38 ± 9	23.31	NC
τ_f (Pa)			87 ± 35	26.69	NC			81 ± 17	20.96	NC			85 ± 20	24.03	NC
G' (Pa)			6783 ± 623	9.18	C			7430 ± 2251	30.30	NC			6961 ± 709	10.18	C
G'' (Pa)			1932 ± 179	9.28	C			2143 ± 712	33.21	NC			1997 ± 263	13.17	C
Tan δ			0.286 ± 0.018	6.44	C			0.288 ± 0.023	7.97	C			0.287 ± 0.021	7.39	C

Key: Compliant – C; Non-compliant – NC.

Regarding temperature effect, the method is generally robust; however, special attention should be regarded for some CAA, which revealed to be more sensitive to this parameter. F_{10} sample testing was conducted at a standard temperature of 32°C (to mimic skin conditions) and at 30°C and 34°C. Under these conditions, a significant decrease on specific CAAs was attained, suggesting a disruption on cream microstructure when exposed to rising temperatures. As displayed in Table 3.6, non-compliant results were accomplished for η_0, η_U, S_R, $\tau_{0.OSC}$ and τ_f responses, with RSD>23.18%.

Both rotational and oscillatory measurements are programmed at isothermal conditions, because of temperature effect on structural properties. Depending on excipients glass transition temperature, molecular weight, melting point and molecular rearrangement, a relationship among temperature and rheology CAAs may be established for non-Newtonian systems, since any change on this parameter may produce significant changes on the network structure rigidity and, thus, on product rheology (Rawat *et al.*, 2019).

F_{10} viscosity endpoints tend to decrease with increasing temperatures. Higher temperatures may impact intermolecular forces breakdown mechanisms, deteriorating the network structure and inducing the establishment of less viscous systems (Lauterbach and Müller-Goymann, 2014; Mezger, 2010).

F_{10} displays a thixotropic behaviour at all investigated temperatures with a typical hysteresis area. A temperature increase induces smaller S_R, since low viscous system offer less resistance to deformation forces, requiring a lower shear rate to deform and less time to structure recovery (Carriço *et al.*, 2019; de Souza Mendes, 2009; Ghica and Hîrj, 2016).

The $\tau_{0.OSC}$ and τ_f values of F_{10} were also highly influenced by temperature. Higher temperatures disrupt intermolecular interactions of the network, resulting in lower $\tau_{0.OSC}$ and τ_f, since a weaker network structure offers low resistance to deformation forces and requires lower shear values to initiate flow and even to structure break (Chanamai and McClements, 2000; Tadros, 2013; Vianna-Filho *et al.*, 2013). This is not in agreement with $\tau_{0.ROT}$ results, a compliant parameter, suggesting that τ_0 determination through oscillatory measurements is highly subject to variability.

Considering the geometry impact, this is a critical method variable that requires prior selection and optimization as confirmed by the lack of method robustness.

In the literature, there is no agreement regarding the most suitable geometry configuration for both oscillatory and rotational measurements. In this context, for the selection of an appropriate configuration, sample viscosity, geometry configuration, angle and radius, and

gap distance should be carefully considered. Generally, cone-plate configuration is used for bulk liquids and dispersions (suspensions and emulsions) with particle size less than 5 μm, whereas plate-plate configuration is used for dispersions containing relatively large particle size (Tricks, 2006).

In this context, geometry impact was assessed in rotational tests pondering distinctive configurations/angles: cone geometry with 2° (C35/2°) and plate geometry with 0° (P35/0°). Note that, when comparing P35-P35 configuration with the standard configuration (C35-P35), higher variability results (RSD>15%) were attained for rotational CAAs intraday measurements. Moreover, as represented in Table 3.6, non-compliant results were observed for the overall CAAs, with RSD exceeding 20.49%.

The results suggest that cone-plate configuration is preferred to perform rotational measurements, since the shear rate is the same throughout the sample, in opposite to plate-plate configuration, where the shear rate varies along the plate radius, increasing from the center to the edge. The non-uniform shear flow observed in plate-plate configuration seems to produce higher apparent viscosities and likewise an increment on the overall CAAs values. When performing rotational tests, rheology results are influenced significantly by this effect, but it is negligible when performing oscillatory test at small deformation values within the LVR plateau (Djalili-Moghaddam *et al.*, 2004; Mezger, 2010).

Considering geometry diameter, its selection is mainly dependent on sample viscosity. For low-viscous materials, it is preferable to use a large geometry diameter, therefore, benefiting from a large shear area. Correspondingly, for high-viscous and rigid materials, a smaller diameter should be selected (Fernanda, 2018).

In this context, geometry impact was assessed in oscillatory tests considering different plate diameter: 35 mm (P35) and 20 mm (P20). Comparing P20-P20 configuration with the standard configuration (P35-P35) (data not shown), higher variability results (RSD>15%) were observed for most intraday measurements. As displayed in Table 3.6, non-compliant results were acquired for $\tau_{0.OSC}$, τ_f, G´ and G'' with an RSD>19.92%. These results indicate that the plate diameter of 35 mm is the suitable geometry to test cream samples with similar F_{10} viscosity.

As previously mentioned, in a plate-plate (P20-20) configuration, shear conditions are not uniform along the plate gap and present a high dependence on geometry radius and gap distance. Amplitude sweep test seems to be independent on plate radius. Despite the non-uniform shear conditions provided by this configuration, if measurements are carried out within the LVR plateau, compliance is not compromised. If measurements are performed

outside the LVR, higher shear stress values are detected, which result in superior $\tau_{0.OSC}$ and τ_f values (Chhabra and Richardson, 2008; Vliet, 2013). In turn, for frequency sweep measurements, this method variable does not impact G' and G'' parameters.

In what concerns geometry radius, the obtained higher viscoelastic results may be related with lack of method sensitivity under low shear stress values (Mezger, 2010).

Regarding sample application, this is a method variable that should be carefully equated, because of its significant effect on rheology results. In the present study, sample application effect was investigated testing distinct devices: syringe and spatula.

When comparing spatula cream application with syringe cream displacement (data not shown), higher variability results (RSD>15%) were achieved for CAAs intraday measurements. Even though proved to be compliant for η_∞, $\tau_{0.ROT}$, LVR plateau, G', G'' and tan δ, rheology method presented higher RSD values for η_0, η_U, η_L, S_R, $\tau_{0.OSC}$ and τ_f (Table 3.6).

With respect to rotational measurements, shear thinning range [η_U - η_L] is highly influenced by this variable due to the irregularities in sample deposition on the plate.

The non-compliant results for S_R confirmed that the syringe device used in this study did not produce sample strain or result in structure loss in contrast with spatula, revealing that this specific CAA is highly influenced by application device. Any non-homogeneity in sample deposition, such as air bubbles, may cause a premature sample rupture and influence the entire rheology profile (Aho *et al.*, 2016; Tricks, 2006). Furthermore, sample amount (a high impacting variable, see Table 3.1) is more carefully monitored with a syringe application.

Even though we obtained compliant results for $\tau_{0.ROT}$, suggesting that this specific CAA is more robust to application changes, this is not in agreement with $\tau_{0.OSC}$ determination through oscillatory measurements, which are non-compliant (Mezger, 2010). τ_f determination seems also to be highly sensitive to sample application variability. These results show a strictly dependence on sample application regarding amplitude sweep tests.

3.3.1.6 Updated risk assessment

According to the previously presented results it was possible to update the REM, see Table 3.7, enlightening the different levels of the main method variables affecting rheology CAAs. The updated levels demonstrate that specific method variables should be carefully pondered due to their significant impact on rheology CAAs.

Table 3.7 – Updated risk estimation matrix (REM) for rheology method validation.

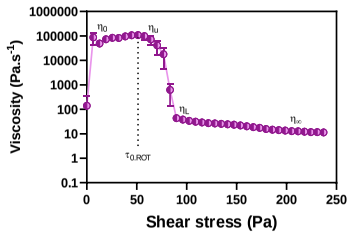
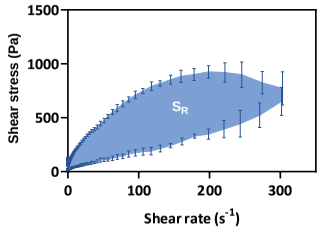
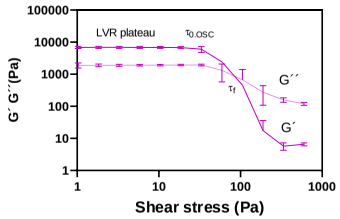
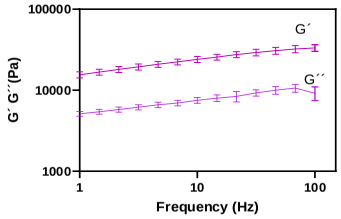
Test	**Viscosity curve**					**Thixotropic behaviour**	**Amplitude Sweep**			**Frequency Sweep**		
CAA	η_0	η_∞	η_U	η_L	$\tau_{0.ROT}$	S_R	**LVR *plateau***	$\tau_{0.OSC}$	τ_f	**G´**	**G´´**	**Tan δ**
CMV												
Temperature	H	L	H	L	L	H	L	H	H	L	L	L
Geometry	H	H	H	H	H	H	L	H	H	H	H	L
Sample application	H	L	H	H	L	H	L	H	H	L	L	L
Gap and trimming	L	L	L	L	L	L	L	L	L	L	L	L
Data acquisition mode	M	M	M	M	M	L	L	L	L	L	L	L
Integration time	M	M	M	M	M	M	M	M	M	M	M	L
Sample amount	M	M	M	M	M	M	M	M	M	M	M	L
Analyst	M	M	M	M	M	M	M	M	M	M	M	L
Shear stress ramp	H	H	H	H	H		H	H	H			
Step duration	M	M	M	M	M	M	M	M	M	M	M	L
Shear rate ramp						H						
Shear load time						H						
Shear recovery time						H						
Frequency value							M	M	M			
Shear stress within LVR plateau										H	H	L
Frequency ramp										M	M	L

Key: Low risk variable – Low; Medium risk variable – Medium; High risk variable – High.

3.3.1.7 Standardizing the procedure

In order to provide a straightforward analysis, the following table summarizes the main outcomes unveiled in this study. In the pursuit of the development and validation of a rheological profile of a semisolid dosage form, the impact of CMVs on specific CAAs should be considered (Table 3.8).

Table 3.8 – Standardizing rheological methodology.

Test	Pre-setting	CMV	Highly relevant CAAs	Interpretation	Typical graphical representation
Rotational: viscosity curve	Acquisition mode Shear stress ramp Step duration	Temperature: medium risk variable Geometry: high risk variable Sample application: medium risk variable	η_0 η_U η_L η_∞ $\tau_{0.ROT}$	Higher η_0, η_U, η_L, η_∞ and $\tau_{0.ROT}$ suggest more structured systems	
Rotational: thixotropic behaviour	Step duration Shear rate ramp Shear load time Shear recovery time		S_R	Larger S_R is indicative of more structured and consistent systems	
Oscillatory: amplitude sweep	Frequency value		LVR *plateau* $\tau_{0.OSC}$ τ_f	Larger LVR and superior τ_f are indicative of more structured systems.	
Oscillatory: frequency sweep	Shear stress within LVR plateau Frequency ramp		G` G`` Tan δ	G' > G'', prevalence of elastic properties Tan δ < 1, Viscoelastic with prevalence of elastic properties, meaning and gel/solid – like structures	

3.4 Concluding remarks

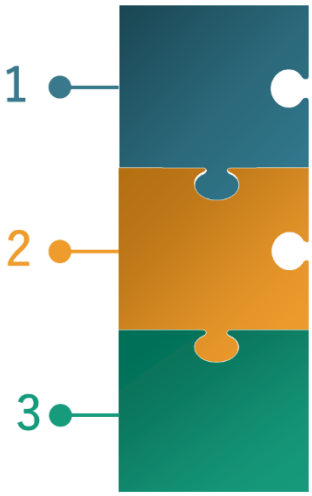
In light of the new regulatory requirements, the importance of a detailed rheological characterization of topical semisolid dosage forms is undeniable. A comprehensive framework for the development and validation of the rheology profile acquisition is herein presented. Even though, a 1%w/w hydrocortisone cream was used as a case study, the same rationale can be transposed to other semisolid products.

The obtained experimental data revealed that the proposed method is accurate, precise, discriminative and robust. Nevertheless, there are critical method variables that should be optimized prior to experiments. These include geometry, sample application mode and temperature. A broad range of rheological critical analytical attributes were identified: zero-shear viscosity, upper shear thinning viscosity, lower shear thinning viscosity, infinite-shear viscosity, rotational yield point, thixotropic relative area, linear viscoelastic region, oscillatory yield point, storage moduli, loss moduli and loss tangent.

According to the updated risk assessment, the following can be considered as more sensitive monitoring responses: thixotropic relative area, oscillatory yield point and viscosity related endpoints. These rheological attributes are crucial to the formulations physical stability, *in vitro* performance and, consequently, spreadability and patient compliance.

Moreover, if rheology methods are applied as PAT tool during product manufacture, a close monitoring of the rotational yield point, linear viscoelastic region, storage and loss moduli, as well as loss tangent, can be highly beneficial. The continuous assessment of these parameters enable an early detection of CPP and CMA, responsible for microstructure fluctuations, which in turn would allow a reduction in out of specifications results and overall batch variability of topical dosage forms.

3.5 Highlights



A broad range of endpoints can be considered throughout a rheology profile analysis:
Zero-shear viscosity, upper shear thinning viscosity, lower shear thinning viscosity, infinite-shear viscosity, rotational yield point, thixotropic relative area, linear viscoelastic region, oscillatory yield point, storage moduli, loss moduli and loss tangent.

A similar validation strategy to that adopted for IVRT/IVPT studies has been transposed to rheology methods. **Overall procedures and respective acceptance criteria regarding traditional validation components** such as equipment qualification, precision, robustness and discriminatory power were herein proposed.

In light of the new regulatory requirements which request a formal validation of each method used for product characterization studies, the proposed strategy can be useful since it regards a **scientific driven platform for both development and validation of rheological methods**.

3.6 Case Study – Establishing rheological equivalence of a clotrimazole 10 mg/g cream

To further illustrate the applicability of the proposed strategy, the rationale herein described was transposed to a distinct case study – a clotrimazole 10 mg/g cream. Apart from the previous method development and the pursuit of a validation strategy, the aim of the present case study was to compare the rheological properties of a test product (TP) with a reference product (RP). Both products are qualitatively and quantitatively equivalent. According to the requirements of the EMA draft guideline, 3 batches *per* product should be used to demonstrate equivalence regarding rheological outputs, and at least 3 replicates per batch are required.

3.6.1 Materials and methods

3.6.1.1 Materials

Three batches of clotrimazole 10 mg/g cream (w/w) (Canesten® - Bayer), hereafter referred to as RP, and three industrial batches of a clotrimazole 10 mg/g cream (w/w) (TP) were used in the present case study. The next table summarizes the product characteristics.

Table 3.9 – General information on the products used in the present study. The batch age during the rheology studies is given in months (M). Both RP and TP have an expiry date of 3 years.

Product	Description	Used batches	Retrieved market	Manufacturing site	Tested at (*M)
Reference Products Bayer®	Canesten® Foot Crème 10 mg/g cream	BXPJR4J – RP1	France	KernPharma – Spain	31 M
	Canesten® Antifungal cream 10 mg/g	BXPJREH – RP2	United Kingdom		28 M
	Canesten® Antifúngico cutâneo creme 10 mg/g	BXPJSWZ– RP3	Portugal		12 M
Test products	Clotrimazole 10 mg/g cream	TP1	N.A	Portugal	27 M
	Clotrimazole 10 mg/g cream	TP2			32 M
	Clotrimazole 10 mg/g cream	TP3			15 M
Excipient	Function				
Benzyl Alcohol	Preservative				
Cetostearyl Alcohol	Emulsifier				
Cetyl Palmitate	Thickener				
Octyldodecanol	Emulsifier				
Polysorbate 60	Emulsifier				
Purified Water	Solvent				
Sorbitan Stearate	Emulsifier				

Key: N.A – Not applicable.

To document the discriminatory power of the proposed rheological methods, a formulation with half the concentration of cetostearyl alcohol was manufactured at a laboratory scale. This excipient was selected due to its thickener characteristics and impact on product viscosity profile. This formulation will be further designated as negative control formulation.

3.6.1.2 Methods

Rotational measurements

The same rheometer, analysis software, and general workflow as in Chapter 3 were herein used (sample hood, 32ºC analysis temperature, syringe application, 0.1 preset gap). For rotational tests, a cone-and-plate (C35/2º/Ti-TMP35) was used. Approximately 0.3 g of the formulation was applied. A linear CS flow ramp from 0.01 to a final value of 100 Pa was

measured for 300 s to trace the viscosity curve. To determine the apparent thixotropy (Pa/s), a shear rate from 0.01 to 300 s^{-1} and return to 0.01, during 300 was also used.

Oscillatory measurements

A parallel plate-and-plate geometry (P35/Ti) was used and approximately 0.3 g of the formulation was applied to the *peltier* plate. First, an amplitude sweep was performed between 0.01 and 100 Pa at 1 Hz to determine the linear viscoelastic region (LVR). Afterwards, a frequency sweep analysis was conducted within the LVR range. The storage modulus G´ and loss modulus G´´ were calculated.

Rheological method validation

The validation of the rheological methods developed here followed a simplified approach compared to that described previously. Rheometer qualification studies were carried out. The intraday precision of the method was evaluated by performing three rheological measurements for each test for all batches of RP. An RSD less than 15% was considered acceptable, consistent with what has been previously established (EMA, 2009; FDA, 2016c). Moreover, the sensitivity and selectivity of the method were evaluated by comparing the rheological profile of the RP with the negative control formulation. The methods were considered sensitive if the rheology outputs obtained with the negative control formulation were lower than those registered in the RP. In addition, the methods were considered selective if the differences between the two formulations (negative control and RP) were statistically significant.

Statistical analysis

To statistically compare the rheological endpoints of TP *vs.* RP, as well as RP *vs*. negative control formulation, the 90% confidence intervals were determined. According to the EMA draft guideline, for quantitative quality attributes, such as the rheological endpoints, the 90% confidence interval for the difference of means of TP and RP should be within the acceptance criteria of +/-10% of the RP mean, assuming a normal distribution of the data. Please note that the 10% maximum difference between quantitative quality characteristics of TP and RP defines the acceptance interval as 90-111%, since 90/100 = 0.9 and 110/100 = 0.11. Although this was not the statistical approach considered in Chapter 3, as this case study focus on equivalence demonstration between an TP and an RP, further efforts were made to best reproduce EMA statistical requirements (EMA, 2018b, 2018c).

First, the data were evaluated to determine if they followed a normal distribution (Shapiro-Wilk test, *p*=0.05).

Afterwards, to perform the equivalence test of quantitative physicochemical parameters, the 90% CI of the ratio of means between the test/reference formulations were determined. The data was natural log transformed. Then, the means and the standard deviations were calculated. This was followed by obtaining the ratio of the two back-transformed averages for rheology and IVRT endpoints. For confidence interval calculations Equations 3.2 and 3.3 were used:

$$\frac{\overline{X_1}}{\overline{X_2}} \pm t_{1-\alpha/2,n_1+n_2-2,} S_p \sqrt{\frac{1}{n_1}+\frac{1}{n_2}} \tag{3.2}$$

$$\text{Eq. (4)} \quad S_p = \sqrt{\frac{(n_1-1)\times s_1^2+(n_2-1)\times s_2^2}{n_1+n_2-2}} \tag{3.3}$$

Where *X* is the mean value to evaluate the test (*X*1) or reference product (*X*2), t1-α/2 is the Student's t value for α = 0.90, s is the standard deviation, and n the number of observations.

Data analysis was performed using Microsoft Office Excel®.

3.6.2 Results and discussion

The next figure summarises the obtained rheological profiles of the RP, TP and negative control clotrimazole 10 mg/g cream formulations.

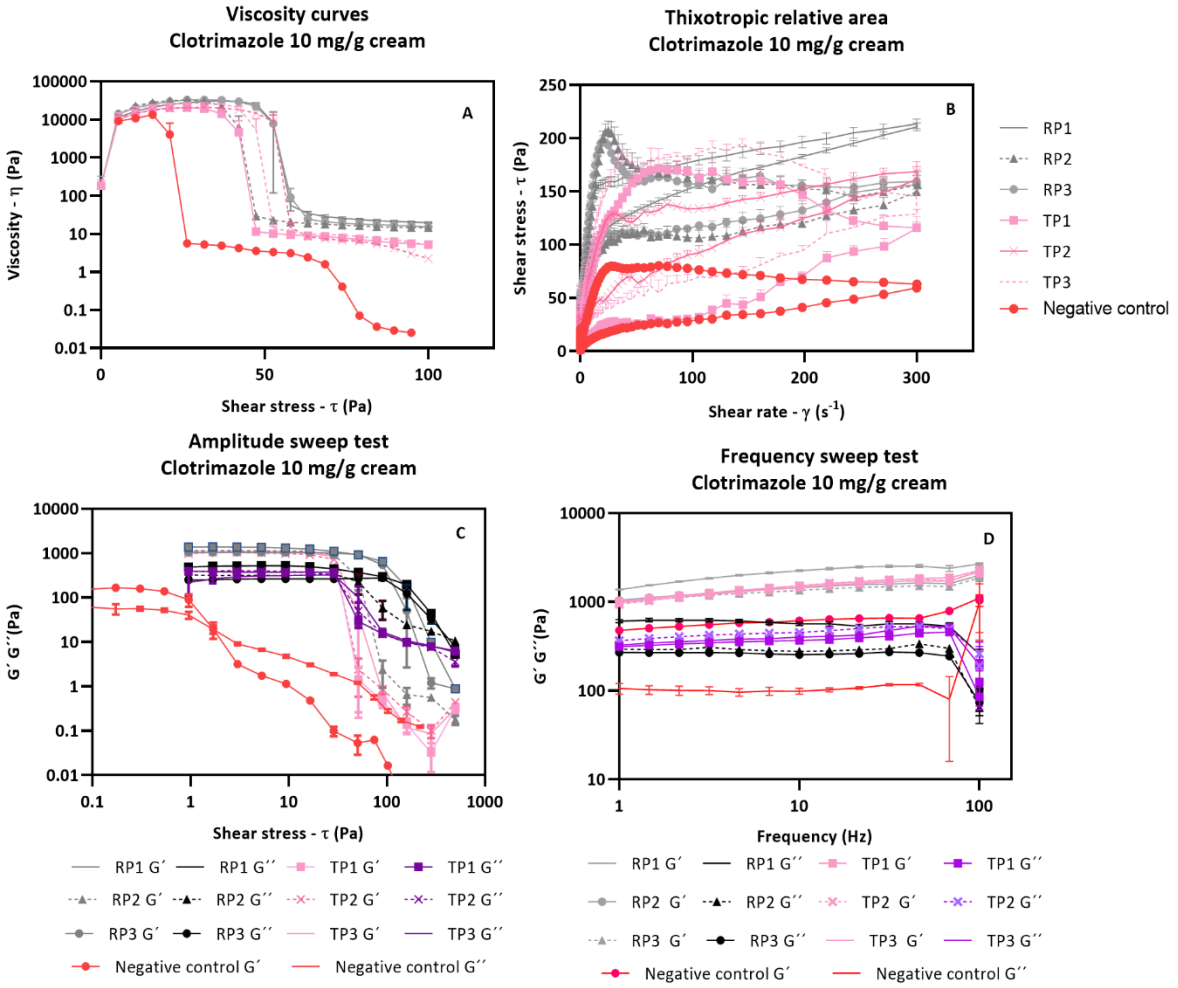


Fig.3.8 – Rheology profiles of clotrimazole 10 mg/g cream formulations. All results report to mean ± SEM. Three replicates were used per batch formulation. A – Viscosity curves; B – Thixotropic behaviour; C – Amplitude sweep test; D – Frequency sweep test.

When representing the viscosity *vs*. shear stress, the three batches of each product showed identical profiles (Fig.3.8A). In all cases, non-Newtonian, and shear thinning behaviour was promptly observed. The acquired viscosity curves clearly present three regions: (i) the 1st Newtonian plateau, from which the zero-shear viscosity (η_0) was derived; (ii) the shear-thinning region, from which the yield point can be estimated; as previously described, this rheological parameter is defined as the minimum shear stress required to initiate the material flow (τ_0), and finally (iii) the 2nd Newtonian plateau, from which the infinite-shear viscosity can be determined (η_∞). Regarding the thixotropic behaviour, the flow curves, depicted in Fig.3.8B, enabled the classification of all formulations as thixotropic, since hysteresis loop areas were observed.

The viscoelastic properties of all formulations were assessed by determining both amplitude and frequency sweep behaviour. Regarding the amplitude sweep test, the plots of the elastic

(G´) and the viscous (G´´) moduli *vs.* the shear stress for each batch, showed a linear viscoelastic region for each batch. This linear trend was likewise observed during the frequency-dependent elastic and viscous moduli in frequency sweep tests (Fig.3.8D). As depicted in Fig.3.8C-D, similar trends were observed for both RP and TP formulations. Moreover, in all cases higher G´ than G´´ values were registered, which is consistent with the predominantly elastic behaviour of semisolid systems.

In order to allow a quantitative comparison between the rheological profiles of all the products, several endpoints were considered in accordance with the intended strategy, obtained from both rotational and oscillatory tests (please see Table 3.10).

The following rotational endpoints were considered: zero (η_0) and infinite (η_∞) shear viscosity, the rotational yield point (τ_{ROT}) and the relative thixotropic area S_R. Although a larger pool of endpoints is considered in the proposed approach, a simplified framework, yet based on the regulatory requirements was applied for this case study. As depicted in Table 3.10, the intra-batch precision results regarding rotational endpoints for the RP were overall adequate, with a maximum CV of 15% being registered for the thixotropic area. Nevertheless, batch variability was observed within the different batches of the RP, as inter-batch precision results far exceed the ones previously registered.

The observed variability does not seem to be correlated with batch age, as the RP2, the batch that exhibited a lower yield point, had a similar batch age to RP1 (31 M).

The selected oscillatory endpoints were as follows: Linear viscoelastic region (LVR), flow point (τ_f), the oscillatory yield point (τ_{OSC}), as well as storage (G´) and loss moduli G´´ at 10 Hz. Overall (3/5) intra-batch precision results met the established criteria. The reported exceptions were for the τ_f and τ_{OSC} endpoints. Nevertheless, when comparing these endpoints retrieved from the different RP batches, inter-batch variability was a point of concern. Again, no obvious correlation was found between batch age and the observed differences. Although the precision of the rheology method can be successfully documented due to the low intra-batch variability, the results obtained raise concerns about RP batch variability when this information is taken into account.

The sensitivity of the rheology method was successfully documented, as the developed methods were able to reflect changes between the RP/TP and a formulation with a distinct quantitative profile (see Figure 3.8 and Table 3.10).

Table 3.10 – Rheological properties of clotrimazole 10 mg/g cream RP, TP and negative control formulations. For the RP and the TP, 3 batches with 3 replicates per batch were considered

(n=9). On the other hand, for the negative control formulation, only 1 batch was considered with triplicate measurements (n=3). An intra-batch coefficient of variation (CV) <15% in the RP was considered appropriate to document the precision of the method.

	Reference Product		Test Product		Negative control formulation
Rheological endpoints	**Mean and CV(%)**	**Overall RP CV (%)**	**Mean and CV(%)**	**Overall TP CV (%)**	**Mean and CV(%)**
Acceptance criteria	**Precision Intra-batch CV of the RP < 15%**				**Sensitivity RP/TP > Altered rheology**
η_0 (Pa.s)	RP1: 16696 (1%) RP2: 22958 (5%) RP3: 20567 (8%)	14	TP1: 14621 (7%) TP2: 14523 (6%) TP3: 20006 (13%)	19	10821 (14%)
τ_{ROT} (Pa)	RP1: 42.2 (2.7%) RP2: 39.5 (1.0%) RP3: 40.2 (8.9%)	6.2	TP1: 48.8 (0.9%) TP2: 36.9 (12.1%) TP3: 45.9 (15.0%)	13.8	12.8 (1.7%)
η_∞ (Pa.s)	RP1: 25.7 (10.9%) RP2: 16.9 (1.0%) RP3: 19.1 (8.9%)	20.4	TP1: 7.76 (17.4%) TP2: 6.83 (2.77%) TP3: 9.27 (7.85%)	16.9	0.4 (16.5%)
S_R (Pa/s)	RP1: 5900 (15%) RP2: 12483 (6%) RP3: 9828 (14%)	31	TP1: 25513 (5%) TP2: 10714 (9%) TP3: 25243 (5%)	34	10243 (2%)
LVR (Pa)	RP1: 1352 (1%) RP2: 1125 (5%) RP3: 1037 (6%)	12	TP1: 1082 (3%) TP2: 1017 (9%) TP3: 1026 (5%)	7	157 (16%)
τ_f (Pa)	RP1: 232 (22%) RP2: 355 (27%) RP3: 295 (14%)	28	TP1: 431 (3%) TP2: 362 (2%) TP3: 374 (9%)	9	24.5 (6.9%)
τ_{OSC} (Pa)	RP1: 17.7 (7.3%) RP2: 25.6 (21.7%) RP3: 48.1 (10.5%)	44.6	TP1: 24.6 (17.2%) TP2: 17.1 (9.30%) TP3: 23.6 (17.6%)	22.4	0.6 (3.2%)
G´ – 10Hz (Pa)	RP1: 2251 (3%) RP2: 1438 (0%) RP3: 1355 (2%)	24	TP1: 1521 (4%) TP2: 1524 (11%) TP3: 1461 (5%)	7	615 (4%)
G´´ – 10Hz (Pa)	RP1: 567 (6%) RP2: 277 (6%) RP3: 255 (6%)	39	TP1: 402 (6%) TP2: 453 (14%) TP3: 369 (9%)	14	99 (6%)

Key: RP – Reference Product; TP: Test product; η_0 (Pa.s) – Zero-shear viscosity; η_∞ (Pa.s) – Infinite-shear viscosity; $\tau_{0.ROT}$ (Pa) – Yield point obtained through rotational methods; S_R (Pa/s – Relative thixotropic Area; $\tau_{0.OSC}$ (Pa) – Yield point obtained through oscillatory methods; LVR plateau (Pa) – Linear Viscoelastic Region plateau; τ_f (Pa) – Flow point; G´ – Storage modulus; G´´ – Loss modulus.

To statistically compare the RP *vs.* TP, as well as the RP *vs.* negative control, the 90% CI were determined, as required by the EMA draft guideline (see Table 3.11).

Table 3.11 – Rheology method validation and equivalence results. Rheology endpoints results pertaining to the RP/TP concern an n=9. For the negative control formulation, an n=3 was considered. To assess method selectivity, the 90% CI of the ratio average (altered rheology/RP) is presented. If the CI of the considered rheological endpoints surpasses the 75-133% interval, selectivity could be inferred. On the other hand, to document rheological equivalence between the RP and TP, the CI must be within the 75-133%.

	Evaluating RP variability RP1 *vs.* RP3 (Best case scenario)		**Validation study**		**Equivalence study**	
Rheological endpoints			**90% CI RP *vs.* Negative control Rheological endpoints Selectivity study**	**Status**	**90% CI RP *vs.* TP Rheological endpoints equivalence study**	**Status**
Acceptance criteria	CI must be within 90-111%		CI fall outside 75-133%		CI must be within 75-133%	
η_0 (Pa.s)	109.6 – 137.7	NC	41.03 – 67.17	C	70.69 – 93.14	NC
τ_{ROT} (Pa)	82.4 – 108.8	NC	29.31 – 33.99	C	99.09 – 120.73	C
η_∞ (Pa.s)	59.9 – 92.9	NC	1.45 – 2.64	C	33.26 – 45.50	NC
S_R (Pa/s)	120.65 –231.2	NC	77.64 – 170.26	C	153.20 – 297.04	NC
LVR (Pa)	70.20 – 83.6	NC	11.26 – 15.84	C	81.92 – 97.46	C
τ_f (Pa)	87.38 – 189.32	NC	6.22 – 12.02	C	113.93 – 164.49	NC
τ_{OSC} (Pa)	223.5 – 326.6	NC	1.21 – 3.59	C	56.81 – 104.05	NC
G´ – 1Hz (Pa)	57.17 – 63.44	NC	28.88 – 48.76	C	78.93 – 106.08	C
G´´ – 1Hz (Pa)	39.55 – 51.30	NC	19.00 – 43.84	C	93.19 – 150.20	NC

Key: RP – Reference Product; TP: Test product; η_0 (Pa.s) – Zero-shear viscosity; η_∞ (Pa.s) – Infinite-shear viscosity; $\tau_{0.ROT}$ (Pa) – Yield point obtained through rotational methods; S_R (Pa/s – Relative thixotropic Area;$\tau_{0.OSC}$ (Pa) – Yield point obtained through oscillatory methods; LVR plateau (Pa) – Linear Viscoelastic Region plateau; τ_f (Pa) – Flow point; G´ – Storage modulus; G´´ – Loss modulus; C – Compliant; NC: Non-compliant.

As reported in Chapter 3, as well as documented by other authors, direct application of the EMA criteria was not possible, because some of the rheological parameters did not follow a normal distribution (EMA, 2018b; Pleguezuelos-Villa *et al.*, 2019; Xu *et al.*, 2020). Given the observed variability of RP batches, it is important to investigate whether the EMA criteria are suitable to realistically infer the rheological equivalence of this specific formulation. To address this question numerically, the 90% CI of the RP batches with closer rheology profile (RP1 *vs.* RP3) were determined. As expected, the 10% maximum difference threshold between the rheological attributes of the RP batches with narrower viscosity characteristics was exceeded in all cases. Since the EMA criteria is not applicable when only the RP is concerned, a wider CI (75-133%) was established here to realistically infer on (i) the selectivity of the proposed methods and (ii) the rheological equivalence between TP and RP. This CI was selected based on previous studies reported in the literature, as well as on the SUPAC-SS criteria. Although this guideline is specific to IVRT studies, these limits take into account the intrinsic variability characteristic of semisolid systems (FDA, 1997; Mangas-Sanjuán *et al.*, 2019). As depicted in Table 3.11, even when considering this wider CI, equivalence pertaining to the RP1 *vs.* RP3 is solely registered for one endpoint (τ_{ROT}). Nevertheless, this CI extension was still considered.

Regarding the validation study, all endpoints successfully documented method selectivity, as the CI obtained with the RP *vs.* negative control formulation fall completely outside the CI limit 75-133%.

As for the equivalence study between RP and TP, 3 out 9 endpoints were found to be compliant with the established criteria. As for the rotational endpoints, equivalence was adequately demonstrated for the rotational yield point. As previously mentioned, this endpoint is highly important from both a technological and patient compliance perspective. The yield point refers to the critical stress at which the formulation starts to be plastically deformed. While increasing the shear stress, the degree of strain exerted in the microstructure increases accordingly, which in turn delays the complete relaxation of the structure in a given timeframe of a respective stress point. Therefore, above a critical value of stress, extreme shear thinning conditions lead to an irreversible change in the microstructure of the product, which is reflected in a drastic reduction in viscosity (Dabbaghi *et al.*, 2021). The equivalence of the viscosity at infinite shear, as well as the range of hysteresis loop area, failed to be documented. Nevertheless, both endpoints also proved to be non-compliant when considering the RP itself with the broader criteria.

Although a clear linear viscoelastic region was observed for both RP and TP during amplitude sweep tests, statistical differences were registered between both products for the flow point

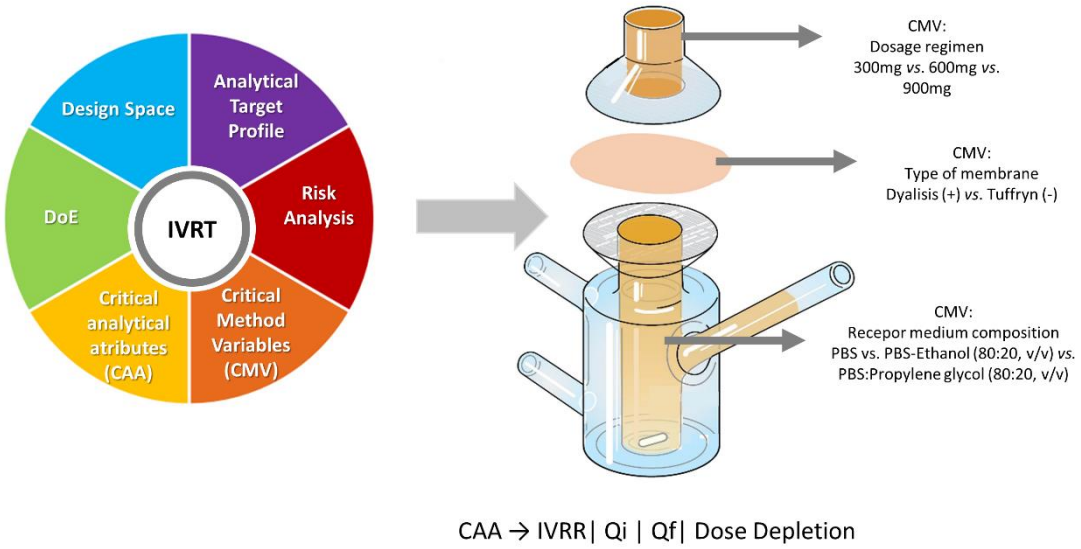
(τ_F) and the yield point (τ_{OSC}). As previously documented for precision, RP inter-batch variability is extremely high in these parameters (τ_F RSD = 28% and τ_{OSC} RSD = 44.6%); therefore, in this specific case study, these parameters may not be suitable to conclude the equivalence of the amplitude sweep test. Nevertheless, the G´ oscillatory endpoint obtained from frequency sweep tests was able to sustain equivalence between the RP and the TP.

3.6.3 Conclusions

The precision, selectivity, and sensitivity of the rheological method were documented in line with the approach previously presented. The comparative rheological studies (RP *vs.* TP) were able to sustain rheological equivalence between both products, as all profiles were found to overlap and 3 out of 9 rheological endpoints were within the established acceptance criteria. Although the remaining parameters presented non-compliant results, these were mainly related with RP inter-batch variability, a constraint that will be further discussed in Chapters 5 and 6 of this thesis.

Taking all the information into account, the strategy developed here was found to be suitable for investigating the rheological behaviour of TP, envisioning its application to support rheological equivalence.

4 aQbD AS A PLATFORM FOR IVRT METHOD DEVELOPMENT: A REGULATORY ORIENTED APPROACH



Key: *In Vitro* Relase testing (IVRT) | Design of Experiments (DoE) | Critical Method Variables (CMV) | Critical Analytical Atributes (CAA) | *In Vitro* Release rate (IVRR) | Cumulative amount released in an initial timepoint (Q_i)| Cumulative Amount Released in final time (Q_f)

Fig.4.1 – Graphical abstract: aQbD as a platform for IVRT method development: a regulatory oriented approach.

This chapter has been adapted from the following publication:

Miranda, M., Pais, A.A.C.C., Cardoso, C., Vitorino, C., 2019. aQbD as a platform for IVRT method development–A regulatory oriented approach. Int. J. Pharm. 118695. https://doi.org/10.1016/j.ijpharm.2019.118695

C.V conceived the idea and established the research program and implementation. M.M performed the experimental parts of the work. A.A.C.C Pais performed the statistical analysis, more specifically the confidence interval calculations according to the Wilcoxon Rank Sum/Mann-Whitney rank test. Supervision, resources and data curation was provided by C.V and A.A.C.C Pais. M.M wrote the first draft of the manuscript and all other authors substantially contributed to revisions. Funding acquisition was provided by C.C and C.V.

KEY CONCEPTS

- To characterize semisolid dosage form performance, the acquisition of the *in vitro* release profile is a fundamental tool, since it is generally responsive to formulation differences. In this context, release testing is an effective approach to monitor batch-to-batch consistency, post-approval changes, scale-up and stability studies. Further applications include TGP development studies, since IVRT, together with Q1-Q3 demonstration, can be used as a **predictive tool to document the extended pharmaceutical equivalence**.
- Regulatory recommendations addressing IVRT method validation are clearly defined. However, there is **not a standardized methodology, based on aQbD principles**, to suport method development.
- A **general workflow can be traced when implementing aQbD**: (i) definition of the ATP and CAA´s; (ii) risk assessment and CMV identification, following a DoE rationale; and finally (iii) through response surface analysis, establishment of the method design space.

4.1 Introduction

Release methods, commonly applied to systemically absorbed solid dosage forms, are a valuable regulatory tool within the generic medicine manufacturing industry. According to the biopharmaceutical classification system, for active substances with a rapid or very rapid dissolution (Class I and Class III), *in vitro* methods can be used to establish BE, thus enabling a biowaiver from clinical trials, one of the most expensive stages during generic drug development (EMA, 2018a).

As extensively reviewed in the introductory chapter of this thesis, IVRT methods, specifically tailored for topical products, share the same scientific principles as the dissolution methods. However, their regulatory status in what concerns BE documentation is still being outlined. Recent efforts by several regulatory agencies are seemingly addressing this issue. In the past 3-4 years, the US-FDA has stated that, for some products, IVRT as well as IVPT (conducted with human skin), can be used as alternative BE assessment tools (FDA, 2016c). More recently, as thoughtfully addressed in the 2nd chapter, EMA emitted a draft guideline on quality and equivalence of topical products in which the concept of pharmaceutical equivalence is highly explored in what regards Q1, Q2, Q3, and also product performance and administration documentation (EMA, 2018b). These documents provide a significant insight into the conditions under which the use of *in vitro* methods (release and permeation) may be acceptable as surrogates for the BE assessment of TGPs (EMA, 2018b; FDA, 2016c).

The release profile of a topical semisolid dosage form, acquired through IVRT, enables the determination of the *in vitro* release rate (IVRR). This kinetic parameter can be regarded as a formulation CQA, as it provides important information on the microstructural characteristics of the product, such as particle size and rheological behaviour (Braddy *et al.*, 2015;

Dandamudi, 2017; FDA, 1997; Flynn *et al.*, 1999; OECD, 2010; Sivaraman and Banga, 2015). As the active pharmaceutical ingredient must be released before it can diffuse and become bioavailable in the skin, the determination of the IVRR is a useful tool to assess product similarity (EMA, 2018b; FDA, 2016c, 1997; Tiffner *et al.*, 2018). Note that an IVRT setup includes an artificial membrane that does not resemble the *stratum corneum* layer in the skin, the main barrier for dermal absorption. According to the new EMA draft guideline, for complex formulations (e.g. multiphasic systems) product efficacy demonstration should be presented by either *in vitro* permeation testing (IVPT), pharmacokinetic studies, or dermatopharmacokinetic methods. Nevertheless, the documentation of product efficacy equivalence must be preceded by pharmaceutical equivalence demonstration with respect to qualitative and quantitative profile, microstructure/physical properties and also to product performance, the latter retrievable from IVRT.

A comprehensive and thorough analysis of the IVRT regulatory requirements clearly indicates that with the progressive acceptance of this method as a BE assessment tool, a solid framework to support the respective analytical development will be needed. In this context, the application of an analytical quality by design (aQbD) approach to the development of IVRT can offer relevant advantages. A plethora of strategies have been used to optimize IVRT conditions but, to our knowledge, none is reported that follows aQbD principles (Baert *et al.*, 2010; Bao *et al.*, 2017; Goebel *et al.*, 2013; Tiffner *et al.*, 2018; Zatz, 1995).

The aQbD approach follows the quality by design (QbD) concepts, defined in ICH guidelines Q8 (R1), pharmaceutical development, Q9, quality risk management and Q10, pharmaceutical quality system (ICH, 2008, 2003a, 2003b). QbD has been introduced in the pharmaceutical industry to strengthen manufacturing processes, enhance product quality, and by doing so, reduce the number of out-of-specification results (Panda *et al.*, 2017; Peraman *et al.*, 2015). In turn, aQbD focuses on the development of a robust and cost-effective analytical method (Peraman *et al.*, 2015). A general workflow can be traced when implementing aQbD: (i) definition of the analytical target profile (ATP) and critical analytical attributes (CAA´s); (ii) risk assessment and identification of the critical method variables (CMV), following a design of experiments (DoE) rationale; and finally (iii) through response surface analysis, establishment of the design space pertaining to the method, also referred to as the method operable design region (MODR) (Basso *et al.*, 2018; Panda *et al.*, 2017; Peraman *et al.*, 2015). The latter element consists of one of the most significant benefits of the implementation of aQbD as a control strategy, since it exactly defines the acceptable ranges of the analytical parameters, which in turn reinforces and assures the robustness of the method (Shao *et al.*, 2018).

aQbD has been mainly applied to chromatographic methods such as high performance liquid chromatography (HPLC), ultra-high performance liquid chromatography, or rapid resolution liquid chromatography (Panda *et al.*, 2017). Nevertheless, the same workflow can be transposed to the development of IVRT.

A workshop on Complex Generic Drug Product Development recently sponsored by FDA addressed the main aspects which frequently impair ANDA applications for TGP (Katragadda, 2018). Several of the reported constraints point out the omission of IVRT components, such as lack of IVRT method development and validation reports, as well as the non-disclosure of IVRT standard operating procedures (SOP) (Katragadda, 2018; Raney, 2018). The absence of such documents conditions *a priori* the submission and approval of the TGP.

Based on the present regulatory background, this work aims at designing a framework applied to the development of a IVRT method for topical products taking into account aQbD principles. To achieve so, the following steps were considered:

- Definition of the IVRT ATP – Since the development of a sensitive and discriminatory IVRT was the main goal, the experimental conditions that enabled the maximization of the release profile were considered, Table 4.1;
- Selection of both CAA and CMV – Made through a risk assessment analysis;
- Preliminary solubility experiments, in order to select appropriate pH range and cosolvents to use as release medium in DoE experiments;
- DoE – Resorting to a 3x2x3 full factorial design, the parameters that better suited the previously defined ATP were determined through the desirability function;
- Finally, validation experiments, a crucial part in every aQbD application. The optimized IVRT conditions were fully validated according to the existing guidelines, as well as other state of the art scientific reports (EMA, 2018b, 2014d; FDA, 2016c; ICH, 2009; Tiffner *et al.*, 2018). Moreover, a critical and reflected, appraisal focusing on the new requirements of IVRT presented in the EMA draft guideline, is presented.

For all experiments, a commercially available diclofenac emulgel formulation was used as “model product”.

4.2 Materials and methods

4.2.1 Drug Products

Hydrocortisone cream 1% (w/w) (Pandermil®, Edol, batch number 170827, Portuguese market) was used as a test product during the laboratory qualification experiments (Tiffner *et al.*, 2018; USP, 2009). Diclofenac emulgel 1% w/w (Voltaren Emulgel®, GSK, batch number WF006, Portuguese market) served as a reference product (RP) for the aQbD experiments. A diclofenac emulgel 2% w/w formulation (Voltaren Emulgelex®, GSK, batch number UO4029A, Portuguese market), a 0.5% w/w diclofenac emulgel and a specifically manufactured placebo were used during IVRT validation studies. The 0.5% w/w diclofenac emulgel was obtained by diluting the RP with ultrapure water (1:1). The formulation was left under magnetic stirring for 30 min until complete homogenization. A placebo emulgel with the same qualitative composition of the RP was used. All the above-mentioned formulations were kindly provided by Laboratórios Basi (Mortágua, Portugal), as well as the sodium diclofenac and hydrocortisone standards.

Propylene glycol was acquired from Merck and phosphate buffered saline was purchased from Sigma. Water was purified (Millipore®) and filtered through a 0.22 µm nylon filter before use. All other chemicals were of analytical grade or equivalent.

4.2.2 Methods

4.2.2.1 Analytical target profile establishment

The establishment of an analytical target profile (ATP) is regarded as the basis of method development. ATP refers to a prospective summary of the quality characteristics desired for the analytical method. Table 4.1 addressed the ATP elements considered for the optimization and validation of the IVRT method.

Table 4.1 – Analytical target profile elements considered for the optimization of the IVRT method for a semisolid topical formulation containing diclofenac.

ATP element	Target	Scientific rationale
Active substance	Diclofenac diethylammonium	Two randomised controlled clinical trials comparing topical diclofenac, in 70 and 155 patients respectively, recorded significant benefit over placebo for pain relief (Jordan *et al.*, 2003). Moreover, large surveillance studies documented topical diclofenac enhanced safety profile, especially when compared to the oral route. Factors which ground this occurrence mainly regard the first pass effect avoidance, implying residual gastrointestinal irritation, as well as metabolic degradation (Khanolkar *et al.*, 2017; Tieppo Francio *et al.*, 2017). According to EULAR (the European League Against Rheumatism) there is evidence of topical diclofenac efficacy in the management of rheumatic diseases, such as knee osteoarthritis (Jordan *et al.*, 2003).
Sample	Diclofenac semisolid dosage form	Development and validation of an IVRT method, especially directed towards the analysis of complex dosage forms.
Analytical technique	IVRT	IVRT is a fundamental tool to characterize the semisolid dosage form performance, since it is generally responsive to physicochemical differences (Bao *et al.*, 2017; Dandamudi, 2017) Release testing is an effective approach to monitor batch-to-batch consistency, post-approval changes, scale-up and stability studies within the pharmaceutical industry (Bao *et al.*, 2017; Shah *et al.*, 2003). Further applications include TGP development studies, where IVRT is recognized as one of the predictive tools to document the extended concept of pharmaceutical equivalence (Dandamudi, 2017; EMA, 2018b; FDA, 1997).
Instrument	Franz Cells	Franz cells were especially developed to study topical delivery by mimicking *in vivo* conditions (temperature, relative humidity and sink conditions). Each diffusion cell is constituted by a donor compartment, where the topical formulation is to be applied, a receptor compartment that entails the receptor solution, and a membrane, from synthetic or biological origin, that separates both chambers (Benson and Watkinson, 2012; OECD, 2010).
IVRT performance attributes	Membrane inertness, sink conditions, linearity, precision, specificity, reproducibility, discriminatory power and robustness.	The method should meet the formal validation criteria (EMA, 2018b).
Application	Release behaviour assessment	The developed method should enable the determination of a sensitive and responsive release profile, able to reflect in detail the formulation physicochemical characteristics.
IVRT critical analytical attributes (CAA)	IVRR, Cumulative amount released at an initial time point ($Q_{initial}$), Cumulative amount released in the end of the experiment (Q_{final}) and dose depletion (DD)	These CAA should reflect the maximization of the release profile.

4.2.2.2 Risk assessment

According to prior knowledge, it was possible to identify several analytical settings, which may have a direct repercussion on the outcome of IVRT. Taken this data into account, the initial step for the development of an aQbD approach to IVRT method development was the construction of a cause-effect diagram, commonly referred to as Ishikawa diagram, see Fig.4.2. The selection of DoE experiments was thus, based on this risk analysis.

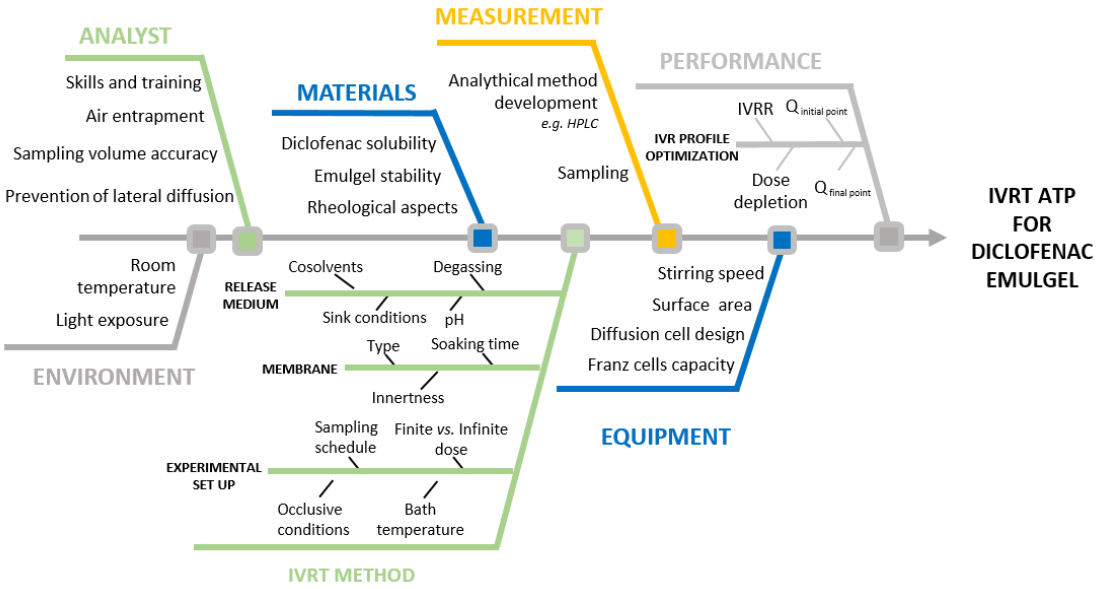


Fig.4.2 – Hypothetical Ishikawa diagram to analytical target development of an IVRT, especially designed for diclofenac emulgel.

4.2.2.3 Franz cell receptor fluid screening

Screening different receptor medium was one of the major considerations of the present study, since their physicochemical characteristics can have a profound impact on the compound solubility. Moreover, the maintenance of sink conditions is crucial throughout the release experiments (Baert *et al.*, 2010). In this context, the effect of pH and cosolvents was screened through preliminary solubility studies. Three pH were selected: 3.6, 5.5 and 7.4, as well as the following three release medium: PBS, PBS-ethanol (80:20, v/v) and PBS-propylene glycol (80:20, v/v) (Baert *et al.*, 2010; Drug Bank, 2018).

Briefly, 60-100 mg of sodium diclofenac were weighted into eppendorfs, and 2 mL of the tested medium added. These suspensions were kept under stirring for 24 hours at 37 ± 0.1ºC, after which they were subjected to centrifugation for 10 min at 11740 x g in a Minispin (Eppendorf Ibérica S.L., Madrid, Spain). The supernatant was collected, filtered by a 0.22 µm

membrane and diluted 160 times with mobile phase. The solubility of diclofenac was then determined using HPLC.

The solubility of the active substance in the different medium was statistically compared using a two-way analysis of variance (ANOVA), with a Tukey multiple comparison test. The differences between the means were considered to be significant for values of $p<0.05$. The statistical test was applied using GraphPad Prism, version 6.01 (GraphPad Software, San Diego, CA, USA).

4.2.2.4 DoE for the IVRT method

A 3x2x3 full factorial design was performed for the optimization of an IVRT for a diclofenac emulgel formulation. The design aimed at assessing the impact of different IVRT critical method variables. The selected CMV include the release medium composition, membrane type and dosage regimen. Having those in mind, three different release medium were selected (PBS-ethanol, PBS-propylene glycol and PBS), two different membranes (Dialysis and Tuffryn) and three dosage regimens (300, 600 and 900mg), that refer to an infinite dose setting. Table 4.2 describes the analytical settings of the IVRT experiments.

Table 4.2 – Experimental design matrix according to a 3x2x3 full factorial design.

Experiment	Dosage regimen	Membrane type	Release medium
1	300 mg	Dialysis	PBS
2	300 mg	Dialysis	PBS-OH
3	300 mg	Dialysis	PBS-PPG
4	300 mg	Tuffryn	PBS
5	300 mg	Tuffryn	PBS-OH
6	300 mg	Tuffryn	PBS-PPG
7	600 mg	Dialysis	PBS
8	600 mg	Dialysis	PBS-OH
9	600 mg	Dialysis	PBS-PPG
10	600 mg	Tuffryn	PBS
11	600 mg	Tuffryn	PBS-OH
12	600 mg	Tuffryn	PBS-PPG
13	900 mg	Dialysis	PBS
14	900 mg	Dialysis	PBS-OH
15	900 mg	Dialysis	PBS-PPG
16	900 mg	Tuffryn	PBS
17	900 mg	Tuffryn	PBS-OH
18	900 mg	Tuffryn	PBS-PPG

A total of 18 autonomous experiments (3 replicates per experiment) were conducted to determine the impact of the selected CMV on the responses. A 3x2x3 full factorial design envisions the in-depth analysis of the impact and interactions between the independent parameters of the IVRT and the selected responses.

The experimental design and the polynomial models were solved resorting to JMP Pro software. These models were used to describe the influence of each factor and to check for potential synergisms between them.

ANOVA and Student's t-test were applied to test pair-wise multiple comparisons. A value of $p<0.05$ was considered statistically significant.

4.2.2.5 *In vitro* release test method

The IVRT method was conducted using static vertical Franz diffusion cells (PermeGear, Inc., PA, USA) with a diffusion area of 0.636 cm^2 and a receptor compartment of 5 mL. IVRT studies were conducted considering a hydrocortisone cream (laboratory qualification purpose), and different strengths of diclofenac emulgel formulations (method optimization).

Qualification

The IVRT method used for the hydrocortisone cream followed the indications provided in the Topical and Transdermal Drug Products — Product Performance Tests section of USP (USP, 2009). Briefly, 300 mg of hydrocortisone cream were applied in the donor compartment, which was separated from the receptor compartment by a polysulfone membrane (HT – 200 Tuffryn diameter 25 mm, pore size 0.45 µm, Pall Corporation, USA), previously soaked in purified water for 30 min. The receptor medium comprised a water:ethanol mixture (70:30, v/v), continuously stirred at 600 rpm and maintained at a temperature of 32^{o}C by means of a circulating water bath. Before the release experiments, the system was allowed to equilibrate at least for 30 min. Samples of the receptor phase (300 µL) were withdrawn at 0.5, 1, 2, 3, 4, 5 and 6 h, and analysed through HPLC. After each collection, the same volume of medium was replaced with preheated receptor solution.

Method optimization

Regarding the IVRT experiments used for diclofenac emulgel, the impact of the dosage regimen, release medium and membrane was evaluated.

Specifically, 300 mg, 600 mg or 900 mg of diclofenac emulgel were evenly applied in the donor compartment. The impact of membranes in the release profile was assessed by using a dialysis cellulose membrane (MWCO 14,000, avg. flat width 33 mm, D9652-100FT, Sigma–Aldrich) or the previously mentioned Tuffryn membranes. The following release medium were considered: PBS, PBS:ethanol (80:20, v/v) and PBS:propylene glycol (80:20, v/v), maintained at 37ºC (assuring 32ºC at membrane surface) and stirred at 600 rpm. Samples (300 µL) were collected at 15, 30, 45, 60, 75, 90, 120, 150 and 180 min, followed by replenishing with the same volume. For the validation studies, additional time points were considered – 15, 30, 45, 60, 75, 90, 120, 150, 180 min, 4 h, 6 h, 8 h, 12 h and 24 h. The main rationale concerning the extended IVRT timeframe was to try to achieve at least 70% of drug released, in agreement with the new EMA draft guideline. (EMA, 2018b). Diclofenac concentration in the receptor medium samples was analysed by HPLC.

Calculations

The cumulative amount of hydrocortisone and diclofenac released as a function of time was calculated in relation to the amount of formulation placed in the donor compartment using:

$$Qn = (Cn \times Vo + \sum_{i=1}^{n=1} Ci \times Vi)/A \quad (4.1)$$

Where C_n corresponds to the drug concentration of the receptor medium at each sampling time, C_i to the drug concentration of the i^{th} sample, A to the effective diffusion area, and V_0 and V_i to the volumes of the receptor compartment and the collected sample, respectively. The release rates were calculated from the slope of the regression line obtained by plotting the cumulative amount of drug diffused per cm^2 versus the square root of time (FDA, 1997; Krishnaiah *et al.*, 2014; Tiffner *et al.*, 2018).

The percentage of drug released was also calculated in order to characterize the extent of dose depletion during IVRT. For this purpose, the average cumulative amount released at the last sampling point (Q_f) was divided by the actual amount of API placed in the diffusion cell, and multiplied by 100.

4.2.2.6 IVRT validation studies

The main purpose of the present work was to establish a framework based on aQbD principles to develop a reliable IVRT method. Therefore, validation studies able to verify the applicability of the purposed strategy were performed.

The general strategy adopted for these validation studies followed the recently published draft guidance on quality and equivalence of topical products (EMA, 2018b), the guideline on

quality of transdermal patches (EMA, 2014d), the draft guidance on acyclovir (FDA, 2016c) and also the work carried out by Tiffner *et al.* (Tiffner *et al.*, 2018). According to the literature, validation studies of IVRT should include IVRT laboratory qualification studies, as well as IVRT method validation studies, in which membrane inertness, linearity, precision, sensitivity, discriminatory power and robustness are to be determined.

Laboratory qualification

Laboratory qualification studies can be further divided into (i) diffusional cell system qualification, and (ii) IVRT method qualification. The qualification of the diffusional system aims at assessing all critical apparatus parameters, which include diffusional area and volume, dispensed sampling volume and temperature at the membrane surface. In addition to these physical parameters, it is also important to qualify the laboratory´s capabilities to perform IVRT. For that, an IVRT using a hydrocortisone cream should be performed and analysed, as described in the USP. The main purpose of this test is to evaluate intra-run, inter-run variabilities and also hydrocortisone sameness testing. If the IVRT procedure is able to establish equivalence of the hydrocortisone cream against itself, the laboratories capabilities to perform IVRT are confirmed (Tiffner *et al.*, 2018; USP, 2009).

In light of the work developed by Tiffner *et al*, and the latest requirements presented by the FDA in the acyclovir draft guidance, all these parameters should have restricted acceptance criteria, since they have a direct repercussion in the cumulative amount released (Q_n), see Equation 4.1 (FDA, 2016c; Tiffner *et al.*, 2018). To assess the receptor chamber capacity of each Franz cell, the donor compartment and membrane were clamped to the receptor chamber and the stirring bar was placed within the cell. The Franz cells were subsequently filled with purified water and the weight increase was recorded. A similar strategy was adopted to evaluate the dispensed sampling volume. The receptor chamber diameters of the receptor compartment were measured using a *vernier caliper* (Vogel, Germany). Moreover, the temperature at the membrane surface was measured in all 18 diffusion cells with a digital thermometer after a 30 min equilibration period. To comply with the guidelines, membrane surface should be at 32ºC, which corresponds to a diffusional system set to 37ºC. All physical parameters were measured in triplicate (FDA, 2016c; Tiffner *et al.*, 2018; USP, 2009).

Hydrocortisone IVRT experiments were conducted as previously described in the section 4.2.2.5. The main purpose of this test was to evaluate the equivalence of two IVRT runs performed on two different days, considering an n=6 (USP, 2009). RSD values below 15% were considered to be indicative of good reproducibility. For product sameness testing, the Wilcoxon Rank Sum/Mann-Whitney statistical test was applied, following SUPAC-SS

recommendations (FDA, 1998). Due to the new EMA draft guidance, a confidence interval of 90 – 111% was established as the acceptance criteria (EMA, 2018b).

IVRT Method validation

Membrane inertness

Possible interactions with the most selective membrane found during optimization studies and diclofenac were screened in membrane inertness studies. Three Tuffryn membranes were incubated in 10 mL of a 35 μg/mL diclofenac solution (PBS-Ethanol pH=7.4) at 32 ± 1°C for 24 h, the selected time frame for the optimized IVRT studies. As control, the same test solution was incubated in the same environmental conditions, without an immersed membrane. To calculate diclofenac recovery, the mean concentration of the membrane samples was divided by the control mean concentration (FDA, 2016c; Tiffner *et al.*, 2018). The membrane was considered to be inert, if at least, a 95% diclofenac recovery was attained.

Linearity, precision and reproducibility

To test linearity, precision and reproducibility, three IVRT runs were conducted, on three different days, each one with a set of 12 vertical diffusion cells (VDC) in order to comply with the new EMA requirements (EMA, 2018b; Tiffner *et al.*, 2018).

A coefficient of determination (R^2) in excess of 0.9 was considered acceptable to demonstrate IVRT method linearity. To determine precision and reproducibility, intra- and inter-run variability were estimated for the release rates (IVRR) and cumulative amount released at the end of the study (Q_f). A RSD of less than 15% was considered acceptable to validate these parameters (Tiffner *et al.*, 2018).

Discriminatory power

One of the most relevant steps during IVRT validation studies is the assessment of the test discriminatory capacity, in other words, the ability of the method to discriminate between similar formulations. This assessment includes the documentation of the following validation indicators: sensitivity, specificity and selectivity (EMA, 2018b, 2014a; FDA, 2016c). For that, the IVRR and the Q_f from three different strength diclofenac emulgel (2%, 1% and 0.5%) were investigated. Please note that the 0.5% dosage was obtained by dilution of the RP. Even though this formulation did not follow the same manufacturing procedures, it enabled the assessment of the method discriminatory ability to products with different critical quality attributes. In this case, it was possible to assess a product with a different rheological profile,

different critical manufacturing variables, different quantitative excipient composition, as well as different active substance strengths (EMA, 2018b).

The IVRT method was considered to be sensitive, if the mean diclofenac IVRR was lower for the 0.5% test cream, when compared to the 1% diclofenac emulgel, and if the mean diclofenac IVRR was higher for the 2% diclofenac cream, when compared to the 1% formulation (FDA, 2016c; Tiffner *et al.*, 2018).

On the other hand, the specificity of the method was evaluated by assessing whether the IVRR of the three formulations (0.5, 1 and 2%) were able to reflect the different concentration levels. A linear regression model of the IVRR as dependent variable by the diclofenac concentration as the independent variable was used to estimate R^2. The method was considered to be specific if the R^2 was larger than 0.9 (FDA, 2016c; Tiffner *et al.*, 2018).

Finally, the selectivity of the method was assessed using the Mann-Whitney statistical test. For selectivity to register, the IVRR attained with each concentration level (0.5, 1 and 2%) could not be within the 90-111% confidence interval, as required by the new guideline on equivalence of topical products (EMA, 2018b). A minimum of 12 replicates were considered for each formulation. Example calculations are provided in Appendix B.

Robustness

To assess method robustness, two IVRT runs (n=12 VDC each) were performed with minor temperature differences, +2ºC and -2ºC, relative to the IVRT pre-established nominal temperature – 37ºC. The method was considered to be robust, if the IVRR and Q_f did not deviate more than 15% from the mean release rate at nominal method parameter settings.

HPLC-UV method validation

The quantification of diclofenac and hydrocortisone was performed by validated HPLC methods. Experimental procedures and results are provided in Appendix A.

4.3 Results and discussion

4.3.1 Establishing a cause-effect relationship, risk assessment and risk management approach to IVRT method optimization

The choice of variables is a task of paramount importance in any aQbD approach, since it conditions both results and interpretation (Vitorino *et al.*, 2011). In order to assess and

characterize the responsiveness of the IVRT method, the following responses, or independent variables, were considered: *in vitro* release rate (IVRR), cumulative amount released at 30 min (Q_i), cumulative amount released at 3 h (Q_f) and dose depletion (percentage of drug released from the system to the medium). Based on these variables and according to prior knowledge, a risk estimation matrix regarding the most prominent CMV which affect IVRT outcomes was equated, see Table 4.3.

Table 4.3 – Initial risk assessment for IVRT method optimization.

Criticality						
Critical method variables (CMV)		**IVRR**	**Q_i**	**Q_f**	**Mass depletion**	**Justification**
Analyst	Prevention of lateral diffusion	Medium	Medium	Medium	Medium	Diffusion cell capacity variability will affect the reproducibility of IVRT. This CMV can be effectively controlled by the use of support disks, placed between the membrane and the receptor compartment, as well as an adequate alignment between the donor and receptor compartment. Therefore, this CMV was ranked as medium impact variable.
	Sampling volume	Medium	Medium	Medium	Medium	Sampling is performed through a positive displacement technique that makes aliquot withdrawal and replacement easier and more effective. A reproducible sampling volume is required to attain an accurate determination of the cumulative drug amount released into the receptor medium. Compliance of this variable is established throughout laboratory qualification studies (Kikwai *et al.*, 2012; Naik *et al.*, 2016). Accordingly, this parameter is bind to regulatory defined acceptance criteria. These should be determined during IVRT laboratory qualification studies. This CMV was ranked as a medium impacting variable.
	Air entrapment	Medium	Medium	Medium	Medium	In general, air bubbles are one of the CMV which account for IVRT variability. The presence of air alters the receptor volume of the diffusion cell, besides hampering the close contact between the formulation loading membrane and the release medium. This, in turn, will introduce bias in the observed drug release pattern. Release medium degassing, as well as periodically inspection of air bubbles throughout the IVRT study, can help controlling this CMV. Nevertheless, this is a medium impact CMV.
Equipment	Surface area	Medium	Medium	Medium	Medium	The surface area is crucial for all drug cumulative amount calculations. Therefore, it is important to assure a low inter variability for the used diffusion cells. In this context, this parameter was ranked as a medium impacting CMV, and will be properly explored during IVRT validation studies.
	Bath temperature	Low	Low	Low	Low	A circulating water bath is used to maintain a temperature of 32 ± 1°C at the membrane surface, to resemble skin surface conditions. As such, water bath temperature should be adjusted to 37°C (Tiffner *et al.*, 2018). Small temperature fluctuations (± 2°C) should not interfere with the selected responses, and for that reason these are commonly tested during IVRT method validation studies, to document the methods robustness. Therefore, this CMV was ranked as a low impact variable.
	Stirring speed	Low	Low	Low	Low	The FDA states that the stirring speed is frequently omitted, being for this reason one of the most common deficiencies present in IVRT method development reports. Several papers report that stirring speed variations do not impose a source of statistically significant alterations in the cumulative amount of drug released. Nevertheless, a very low rotation speed can lead to the presence of stationary layers, resulting in a deficient homogenization and variability in diffusion behaviour (Katragadda, 2018; Kikwai *et al.*, 2012; Naik *et al.*, 2016; Tiffner *et al.*, 2018). Vertical diffusion Franz cells are set up to a fixed

Criticality						
Critical method variables (CMV)		**IVRR**	**Q_i**	**Q_f**	**Mass depletion**	**Justification**
						stirring speed of 600 rpm, which provides an adequate receptor medium agitation. Therefore, this CMV was also ranked as a low impact variable.
	Diffusion cell design	Low	Low	Low	Low	Vertical diffusion cell is a highly prevalent configuration to warrant an unidirectional flux to resemble *ex vivo* permeation conditions (Benson and Watkinson, 2012).
Method conditions	Sampling times	Medium	Medium	Medium	Medium	Should be frequent enough to allow the assessment of the IVRR steady state or pseudo steady state (Benson and Watkinson, 2012).
	Finite *vs.* Infinite dose	High	High	High	High	Finite dose conditions are designed to reproduce in-use conditions, involving the application of a dose that may exhibit marked depletion throughout an experiment. Depletion occurs when the proportion of permeant entering the membrane is larger when compared to the amount applied. Typically, the permeation profile may exhibit a characteristic plateauing effect that accompanies donor depletion (Brain *et al.*, 2002). Per definition of the OECD, finite dose absorption experiments are characterized by the application of 1-10 mg/cm^2 of semisolid formulations. On the other hand, infinite-dose techniques involve the application of a large amount of formulation. As such, changes in the donor compartment, caused by diffusion or evaporation, are considered to be negligible. This is desirable when the experimental objectives include calculation of diffusional parameters or for investigation of mechanisms of penetration enhancement (Brain *et al.*, 2002; Selzer *et al.*, 2013).
	Dose application techniques	Low	Low	Low	Low	A homogeneous layer of drug formulation over the whole diffusion area is desirable to obtain a reliable IVRR estimation and reduce the variability among diffusion cells. Semisolid formulations are usually applied employing a metal, glass or plastic spatula. Nevertheless, the application of a small amount of formulation, in a restricted area, might hamper this procedure. To reduce the impact of this CMV, it is common practice to use a plastic syringe. In this case, the application device should be weighted, before and after, formulation distribution to account for possible losses (Selzer *et al.*, 2013). Since this was a fixed parameter, a low risk is expected.
Medium	Sink conditions maintenance	High	High	High	High	To guarantee that the drug release through the membrane will not be limited by the solubility, sink conditions have to be ensured in the receptor medium (Selzer *et al.*, 2013). Typically, the receptor medium comprises physiological saline or PBS solutions. Cosolvents may be added to release medium to ensure an adequate solubility, especially for lipophilic active substances. This CMV has to be carefully considered, since it will affect all the CAA (Baert *et al.*, 2010).
	Co-solvents	High	High	High	High	
	pH	High	High	High	High	pH adjustment in the receptor medium may increase the solubility of the drug. However, changing the pH value far from the physiological pH of the skin should not be performed, since it will not be predictive of an *in vivo* situation (Selzer *et al.*, 2013).

Criticality						
Critical method variables (CMV)		**IVRR**	**Q_i**	**Q_f**	**Mass depletion**	**Justification**
	Degassing effect	Medium	Medium	Medium	Medium	See air entrapment justification.
Membrane	Type	High	High	High	High	Should provide an inert holding surface for the formulation, but not a barrier for drug release. Should not contain any leachables. Should provide good linearity and precision of the IVRR. Since there are various types of commercial porous membranes on the market, each type may have different drug diffusion properties. For this reason, this CMV should be carefully selected (Katragadda, 2018; Klein *et al.*, 2002; Ng *et al.*, 2012).
	Pre-soaking time	Low	Low	Low	Low	Membrane should be perfectly saturated before the beginning of the IVRT experiment. A soaking time of 30 min is generally considered acceptable (Kikwai *et al.*, 2012). This parameter was kept constant; therefore, it is considered a low risk CMV.

Key: CAA – critical analytical attribute; CMV – critical method variables; IVRR – *in vitro* release rate; Q_i – cumulative drug amount released at 0.5 h; Q_f – cumulative drug amount released in the end of the experiments.

4.3.2 Franz cell receptor fluid screening

The choice of the receptor solution is of the utmost importance when designing an IVRT experiment, as the receptor solution is used as a potential alternative to biorelevant medium (Bou-Chacra *et al.*, 2017). Therefore, determining the solubility of the compound of interest in the potential receptor solutions should be a primary consideration prior to the IVRT study (Benson and Watkinson, 2012).

As previously mentioned, the impact of 2 cosolvents (ethanol and propylene glycol), and three pH values (3.6, 5.5 and 7.4) in diclofenac solubility was assessed. Ethanol was herein selected due to its prevalence in the literature, whilst the selection of propylene glycol was mainly related with the presence of this component in the qualitative formulation of Voltaren emulgel®. Regarding pH values, these were mainly selected taking into account diclofenac pKa (4.00) and the diclofenac pH-solubility profile (Chemaxon, 2019; Drug Bank, 2018). At a 3.6 pH, approximately 50% of diclofenac is in the ionized form. The most consistent pH for topical administration is 5.5, therefore this value was included in these preliminary studies. Since diclofenac solubility is favoured by alkaline medium, a pH of 7.4 was also tested. Solubility results are summarized in Table 4.4.

Table 4.4 – Mean amount of sodium diclofenac dissolved in each medium (mg/mL). The results are expressed as mean ± standard error of the mean (SEM) (n=7). Since the data is normally distributed (Shapiro-Wilk normality test), a two-way ANOVA, with a Tukey multiple comparison test was used to statistically compare the means. The differences among the means were considered significant for values of $p<0.1$.

PBS			PBS-Ethanol			PBS-PPG		
pH = 3.6	**pH = 5.5**	**pH = 7.4**	**pH = 3.6**	**pH = 5.5**	**pH = 7.4**	**pH = 3.6**	**pH = 5.5**	**pH = 7.4**
6.5 ± 0.5	6.5 ± 0.4	8.2± 0.7	16 ± 2	18 ± 1	14.5 ± 0.5	14 ± 2	13 ± 2	12 ± 2

Key: PBS – Phosphate buffered saline; PBS-OH – Phosphate buffered saline: ethanol (80:20, v/v); PBS-PPG – Phosphate buffered saline: propylene glycol (80:20, v/v).

The use of cosolvents significantly increased the solubility of diclofenac, with ethanol based medium displaying the highest solubility results. Regarding the three tested pH values, these do not seem to have a significant impact on diclofenac solubility, even though, theoretically, the solubility of diclofenac is favoured by more alkaline medium.

Even though the use of cosolvents in *in vitro* permeation testing is not advisable by the regulatory authorities, the same regulatory reluctance does not apply to IVRT. Reasons which might ground this occurrence relate with the very low solubility of several active substances (Baert *et al.*, 2010; Montenegro *et al.*, 2016). Historically, ethanol-based receptor medium are

often used in IVRT. However, the use of other surfactants/cosolvents, such as propylenoglycol, Tween 20® or Brij98® are also described (Baert *et al.*, 2010; Barradas *et al.*, 2016; Bou-Chacra *et al.*, 2017; Narkar, 2010; Vitorino *et al.*, 2015).

4.3.3 aQbD-based IVRT method optimization

Based on the risk assessment analysis, there are clearly three main CMV that require further investigation when implementing an IVRT method: release medium, membrane and dosage regimen.

To assess the impact of these factors on IVRT, the 3x2x3 full factorial design was performed. For release medium, three different conditions were selected (PBS-ethanol, PBS-propylene glycol and PBS). Regarding membrane, both dialysis and Tuffryn membranes were chosen. Finally, the impact of three dosage regimens was assessed: 300 mg, 600 mg and 900 mg.

Per definition of the OECD, finite dose experiments are meant to mimic 'in-use' conditions and require a product application up to 10 mg/cm^2 (OECD, 2004). On the other hand, infinite-dose techniques involve the application of a large amount of formulation (more than 10 mg/cm^2 of formulation) (OECD, 2004). As such, changes in the donor compartment, caused by diffusion or evaporation, are considered to be negligible. This is desirable when the experimental objectives include the determination of the steady-state conditions from which the IVRR (µg/cm^2/√h) is retrieved. As previously mentioned, the determination of the IVRR is one of the most relevant outputs retrieved from IVRT studies, since it provides important information on the formulation microstructure characteristics. Therefore, all studies were performed under infinite dose conditions.

The obtained DoE results are summarized in Table 4.5.

After collecting responses, suitable mathematical models were fitted to calculate the coefficient values, see Table 4.6. Note that the present factorial design considers categorical as well as continuous variables. Moreover, different levels of each variable were included, 2 referring to the membranes, and 3 pertaining both to the release medium and dosage regimen.

Table 4.5 – Observed and predicted values for the 3x2x3 DoE. Observed values were presented as the mean of (n=3).

Runs	Dose	Membrane	Release medium	Observed Qi	Predicted Qi	Observed IVRR	Predicted IVRR	Observed Q_f	Predicted Q_f	Observed DD	Predicted DD
1	300	Dialysis	PBS	103.28	82.50	323.98	325.92	448.54	430.08	9.18	8.88
2	300	Dialysis	PBS-OH	129.29	125.09	422.75	384.90	574.82	529.25	11.40	10.66
3	300	Dialysis	PBS-PPG	105.46	97.93	408.44	347.15	544.03	466.45	10.85	9.33
4	300	Tuffryn	PBS	144.95	137.04	466.97	411.99	624.47	607.62	13.19	12.24
5	300	Tuffryn	PBS-OH	213.64	198.62	439.71	435.56	660.86	637.11	14.03	13.11
6	300	Tuffryn	PBS-PPG	153.58	140.00	448.57	403.78	621.79	558.10	11.82	11.21
7	600	Dialysis	PBS	73.58	101.07	342.29	346.31	437.19	467.43	4.72	5.85
8	600	Dialysis	PBS-OH	155.82	185.88	421.64	487.44	595.67	695.57	6.43	8.18
9	600	Dialysis	PBS-PPG	102.84	110.31	384.60	509.16	504.29	657.37	5.28	7.54
10	600	Tuffryn	PBS	200.87	175.73	459.02	452.76	658.18	681.39	6.82	8.55
11	600	Tuffryn	PBS-OH	271.13	279.53	540.28	558.47	801.12	839.85	8.40	9.97
12	600	Tuffryn	PBS-PPG	137.78	172.50	498.57	586.17	655.96	785.43	6.75	8.76
13	900	Dialysis	PBS	126.35	119.65	372.66	366.70	516.57	504.79	3.66	2.83
14	900	Dialysis	PBS-OH	272.53	246.67	617.92	589.97	916.22	861.89	6.70	5.69
15	900	Dialysis	PBS-PPG	122.64	122.69	734.45	671.17	923.78	848.28	6.49	5.75
16	900	Tuffryn	PBS	236.41	214.43	540.61	493.53	778.68	755.16	5.31	4.87
17	900	Tuffryn	PBS-OH	353.82	360.44	695.44	681.39	1057.57	1042.58	7.47	6.82
18	900	Tuffryn	PBS-PPG	226.15	205.00	811.37	768.57	1078.54	1012.76	7.70	6.31

Key: IVRR – *In vitro* release rate; Q_i – Cumulative amount released at 0.5 h; Q_f – Cumulative amount released in the end of the experiments; DD – Dose depletion; PBS – Phosphate buffered saline; PBS-OH – PBS Ethanol: (80:20; PBS-PPG – PBS-Propylene glycol (80:20).

Table 4.6 – Coefficient values of IVRR, cumulative amount of diclofenac released at an initial time point (Q_i), cumulative amount of diclofenac released in the final time point (Q_f) and dose depletion (DD).

$IVRR = 284.93 + 0.35 \times Dose + Membrane\begin{Bmatrix} Tuffryn = 47.63 \\ Dyalisis = -47.63 \end{Bmatrix} + \begin{Bmatrix} PBS:PPG = 52.4 \\ PBS = -80.08 \\ PBS:OH = 27.68 \end{Bmatrix} + Dose \times Membrane\begin{Bmatrix} Tuffryn = 0.04 \\ Dyalisis = -0.04 \end{Bmatrix} + Dose \times \begin{Bmatrix} PBS:PPG = 0.22 \\ PBS = -0.24 \\ PBS:OH = 0.025 \end{Bmatrix} + Membrane\begin{Bmatrix} Tuffryn \times \begin{Bmatrix} PBS:PPG = -9.12 \\ PBS = 21.24 \\ PBS:OH = -12.14 \end{Bmatrix} \\ Dyalisis \times \begin{Bmatrix} PBS:PPG = -9.12 \\ PBS = -21.24 \\ PBS:OH = 12.14 \end{Bmatrix} \end{Bmatrix}$
$Q_i = 92.46 + 0.135 \times Dose + Membrane\begin{Bmatrix} Tuffryn = 41.33 \\ Dyalisis = -41.33 \end{Bmatrix} + \begin{Bmatrix} PBS:PPG = -32.45 \\ PBS = -26.61 \\ PBS:OH = 58.95 \end{Bmatrix} + Dose \times Membrane\begin{Bmatrix} Tuffryn = 0.0335 \\ Dyalisis = -0.033 \end{Bmatrix} + Dose \times \begin{Bmatrix} PBS:PPG = 0.06 \\ PBS = -0.04 \\ PBS:OH = 0.1 \end{Bmatrix} + Membrane\begin{Bmatrix} Tuffryn \times \begin{Bmatrix} PBS:PPG = -10.23 \\ PBS = 4.73 \\ PBS:OH = 5.5 \end{Bmatrix} \\ Dyalisis \times \begin{Bmatrix} PBS:PPG = 10.23 \\ PBS = -4.74 \\ PBS:OH = -5.5 \end{Bmatrix} \end{Bmatrix}$
$Q_f = 388.36 + 0.499 \times Dose + Membrane\begin{Bmatrix} Tuffryn = 81.05 \\ Dyalisis = -81.05 \end{Bmatrix} + \begin{Bmatrix} PBS:PPG = 33.59 \\ PBS = -113.43 \\ PBS:OH = 79.89 \end{Bmatrix} + Dose \times Membrane\begin{Bmatrix} Tuffryn = 0.06 \\ Dyalisis = -0.06 \end{Bmatrix} + Dose \times \begin{Bmatrix} PBS:PPG = 0.197 \\ PBS = -0.32 \\ PBS:OH = 0.12 \end{Bmatrix} + Membrane\begin{Bmatrix} Tuffryn \times \begin{Bmatrix} PBS:PPG = -17.02 \\ PBS = 25.93 \\ PBS:OH = -8.911 \end{Bmatrix} \\ Dyalisis \times \begin{Bmatrix} PBS:PPG = 17.02 \\ PBS = -25.93 \\ PBS:OH = 8.91 \end{Bmatrix} \end{Bmatrix}$
$DD = 13.68 + (-0.009 \times Dose) + Membrane\begin{Bmatrix} Tuffryn = 0.95 \\ Dyalisis = -0.95 \end{Bmatrix} + \begin{Bmatrix} PBS:PPG = 0.006 \\ PBS = -0.94 \\ PBS:OH = 0.93 \end{Bmatrix} + Dose \times Membrane\begin{Bmatrix} Tuffryn = -0.001 \\ Dyalisis = 0.001 \end{Bmatrix} + Dose \times \begin{Bmatrix} PBS:PPG = 0.002 \\ PBS = -0.002 \\ PBS:OH = -0.00017 \end{Bmatrix} + Membrane\begin{Bmatrix} Tuffryn \times \begin{Bmatrix} PBS:PPG = -0.34 \\ PBS = 0.39 \\ PBS:OH = -0.056 \end{Bmatrix} \\ Dyalisis \times \begin{Bmatrix} PBS:PPG = 0.341 \\ PBS = -0.39 \\ PBS:OH = 0.056 \end{Bmatrix} \end{Bmatrix}$

To correctly interpret the equations that describe the behaviour of the CAA, it is important to consider that a higher coefficient magnitude indicates a stronger effect on the system, whilst a negative coefficient bears the opposite system trend. In other words, the higher the coefficient, the higher is the influence of that variable, either positively or negatively (Basso *et al.*, 2018; Carla Vitorino *et al.*, 2013).

As an example for the use of DoE expressions considering Table 4.6, the calculation of the mean IVRR for dose 300 mg, Tuffryn membranes and solvent PBS-OH, requires:

$$\boldsymbol{IVRR} = 284.96 + 0.35 \times 300 + 47.63 + 27.68 + 300 \times 0.04 + 300 \times 0.025 - 12.14$$

4.3.3.1 Analysing the CAA

As aforementioned, IVRR is one of the crucial outcomes of IVRT. This parameter corresponds to the slope of the regression line obtained by plotting the cumulative amount of drug diffused per cm^2 versus the square root of time (FDA, 1997; Krishnaiah *et al.*, 2014; Tiffner *et al.*, 2018). The release rate is formulation specific and therefore a critical quality attribute of the formulation (Dandamudi, 2017).

Taking into account that the "dose regimen" has to be multiplied by the corresponding value (300, 600 or 900 mg), this CMV plays a major role in the IVRR. As expected, increasing the amount of formulation in the system translates into a maximization of the IVRR.

In what concerns the membrane and release medium, Tuffryn membranes and the addition of cosolvents (either ethanol or propylene glycol), promote a maximization of the IVRR, as suggested by the positive magnitude of both coefficients.

Q_i and Q_f, as previously mentioned, represent the cumulative amount of diclofenac released at 0.5 h and 3 h, respectively. In line with the requirements for *in vitro* drug release/dissolution methods, the selection of these variables as CAA has taken into account the draft guideline on quality and equivalence of topical products, the guideline on quality of oral modified release products and the guideline on the quality of transdermal patches (EMA, 2014d, 2014a). Accordingly, it is stated that whenever carrying out dissolution studies, the inclusion of both an early and later point should be made. The first intends to exclude the possibility of dose dumping and to characterize the loading dose. The former intends to demonstrate that the majority of the active substance has been released. Both responses are obviously interconnected with IVRR, nevertheless, in Q_f the magnitude of the coefficients is higher, see Table 4.6.

In both Q_i and Q_f, the dosage regimen is once again the main CMV, and Tuffryn membranes continue to display a higher coefficient magnitude over the dialysis ones, thus enabling a superior diclofenac release. In what concerns the release medium effect, the PBS-OH medium promotes a higher magnitude for both endpoints, when compared to PBS or PBS-PPG medium. This trend is consistent with the solubility assumptions previously presented, exerting a marked positive effect in an early stage.

Mass depletion refers to the percentage of diclofenac released by the end of the IVRT experiment. In line with the previously selected CAAs, this parameter was included since it regards mass balance studies, as reported in the draft guideline on quality and equivalence of topical products and the guidance notes on dermal absorption provided by OECD (EMA, 2014d; OECD, 2004). This is the less impacted response as reflected by the reduced magnitude of the coefficients.

A *t*-test analysis of coefficients indicated that, in the vast majority, parameters are highly significant, see Fig.4.3. Moreover, Table 4.5 presents the observed *vs.* predicted values for each of the CMV and Table 4.7 displays the coefficient terms estimated for the different responses, and respective statistical information.

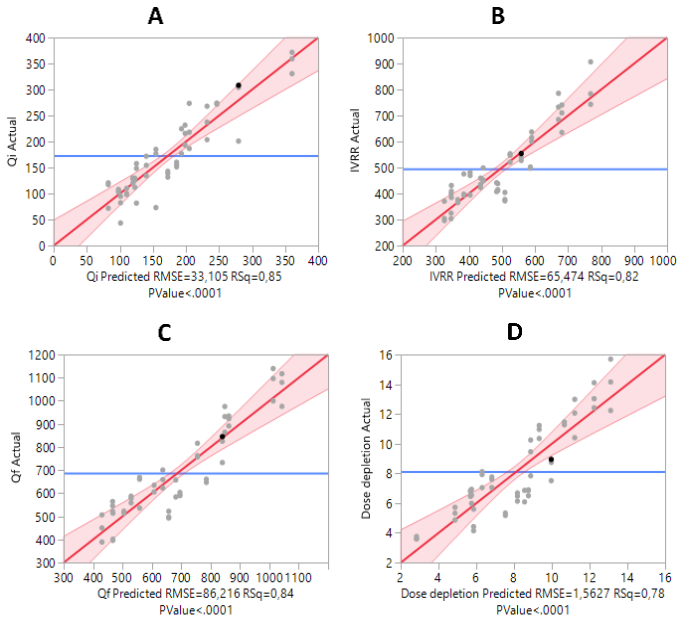


Fig.4.3 – Actual by predicted plots A=Q_i; B=IVRR; C=Q_f and D=Dose depletion. The models for each CMV are well estimated since the confidence curves cross the horizontal line at the mean of the response, and that the r-squared values (R^2) are higher than 0.78 for all responses.

Table 4.7 – Scaled parameters of the response surface for IVRR, Q_i, Q_f and dose depletion obtained from a 3x2x3 full factorial design planning, respective significance and t values.

	IVRR				Qi				Qf				Dose depletion			
Term	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Intercept	495.3	9.0	55.0	<.0001	173.8	4.6	38.2	<.0001	687.8	11.9	58.0	<.0001	8.1	0.2	37.9	<.0001
Dose	105.2	10.9	9.6	<.0001	40.6	5.5	7.4	<.0001	149.7	14.4	10.4	<.0001	-2.8	0.3	-10.6	<.0001
Membrane type[Tuffryn]	47.6	9.0	5.3	<.0001	41.3	4.6	9.1	<.0001	81.1	11.9	6.8	<.0001	1.0	0.2	4.4	<.0001
Membrane type[Dyalisis]	-47.6	9.0	-5.3	<.0001	-41.3	4.6	-9.1	<.0001	-81.1	11.9	-6.8	<.0001	-1.0	0.2	-4.4	<.0001
Release medium[PBS-PPG]	52.4	12.7	4.1	0.0	-32.3	6.4	-5.1	<.0001	33.6	16.7	2.0	0.1	0.0	0.3	0.0	1.0
Release medium[PBS]	-80.1	12.9	-6.2	<.0001	-26.6	6.5	-4.1	0.0	-113.4	16.9	-6.7	<.0001	-0.9	0.3	-3.1	0.0
Release medium[PBS-OH]	27.7	12.7	2.2	0.0	59.0	6.4	9.2	<.0001	79.9	16.7	4.8	<.0001	0.9	0.3	3.1	0.0
(Dose-600)*Membrane type[Tuffryn]	10.2	10.9	0.9	0.4	10.1	5.5	1.8	0.1	18.2	14.4	1.3	0.2	-0.3	0.3	-1.3	0.2
(Dose-600)*Membrane type[Dyalisis]	-10.2	10.9	-0.9	0.4	-10.1	5.5	-1.8	0.1	-18.2	14.4	-1.3	0.2	0.3	0.3	1.3	0.2
(Dose-600)*Release medium[PBS-PPG]	67.0	15.4	4.3	<.0001	-18.2	7.8	-2.3	0.0	59.4	20.3	2.9	0.0	0.6	0.4	1.7	0.1
(Dose-600)*Release medium[PBS]	-74.6	15.4	-4.8	<.0001	-12.0	7.8	-1.5	0.1	-94.2	20.3	-4.6	<.0001	-0.6	0.4	-1.6	0.1
(Dose-600)*Release medium[PBS-OH]	7.6	15.4	0.5	0.6	30.2	7.8	3.9	0.0	34.8	20.3	1.7	0.1	-0.1	0.4	-0.1	0.9
Membrane type[Tuffryn]*Release medium[PBS-PPG]	-9.1	12.7	-0.7	0.5	-10.2	6.4	-1.6	0.1	-17.0	16.7	-1.0	0.3	-0.3	0.3	-1.1	0.3
Membrane type[Tuffryn]*Release medium[PBS]	21.2	12.9	1.7	0.1	4.7	6.5	0.7	0.5	25.9	16.9	1.5	0.1	0.4	0.3	1.3	0.2

	IVRR				Qi				Qf				Dose depletion			
Term	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**	**Scaled Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Membrane type[Tuffryn]*Release medium[PBS-OH]	-12.1	12.7	-1.0	0.3	5.5	6.4	0.9	0.4	-8.9	16.7	-0.5	0.6	-0.1	0.3	-0.2	0.9
Membrane type[Dyalisis]*Release medium[PBS-PPG]	9.1	12.7	0.7	0.5	10.2	6.4	1.6	0.1	17.0	16.7	1.0	0.3	0.3	0.3	1.1	0.3
Membrane type[Dyalisis]*Release medium[PBS]	-21.2	12.9	-1.7	0.1	-4.7	6.5	-0.7	0.5	-25.9	16.9	-1.5	0.1	-0.4	0.3	-1.3	0.2
Membrane type[Dyalisis]*Release medium[PBS-OH]	12.1	12.7	1.0	0.3	-5.5	6.4	-0.9	0.4	8.9	16.7	0.5	0.6	0.1	0.3	0.2	0.9

Evaluation of ANOVA was also performed for model fitness (Table 4.8, Table 4.9 and Fig.4.4).

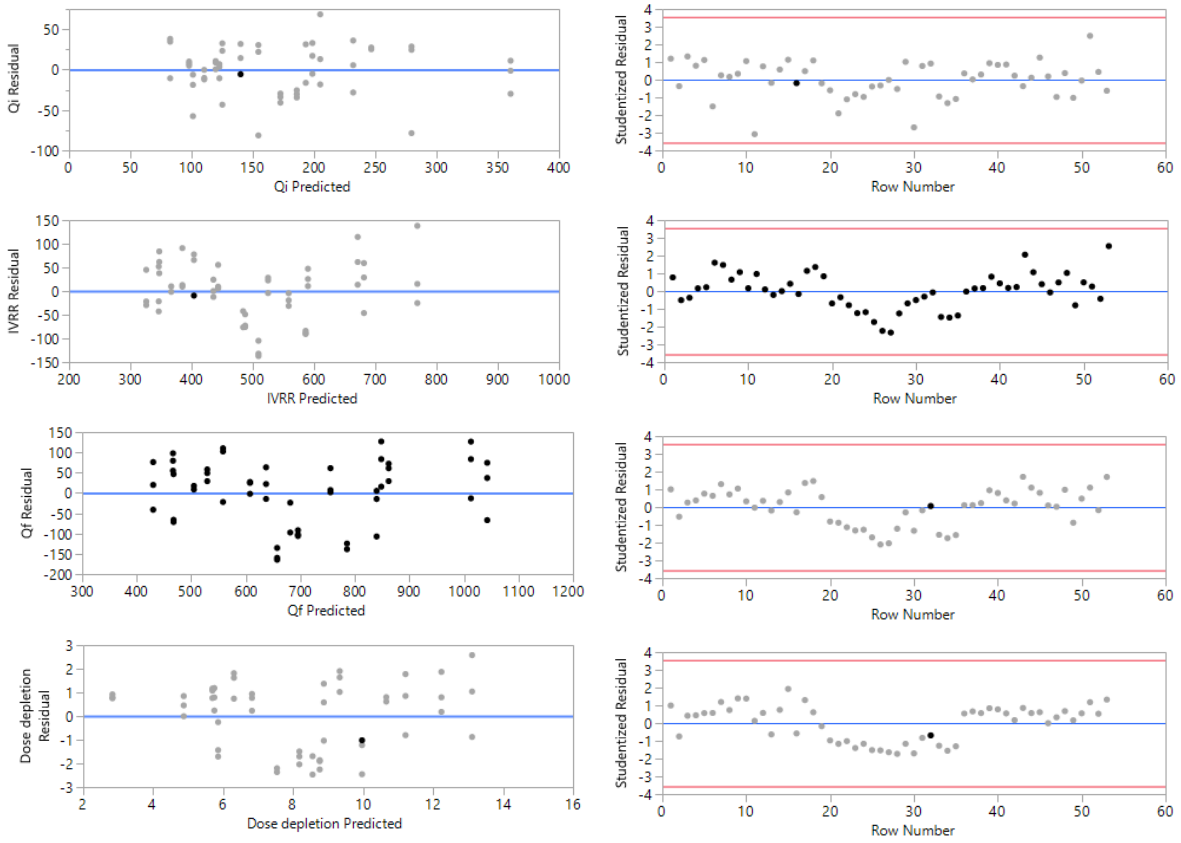


Fig.4.4 – (A) Residual plots according to the response evaluated. A1 = Q_i; A2 = IVRR; A3 = Q_f and A4 = Dose depletion. Ideally, the residual plots against the predicted values should be scattered randomly about zero, however, these value is proportional to the coefficient magnitude. (B) Studentized residuals. B1 = Q_i; B2 = IVRR; B3 = Q_f and B4 = Dose depletion. This statistical test conducts a *t* test for each residual, being for this reason a more effective way to determine outliers and assess the equal variance assumption. Since there are no studentized residuals falling outside the red limits, the model is considered to be suitable.

Table 4.8 – Summary of fit of the selected CMV.

	IVRR	**Qi**	**Q_f**	**Dose Depletion**
R^2	0.82	0.85	0.844	0.78
R^2 adj	0.78	0.82	0.81	0.73
Root Mean Square Error	65.5	33.1	2	1.56
Mean of Response	495	173.4	687	8.13
Observations (or Sum Wgts)	53	53	53	53

Table 4.9 – ANOVA analysis for the selected CMV.

	IVRR			Qi			Q_f			Dose depletion		
Source	**Model**	**Error**	**C. Total**	**Model**	**Error**	**C. Total**	**Model**	**Error**	**C. Total**	**Model**	**Error**	**C. Total**
DF	9	43	52	9	43	52	9	43	52	9	43	52
Sum of Squares	836608	184332	1020941	268595	47125	315720	1722501	319625	2042126	373	105	478
Mean Square	92956	4286		29843	1095		191389	7433		41	2	
F Ratio	21.7			27.23			25.7			16.9		
Prob > F	<0.0001*			<0.0001			<0.0001			<.0001		

All these statistical parameters confirmed the suitability of the selected mathematical model for predicting the responses.

4.3.3.2 What DoE tells us

Aiming at a maximization of all IVRT responses, the desirability approach was used for CMV optimization. Desirability (D) function is defined as the weighted geometric mean for several responses, or alternatively, a value between 0 and 1 per response. A value of D different from zero means that all responses are in a desirable range, and a value close to 1 indicates that the combination of the different criteria is globally optimal. In other words, if D = 1, the response values are close to the target ones (Kalariya *et al.*, 2017; Kamboj and Rana, 2016).

The maximum desirability was found to be 0.62, see Fig.4.5. This refers to a 900 mg dosage regimen, Tuffryn membranes and a PBS-OH release medium.

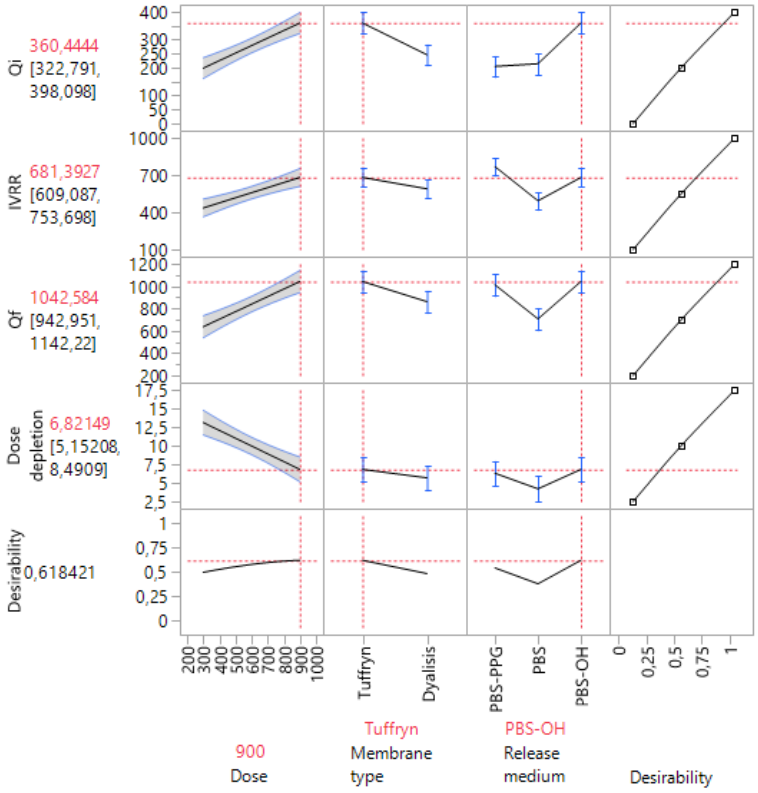


Fig.4.5 – Maximum desirability for IVRT for diclofenac emulgel.

As previously mentioned during coefficient analysis, dialysis membranes offer more resistance to diclofenac release, therefore, tuffryn membranes better fit the purpose of inertness support formulation. A release medium comprising ethanol as cosolvent (PBS-OH, 80:20, v/v), warrants sink conditions, as reflected in the release behaviour which was also previously documented during solubility studies, see Table 4.4.

Regarding the dose, according to the EMA draft guideline on quality and equivalence of topical products, the amount of formulation applied should be consistent (±5% between samples) and validated.

In this work, solely infinite dose conditions were considered, since the attainment of steady-state conditions was mandatory to calculate the kinetic parameters considered throughout the study. This parameter should be carefully addressed while establishing IVRT method development and validation reports. Another aspect that the guideline refers to is the maintenance of pseudo-infinite dose conditions. Therefore, even though a 900 mg dosage regimen replicates optimal settings, a 300 mg depicts a more usable dose. Moreover, according to pharmacopoeial requirements (USP), typically, 200-400 mg of formulation are to be applied for topical drug products performance test (IVRT studies). For these reasons, a 300 mg dosage regimen was considered for validation studies (EMA, 2018b). Note, however, that

testing different donor drug loading amounts is of paramount importance for evaluating method responsiveness. In what follows, the method overall validation is to be addressed.

4.3.4 IVRT Validation Studies

4.3.4.1 Laboratory qualification

The acceptance criteria and results of the laboratory qualification studies are summarized in Table 4.10.

Table 4.10 – Acceptance criteria for laboratory qualification studies based on the USP general chapter, the acyclovir draft guidance and studies from Tiffner *et al* (FDA, 2016c; Tiffner *et al.*, 2018; USP, 2009).

Parameter	Acceptance criteria	Results	Status
Diffusional cell volume	5 ± 0.75 mL Precision (RSD) < 15% Accuracy (Bias) < 15%	Mean = 5.1 ± 0.1 mL RSD = 2.2% Bias = 1.5%	Complies
Receptor compartment diffusional area	0.636 cm^2 RSD and Bias < 15%	Mean = 0.649 cm^2 RSD = 1.2% Bias =2.19%	Complies
Temperature at membrane surface	32 ± 1ºC RSD and bias < 15%	Mean = 32.7 ± 0.4ºC RSD = 1.16% Bias =2.22%	Does not comply
Dispensed sampling volume	0.3 mL RSD and bias < 15%	Mean = 0.29 mL RSD = 1.69% Bias = 3.31%	Complies
Intra-run variability	RSD for each of the hydrocortisone runs (n=6) < 15%	RSD Run 1 = 4.14% RSD Run 2 = 4.42%	Complies with USP requirements
Inter-run variability	RSD for both hydrocortisone runs (n=6) < 15%	RSD = 4.47%	Complies with USP requirements
Hydrocortisone sameness testing	The 90% confidence interval falls with the new EMA requirements – 90-111%	Lower limit: 94.7% Upper limit: 106.5%	Complies with USP requirements

Regarding apparatus parameters (capacity of Franz cells, diffusional area, sampling volume and temperature at membrane surface), most exhibited the predefined acceptance criteria, with the exception of temperature at the membrane surface, which did not met the requirements (FDA, 2016c). Nevertheless, the variation observed (0.08ºC) was not deemed significant. Moreover, since a low inter-cell variability was assured for all parameters, the Franz cells equipment was considered suitable for IVRT.

The results from the hydrocortisone IVRT were also satisfactory, with reduced inter and intra variability. Additionally, from the product sameness test, a confidence interval of 94.7-106.5%

was registered, which is consistent with the new EMA draft guideline requirements (EMA, 2018b; Tiffner *et al.*, 2018; USP, 2009). Please note that these results were attained with the same hydrocortisone cream batch.

4.3.4.2 IVRT method validation

Membrane inertness

Regarding Tuffryn membrane inertness, and similarly to what has been established for other active substances, obtained results indicate that the membrane does not constitute a rate limiting barrier for diclofenac diffusion with an attained 99.4% recovery (Ciolan *et al.*, 2015; Thakker and Chern, 2003; Tiffner *et al.*, 2018).

Linearity, precision and reproducibility

The method proved to be linear (R^2 = 0.98), in accordance to the recent recommendations of both FDA and EMA (EMA, 2018b; FDA, 2016c).

Two outcomes/CAA were considered to assess the precision and reproducibility of the method – IVRR and Q_f. These were screened during three independent IVRT runs (n=12 per analysis) of the same diclofenac formulation batch. Even though the method presented an acceptable intra-run precision and reproducibility (RSD IVRR = 7.8% and RSD Q_f = 7.5%), a slightly different scenario was observed in the inter-run variability (RSD IVRR = 9.8% and RSD Q_f = 10.6%). These results would have been acceptable for HPLC validation studies and also for the FDA IVRT recommendations provided in the acyclovir guidance (RSD < 15%) (FDA, 2016c). However, considering the limits recommended by the new EMA requirements, these inter-variability results are borderline, since only a maximum of 10% inter-run variability is acceptable: "Method intermediate precision for the same batch should be studied with different operators on different days (CV<10%)" (EMA, 2018b).

In addition to this restrictive CV requirement, there are other parameters in the new EMA draft guideline regarding IVRT performance that may condition the translation of the guidance into the practice, such as:

- Attaining 70% release for topical drug products – The majority of topical products do not attain these values. To achieve so, prolonged assays that do not mimic *in vivo* conditions would be in order (Al-Ghabeish *et al.*, 2015; Bao *et al.*, 2017; Fernández-Campos *et al.*, 2017; Goebel *et al.*, 2013; Khanolkar *et al.*, 2017; Krishnaiah *et al.*, 2014; Lauterbach and Müller-

Goymann, 2014; Leal *et al.*, 2017; Nallagundla *et al.*, 2014; Petró *et al.*, 2013; Xu *et al.*, 2015b, 2015a).

- Restrictive confidence intervals (CI). Limiting the CI to 90-111%, instead of 75-133%, may compromise the acceptance of many topical generic products already market approved, as well as many topical reference products due to their intrinsic variability.
- One additional challenge that the guideline purposes is the inclusion of the IVRR as a critical quality attribute (CQA) of the topical product. In light of this requirement there would be the necessity to conduct release experiments on a daily routine basis, which may prove to be too demanding for generic manufacturers.

It is necessary to take into account the intrinsic variability linked to IVRT. As suggested by multiple literature reports, IVRT sources of variability may be caused by a plethora of factors, such as air entrapment, inability to uniformly spread the formulation upon the membrane in Franz cells, difficulty to reproduce the exact amount of formulation loaded in the system (Bao and Burgess, 2018). In order to promote a practical applicability of the extended pharmaceutical equivalence concept, as desired in this European draft guideline, it is imperative to establish more realistic criteria.

Discriminatory power

The documentation of the discrimination ability of IVRT is progressively being recommended by the regulatory authorities, since it is critical to prove that the method assures a proper distinction between batches with acceptable and non-acceptable release characteristics (EMA, 2014d).

The method was able to detect different IVRR according to the strength of the formulations (IVRR 0.5% = 456.74; IVRR 1% = 676.35; IVRR 2% = 913.60) and, for this reason, it proved to be sensitive, see Table 4.11.

The developed IVRT method is specific, since it was possible to establish a linear relationship (R^2 = 0.9721) between strength and IVRR, as illustrated in Fig.4.6.

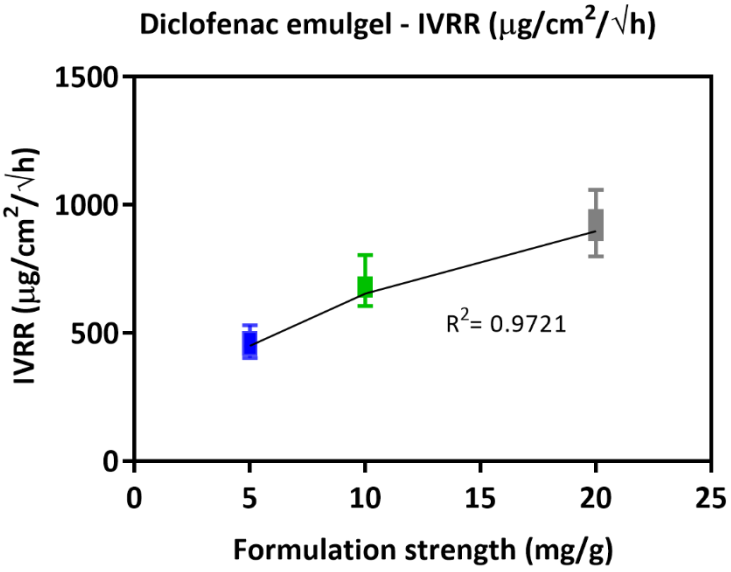


Fig.4.6 – IVRT specificity. Box and Whiskers plot of the measured release rates for the three test diclofenac emulgels.

By calculating the 90% confidence intervals of the 0.5% and the 2% formulations against the reference product (1%), it is possible to establish product inequivalence, which in turn highlights the selectivity of the method. The confidence intervals were calculated based on the Wilcoxon Rank Sum/Mann-Whitney rank test, previously described in the SUPAC-SS guidance, with the proper adaptation to meet the n=12 matrix (FDA, 1997), see table 6. For example calculations please refer to Appendix B.

The test/reference percentages of both test products (2% and 0.5%) fall outside the range 90-111%, when compared to the RP. This highlights the significant differences between the formulations, and the selectivity of this method (EMA, 2018b).

Robustness

The robustness of the method was evaluated by conducting two IVRT runs (n=12 each) with alteration in the temperature (35ºC and 39ºC). Since Franz cells are static, it was not possible to investigate the effect in the IVRR of different mixing rates. In the EMA draft guideline, IVRT robustness assessment not only contemplates evaluating the system response to changes in the temperature and mixing rate, but also regards the evaluation of different amounts of applied formulation and different receptor medium. These latter variables were contemplated in the aQbD strategy developed in this work.

The IVRT was considered to be robust if the resulting mean IVRR for each temperature scenario (35ºC or 39 ºC) did not deviate by more than 15% from the mean release rate at

nominal method parameter settings (37 ºC). The obtained results, see Table 4.11 and Fig.4.7 portrait that the method is able to withstand minor temperature fluctuations without compromising the analysis, thus establishing its robustness.

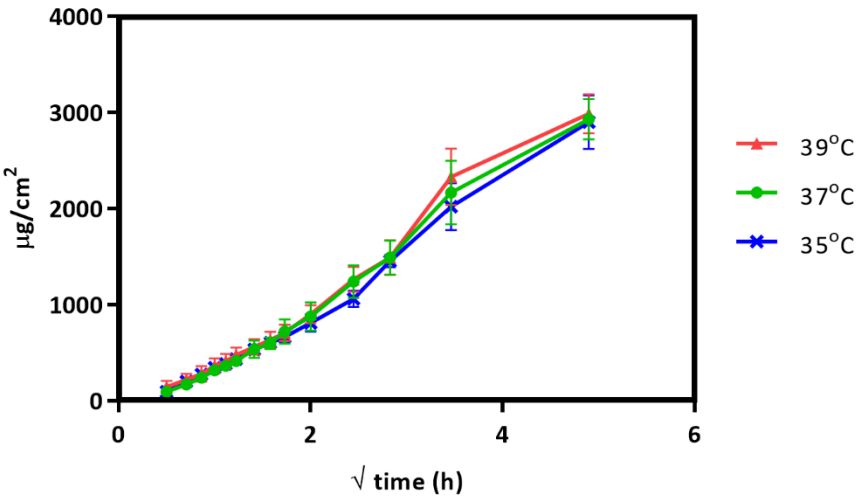


Fig.4.7 – Robustness of IVRT. Mean amount of sodium diclofenac released in each sampling point (µg/mL). The means are expressed as mean ± standard deviation (SD) (n=12).

Table 4.11 – Acceptance criteria for IVRT method validation studies based on the acyclovir draft guidance, the new EMA draft guideline and studies from Tiffner *et al* (FDA, 2016c; Tiffner *et al.*, 2018; USP, 2009).

Parameter	Acceptance criteria	Results	Status
Membrane inertness studies	Recovery ≥ 95%	Recovery = 99.4%	Tuffryn membranes are inert
Linearity	$R^2 > 0.90$	$R^2 = 0.98$	IVRT is linear
Precision and reproducibility	**Intra-run variability:** RSD $Q_f \leq 10\%$ RSD IVRR ≤ 10% **Inter-run variability:** RSD $Q_f \leq 10\%$ RSD IVRR ≤ 10%	**Intra-run variability:** RSD $Q_f = 7.56\%$ RSD IVRR = 8.02% **Inter-run variability:** RSD $Q_f = 9.93\%$ RSD IVRR = 9.98%	
Sensitivity	Different IVRR for different formulations strength RSD ≤ 10%	0.5% Diclofenac IVRR = 456.74 RSD = 9.84 1% Diclofenac (reference product) IVRR = 676.35 RSD = 8.64 2% Diclofenac IVRR = 913.60 RSD = 7.97	IVRT is sensitive
Specificity (IVRR)	$R^2 > 0.90$	$R^2 = 0.9721$	IVRT is specific
Selectivity	Confidence interval (CI) between different strength products, in independent IVRT runs, falls outside the limits [90-111] %	0.5% *vs.* 1% (n=12) CI = [62.71 – 78.61%] 2% *vs.* 1% (n=12) CI = [139.10 – 158.9%]	IVRT is selective
Robustness	Mean IVRR of runs with minor temperature fluctuations should not deviate more than 15% from the IVRR of the nominal method parameter settings	Mean IVRR 37ºC = 676 (8.6%) Mean IVRR 35ºC = 645 (8.6%) Mean IVRR 39ºC = 685 (7.1%)	IVRT is robust

4.3.5 Updated risk assessment

An overall analysis and critical appraisal of the information retrieved from DoE and validation studies, enable to update the previously presented risk matrix, see Table 4.12.

Table 4.12 – Updated risk assessment matrix after IVRT method optimization.

	Criticality					
	Critical analytical attributes (CAA)					Justification
Critical method variables (CMV)		**IVRR**	**Q initial point**	**Q final point**	**Mass depletion**	
Analyst	Prevention of lateral diffusion	Low	Low	Low	Low	According to laboratory qualification studies.
	Sampling volume	Low	Low	Low	Low	
	Air entrapment	Medium	Medium	Medium	Medium	
Equipment	Bath temperature	Low	Low	Low	Low	According to IVRT validation studies.
	Surface area	Low	Low	Low	Low	
	Stirring speed	Low	Low	Low	Low	Fixed parameter.
	Diffusion cell design	Low	Low	Low	Low	
Method conditions	Sampling times	Medium	Low	Low	Low	
	Dosage regimen	Low	Low	Low	Low	According to DoE studies.
	Dose application techniques – pippete. vial. spatula. finger	Low	Low	Low	Low	Fixed parameter.
Medium	Co-solvents	High	High	High	High	According to DoE studies.
	pH	Medium	Medium	Medium	Medium	According to solubility studies.
	Degassing effect	Low	Low	Low	Low	Fixed parameter.
	Sink conditions maintenance	High	High	High	High	According to DoE studies.
Membrane	Type	High	High	High	High	According to DoE studies.
	Presoaking time	Low	Low	Low	Low	Fixed parameter.
	Inertness	Medium	Medium	Medium	Medium	According to IVRT validation studies.

Key: CAA – Critical analytical attribute; CMV – Critical method variables; IVRR – *In vitro* release rate; Q initial point – Cumulative amount released at 0.5h; Q final point – Cumulative amount released at 3h.

4.4 Concluding remarks

The results reported in the present work underline the multiaddressable characteristics of aQbD as a framework for IVRT method development. To our knowledge, this is the first work based on that framework. Since aQbD requires the definition of the analytical target profile, one must take into account a variety of parameters and their respective synergisms. This extensive and comprehensive analysis would normally not be considered while developing standard IVRT methods (Kochling *et al.*, 2016).

As shown in the present work, the application of aQbD as a systematic approach strategy to IVRT development enabled the determination of CMV and CAA, and by doing so, a deep understanding of the main risks intrinsic to the method.

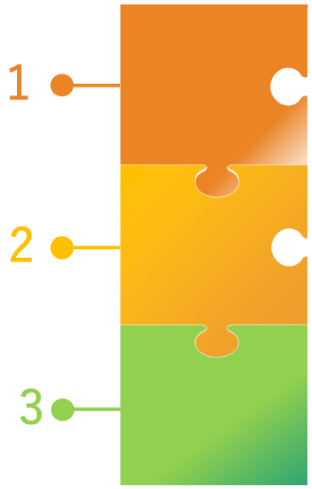
A 3x2x3 full factorial design experiment was employed to assess the impact of the dosage regimen, membrane and release medium in the predefined critical analytical attributes, IVRR, $Q_{initial}$, Q_{final} and dose depletion. The quantitative models that portrait these influences were properly constructed and validated through *t*-tests and ANOVA. Moreover, the predictive capabilities of the model were confirmed by establishing comparative analysis of the actual and predictive values.

Tuffryn membranes, PBS:Ethanol release medium (80:20) and a dose of 300 mg were found to be suitable parameters for the maximization of the release profile without compromising the discriminatory capacity of the method.

The optimized IVRT conditions were subsequently evaluated in terms of membrane inertness, linearity, precision, robustness and the indicators of discriminatory power, following the recent guidelines of both European and US regulatory authorities. Validation of the HPLC method was also carried. Both validation studies were found to be compliant.

Applying an aQbD rationale to IVRT will highly reduce the method development time and cost, besides offering a robust and regulatory-oriented platform for its predictive development. Frequently, the absence of IVRT method development protocols often impairs TGP submissions, and therefore this work can be a reliable strategy to overcome such deficiencies.

4.5 Highlights



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Applying an aQbD rationale to IVRT offers a robust and regulatory-oriented platform for its predictive development. The absence of development protocols often impairs TGP submissions, and therefore this framework can be a reliable strategy to overcome such deficiencies.

5 DIVING INTO BATCH-TO-BATCH VARIABILITY OF TOPICAL PRODUCTS: A REGULATORY BOTTLENECK

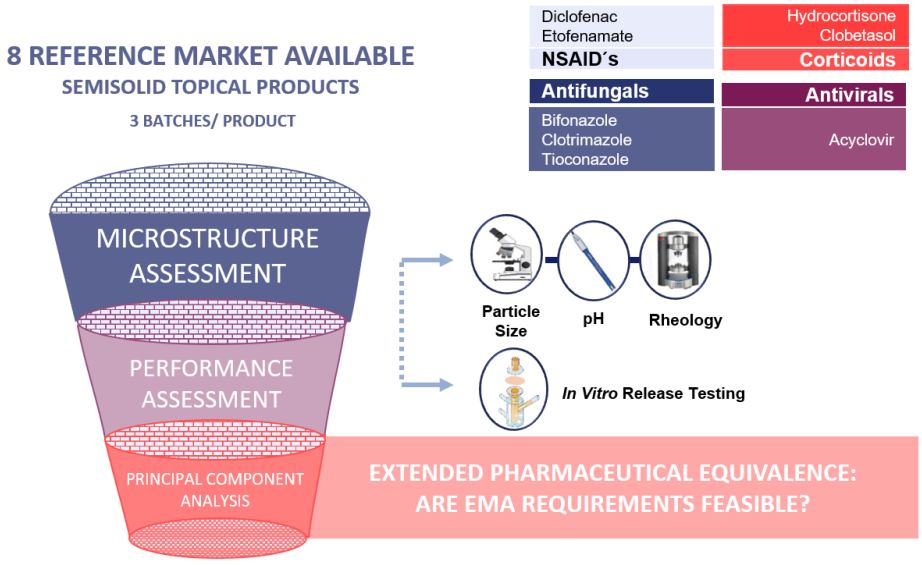


Fig.5.1 – Graphical abstract: Diving into batch-to-batch variability of topical products: A regulatory bottleneck.

This chapter has been adapted from the following publication:

Miranda, M., Cova, T., Augusto, C., Pais, A.A.C.C., Cardoso, C., Vitorino, C., 2020. Diving into Batch-to-Batch Variability of Topical Products-a Regulatory Bottleneck. Pharm. Res. 37, 218. https://doi.org/10.1007/s11095-020-02911-y

M.M and C.V. conceived the presented idea and established the research program and implementation. A.A.C.C Pais assisted in the statistical analysis, more specifically in confidence interval calculations pertaining to IVRT and microstructure data. T.C performed the HCA and PCA analysis in R software. Supervision, resources, data curation, review and editing was provided by C.V and A.A.C.C. Pais. M.M performed the experimental part of the work and wrote the first draft of the manuscript. All the other authors substantially contributed to revisions. Funding acquisition was provided by C.C and C.V.

KEY CONCEPTS

- As extensively discussed in chapters 2 and 4, the extended pharmaceutical equivalence demonstration is a crucial requirement during an abridged TGP BE assessment. To document so, an in-depth RP characterization is mandatory.
- In spite of negligible batch to batch differences being actively pursuit by both manufacturers and regulators, there are still products in which batch variability is deeply observed. These variations are essentially prompted by raw materials / manufacturing process fluctuations. Semisolid dosage forms, which account for the majority of topically applied products, are particularly prone to batch variations, as their microstructure is highly sensitive to the above mentioned variability sources.
- Performance endpoints, according to EMA requirements, should be within a 90-111% confidence interval pertaining to the RP. Inter-run variability should not surpass 10% and at least 12 replicates should be considered. These criteria are different from those proposed by USA regulators, where only 6 replicates should be regarded, IVRT confidence intervals should be within 75-133% and inter-run variability should not exceed 15%.

5.1 Introduction

The global dermatological market for skin drug delivery is expected to register a compound annual growth rate of 7% between 2019 and 2024 (Mordor, 2019a; Patere *et al.*, 2018). There are several identifiable market drivers: growing geriatric population, high prevalence of osteoarticular diseases, rising consumption of topical antifungal drugs, forthcoming major drugs patent expiries, among other factors (Mordor, 2019a, 2019b, 2019c). In line with this tremendous market potential, as well as concrete medical needs, the regulatory processes concerning TGP development and approval have been subject to several noteworthy amendments, as extensively reviewed in the introductory chapters of the present thesis. Regulatory agencies, such as EMA and FDA, have "sharpen" the TGP development process by promoting a rational and modular framework to its approval. According to this strategy, the extended pharmaceutical equivalence of the products should be evidenced. Afterwards, equivalence regarding product performance, administration and efficacy should also be adequately provided (Flühmann *et al.*, 2018). The basis of this workflow is, understandably, an in-depth characterization of the RP (Chang *et al.*, 2013b; Fernández-Campos *et al.*, 2017; Murthy, 2017; Raghavan *et al.*, 2019; Roberts *et al.*, 2017; Sinamora, 2017).

Semisolid formulations, such as creams, ointments, gels and emulsions, represent the majority of topical dosage forms intended for a local action. These are complex, homogenous or heterogeneous systems, in which the drug is dissolved or dispersed into the vehicle (Shah *et al.*, 2016). Their production is usually performed in large homogenisers as a batch process (Qwist *et al.*, 2019). A batch can be defined based on production time, amount of material,

maintenance cycles or changes in lots of feed raw materials (Bostijn *et al.*, 2019). Even though pharmaceutical development is presently entering a new era of quality build, where the regulatory agencies tend to impose strict product specifications for new drug / abbreviated new drug applications, there are still RP in which batch-to-batch variation is a reality (van Heugten *et al.*, 2017). There are several contributing factors for variability, often referred to as the 6 "M´s": machine, manpower, materials, measurements, manufacturing processes and mother nature (Sayeed-Desta *et al.*, 2017). The microstructure of semisolid drug products is highly sensitive to these variability sources, especially to interchanges between suppliers (sourcing) and manufacturing processes (Bao *et al.*, 2020; Patere *et al.*, 2018; Zarmpi *et al.*, 2017). Microstructure is assessed by several parameters, such as rheology profile, globule/particle size, pH, phase homogeneity, polymorphism, among others (Raghavan *et al.*, 2019). These attributes have a direct impact on product performance, mainly supported through IVRT studies (Hauck *et al.*, 2007; Raghavan *et al.*, 2019; Shah *et al.*, 2016). The recently issued EMA draft guideline on quality and equivalence of topical products represents a long awaited regulatory advance concerning the establishment of analytical surrogates to CES. Nevertheless, there are several criteria imposed by the guideline that may undermine its translation into practice (EMA, 2018b). These mainly relate with the restrictive statistical parameters regarding the extended pharmaceutical equivalence, product performance and efficacy documentation.

The present chapter aims to highlight the intrinsic variability of topical semisolid dosage forms. For that purpose, eight reference blockbuster topical drug products were selected based on the active substance pharmaceutical profile as well as market relevance. For each product, a total of 3 batches was considered. All products, retrieved from the European market, concern well established used molecules, having in the majority of cases a generic/hybrid product. Table 5.1 displays the study products and respective batch manufacturing sites, whilst Table 5.2 portraits the qualitative composition for each RP.

All formulations were characterized in terms of pH, globule or particle size, full rheological and IVRT profile. Statistical evaluation regarding IVRR comparison was performed following FDA and EMA guidelines, in order to clarify the underlying statistical models and further illustrate the subtle but high impacting differences between both criteria. Finally, to summarize all the considered variables regarding product microstructure and performance, a multivariate approach resorting to a hierarchical clustering and principal component analyses is thoroughly presented.

Table 5.1 – Studied RP.

Reference product	API	Dosage form	Strength	Code	Manufacturing site
RP_1	Hydrocortisone	Cream	10 mg/g	HC_1	1
				HC_2	
				HC_3	
RP_2	Etofenamate	Gel	100 mg/g	ETF_1	1
				ETF_2	
				ETF_3	
RP_3	Bifonazole	Cream	10 mg/g	BFZ_1	1
				BFZ_2	
				BFZ_3	
RP_4	Clotrimazole	Cream	10 mg/g	CLT_1	1
				CLT_2	
				CLT_3	
RP_5	Acyclovir	Cream	5 mg/g	ACV_1	1
				ACV_2	
				ACV_3	
RP_6	Tioconazole	Cream	10 mg/g	TCZ_1	1
				TCZ_2	2
				TCZ_3	3
RP_7	Clobetasol	Cream	0.5 mg/g	CLB_1	1
				CLB_2	2
				CLB_3	
RP_8	Diclofenac	Emulgel	23.2 mg/g	DF_1	1
				DF_2	
				DF_3	

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac.

Table 5.2 – Qualitative composition of the RP.

Excipient	Function	HC	ETF	BFZ	CLT	ACV	TCZ	CLB	DF
Beeswax substitute 6621	Stiffening agent							X	
Benzyl alcohol	Preservative			X	X		X		
Butylhydroxytoluene	Preservative								X
Carbomers	Gelling agents								X
Carbopol 940	Gelling agents		X						
Cetostearyl alcohol	Emulsifier	X		X	X	X	X	X	
Cetyl palmitate	Thickener			X	X				
Chlorocresol	Preservative							X	
Citric acid monohydrate	Buffering agent							X	
Cocoyl caprylocaprate	Solubilizer								X
Dexpantenol	Moisturizer	X							
Diethylamine	Buffering agent								X
Dimethicone 20	Emollient					X			
Eumulgin M8	Emulsifier		X						
Glycerol	Humectant	X							
Glycerol monostearate	Emollient					X		X	
Isopropanol	Solvent		X						X
Isopropyl myristate	Emollient / permeation enhancer	X						X	
Mineral oil	Emollient / emulsifier					X			X
Macrogol 400	Emulsifier / solubilizer	X	X						
Polyoxyethylene Alkyl Ethers	Emulsifier								X
Methyl parahydroxybenzoate	Preservative	X							
Octyldodecanol	Emulsifier			X	X		X		
Oleyl alcohol	Permeation enhancer								X
Parfum	Organoletic agent								X
Poloxamer 407	Emulsifier					X			
Polyoxyethylene stearate	Emulsifier			X		X			
Polysorbate 60	Emulsifier				X		X		
Propyl parahydroxybenzoate	Preservative	X			X				
Propylene glycol	Solvent / humectant		X			X		X	X
Sodium citrate	Buffering agent							X	
Sodium Hydroxide	Buffering agent		X						
Sodium lauryl sulfate	Emulsifier					X			
Sorbitan stearate	Emulsifier			X	X		X		
Stearic acid	Emollient	X							
Synthetic sperm oil	Stiffening agent						X		
Purified water	Solvent	X	X	X	X	X	X	X	X
Petrolatum	Emollient					X		X	

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac.

5.2 Materials and Methods

5.2.1 Drug Products

Eight reference topical products were included in this study. For each product, 3 batches were considered: 1% (w/v) hydrocortisone cream (Pandermil®, Edol); 10% (w/v) etofenamate gel (Traumon®, Meda); 1% (w/v) bifonazole cream (Canespor®, Bayer); 1% (w/v) clotrimazole cream (Canesten®, Bayer); 5% (w/v) acyclovir cream (Zovirax®, GSK); 2% (w/v) diclofenac emulgel (Voltaren emulgelex®, GSK). All the above products were manufactured in one manufacturing site. Conversely, 0.5% (w/v) clobetasol cream (Dermovate® /Dermoval, GSK) batches were produced in two manufacturing sites, and finally the 1% (w/w) tioconazole cream batches (Trosyd®, Pfizer) were produced in three different manufacturer sites.

All the batches, as well as the respective standards were kindly supplied by Laboratórios Basi.

Propylene glycol was acquired from Merck, phosphate buffered saline (PBS) was acquired from Sigma. Water was purified with a Millipore MILLI-Q reagent water system and filtered through a 0.22 µm nylon filter before use. All other chemicals were of analytical grade or equivalent.

5.2.2 Methods

5.2.2.1 Topical product microstructure

According to the new EMA guideline, to establish BE between two topical products, evidence should be provided on microstructure sameness. Product microstructure is regarded as a formulation CQA, due to its impact on drug bioavailability, stability and usability (EMA, 2018b). Product microstructure analysis entailed the following parameters: pH, droplet size and rheological behaviour.

pH values were determined at room temperature (25 ^{o}C), in triplicate, using a digital pH meter Consort C3010 (Consort bvba, Turnhout, Belgium), previously calibrated using standard buffer solutions (pH of 4.00, 7.00 and 10.01). About 0.6-1 g of each sample were weighed and dispersed in 10 times the volume of purified water. Afterwards, the respective pH value was recorded.

Microscopic analysis was performed to study and compare the droplet/particle size distribution of the different batches. For this, a Leica DM IL with a Nikon DS-Fi1 camera (Nikon Instruments Europe BV, Amsterdam, The Netherlands) at a 100-fold magnification was used.

A thin layer of each formulation was carefully dispersed on a slide, and the cover slip was then gently placed on the top to avoid sample shearing and prevent microscopic alterations of the sample. Five representative images were acquired for each sample, and droplet length was measured using an imaging software (NIS Elements, version 3.10). Approximately 100 ≤ n≤ 400 droplet/particle sizes were considered per batch formulation.

5.2.2.2 Thermal analysis

Taking into account the microscopic studies, acyclovir samples due to the observed crystallization phenomena were also thermally studied through differential scanning calorimetry (DSC). DSC analysis was performed using a DSC-60 differential scanning calorimeter (Shimadzu, Japan). Acyclovir standard (about 1 mg) and the three acyclovir batches (20-24 mg) were placed in aluminium pans hermetically sealed. Empty pans were used as reference. Each sample was submitted to a heating cycle from 25 to 270°C, at the rate of 10°C/min, with a nitrogen purge of 30 mL/min. The onset temperature (T_{on}), melting point (T_{peak}), and enthalpy (ΔH) were evaluated using the TA Software (Shimadzu, Japan).

5.2.2.3 Rheological properties

The rheological profile of a semisolid, as reviewed in Chapter 3, is highly linked with the product sensory qualities, such as consistency, spreadability and feel, which strongly impact patient compliance. The rheological profile of all products was investigated using the same rheometer and analysis software of Chapter 3. The following paragraphs describe the sequence of procedures performed for each sample. All measurements were performed in triplicate and conducted at 32°C, the physiological skin temperature, and a sample hood was used to minimize sample volatilization.

Rotational measurements

Rotational tests were performed with a C35/2°/Ti cone geometry at 32°C. Approximately 0.3 g of formulation were placed on a lower plate TMP35 using a positive displacement syringe. A preset gap of 0.1 mm was considered.

A linear CS flow ramp from 0.01 to a final 100 Pa was measured for 300 s, to trace the viscosity curve. The main objective of the proposed settings was to enable a detailed acquisition of the shear stress viscosity profile of all the studied products with the zero-shear plateau, the shear thinning region and the infinite shear plateau. For that, for some products, slight method adjustments had to be carried out: diclofenac batches (0.001 – 100 Pa) and for clobetasol

batches (50 – 500 Pa for 450 s). Additionally, to determine the apparent thixotropy (Pa/s), a shear stress from 0.01 to 300 s^{-1} and again down to 0.01, during 300 s, was used.

Oscillatory measurements

The viscoelasticity of all products was tested by applying an oscillating shear stress (Li *et al.*, 2011). A parallel plate-and-plate geometry (P35/Ti) was used and approximately 0.3 g of the formulation were applied to the *peltier* plate. An amplitude sweep between 0.01 and 100 Pa at $1s^{-1}$ was firstly conducted in order to estimate the linear viscoelastic region (LVR). Afterwards, a frequency sweep analysis was conducted within the LVR range. The storage modulus (G´), loss modulus (G´´) and loss tangent were calculated.

5.2.2.4 Performance attributes – IVRT studies

According to the new EMA requirements, IVRT outputs are considered as a CQA of the formulation. Therefore, the assessment of product performance made through the determination of kinetic parameters, such as the IVRR, is one of the most important parameters to establish the extended pharmaceutical equivalence.

5.2.3 *In vitro* release testing

Release studies of the different drug products were conducted using the same Franz cell diffusion system used in Chapter 4. Moreover, the indications provided in the Topical and Transdermal Drug Products — Product Performance Tests section of USP were likewise followed (USP, 2009). Briefly, infinite dose conditions (300 or 150 mg) of the semisolid formulations were applied in the donor compartment, separated from the receptor compartment by a polysulfone membrane (SUPOR 450 pore size 0.45 µm, Pall Corporation, USA), previously soaked in purified water for 30 min. Despite the membrane characteristics, such as thickness, pore size, surface morphology, and diameter may prompt kinetic changes in the release profile, note that, since a formulation comparison is intended, the type of membrane was kept constant, so that this fixed parameter was not considered a concern.

The receptor medium was carefully selected in order to assure the maintenance of sink conditions throughout the IVRT experiment. The rationales concerning the suitability of co-solvents and pH for the specific physicochemical profile of the active substance panel herein presented, are carefully debated in Appendix A. Table 5.3 presents the release medium used for each drug product.

Table 5.3 – Release medium composition used for IVRT studies.

Drug	Release Medium
Hydrocortisone	Water-ethanol (70:30, v/v), adopted from (USP, 2009)
Etofenamate	PBS-ethanol (70:30, v/v)
Bifonazole	PBS-ethanol (50:50, v/v, pH=7.4)
Clotrimazole	PBS-ethanol (50:50, v/v, pH=7.4)
Acyclovir	PBS
Tioconazole	PBS- ethanol (50:50, v/v, pH=4.5)
Clobetasol	PBS-ethanol (50:50, v/v, pH=7.4)
Diclofenac	PBS- ethanol (80:20, v/v, pH=7.4)

The receptor medium was continuously stirred at 600 rpm and maintained at a temperature of 37^{o}C by means of a circulating water bath. Before the release experiments, the system was allowed to equilibrate for at least 30 min. Throughout the release studies, the donor compartment, as well as the receptor sampling arm, were carefully covered with Parafilm® to avoid unnecessary release medium volatilization and to conduct all the release experiments under occlusive conditions. Samples of the receptor phase (300 µL) were withdrawn at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h, since according to the EMA draft guideline: "At least 6 time points should be obtained in the linear portion of the drug release profile" (EMA, 2018b). All IVRT samples were analysed by regulatory compliant HPLC methods, please see Appendix A. After each collection, the same volume of medium was replaced with preheated receptor solution. In order to determine the cumulative amount of drug released as a function of time, the same procedures as section 4.2.2.5 were used. According to the recent regulatory requirements an n=12 was considered for each batch (European Medicines Agency, 2018).

5.2.4 Multivariate analysis

Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed using a total of 8 reference products x 3 batches, as objects, and 13 variables extracted from both physicochemical and pharmaceutical characterization. These analyses were carried out in R software version 3.6.1, using the FactoMineR and the factoextra packages (Kassambara, 2017; Lê *et al.*, 2008).

5.2.5 Data analysis and statistics

The confidence intervals (CI) regarding IVRR comparisons were calculated according to EMA criteria (parametric test) and FDA criteria (non-parametric test), so that the differences between these approaches and their possible impact on BE could be carefully assessed.

As previously mentioned in Chapter 4, the FDA recognized approach, based on the Wilcoxon Rank Sum/Mann-Whitney U test, was adapted to meet the 12 x 12 T/R matrix (Food and Drug Administration, 1997). On the other hand, to calculate CI according to EMA directives, the procedures described in the bioequivalence guideline, as well as in the EMA draft guideline of topical products, were followed (EMA, 2018b, 2010). For this, one should take into account that the terms sequence, subject within sequence, period and formulation do not apply, since we are considering *in vitro* studies. According to the above mentioned guidelines, to perform the equivalence test of quantitative physicochemical parameters, the 90% CI of the ratio of means between the test/reference formulations should be determined. For that, the data was natural log transformed. Then, the means and the standard deviations were calculated. This was followed by obtaining the ratio of the two back-transformed averages for IVRT endpoints. For confidence interval calculations Equations 5.1 and 5.2 were used.

$$S_p = \sqrt{\frac{(n_1-1)\times s_1^2+(n_2-1)\times s_2^2}{n_1+n_2-2}} \tag{5.1}$$

$$\frac{\overline{X_1}}{\overline{X_2}} \pm t_{1-\alpha/2,n_1+n_2-2,} S_p \sqrt{\frac{1}{n_1}+\frac{1}{n_2}} \tag{5.2}$$

Where $\bar{X}$ is the mean value to evaluate the test ($\overline{X_1}$) or reference product ($\overline{X_2}$), t1-α/2 is the Student's t value for α = 0.90, s is the standard deviation, and n the number of observations.

For example calculations please refer to Appendix B. All the procedures involved in the calculation of the CI according to FDA and EMA directives were repeated to obtain a CI for all batch combinations.

Confidence intervals regarding the other microstructure parameters were calculated based on EMA approach, please see Appendix B.

5.3 Results

5.3.1 Microstructure evaluation

To characterize all products regarding their microstructure, the globule/particle size, pH and rheology profile were determined, see Table 5.4.

Table 5.4 – pH and globule/particle size assessment. Results are expressed as mean ± SD. The means were statistically compared by a one-way ANOVA using a Tukey multiple comparison test. The differences among the means were considered significant for values of * p<0.1, ** p<0.01 and **** p<0.0001. For microscopic studies, 100≤n≤400 was considered. For pH, an n=3 was considered.

Products	Batch	Manufacturing site	Globule / Particle size (µm)	SS	pH
HC	HC_1	1	4.9±1.4	****	7.33
	HC_2		9.6±2.2		7.15
	HC_3		2.8±0.7		7.15
ETF	ETF_1	1	7.8±2.4	****	4.69
	ETF_2		10±4		5.02
	ETF_3		8±2.4		4.79
BFZ	BFZ_1	1	6.3±5.2	*	6.00
	BFZ_2		5.4±4.5		7.08
	BFZ_3		5.1±3		8.02
CLT	CLT_1	1	0.91±0.27	****	6.93
	CLT_2		1.42±0.6		7.35
	CLT_3		0.96±0.25		7.06
ACV	ACV_1	1	2.4±0.6	****	7.44
	ACV_2		2.4±0.7		7.80
	ACV_3		4±2.3		7.07
TCZ	TCZ_1	1	6.6±2.1	****	6.07
	TCZ_2	2	5.4±4.6		5.50
	TCZ_3	3	4.4±1.2		5.32
CLB	CLB_1	1	5.8±3.8	****	4.77
	CLB_2	2	3.7±1.9		4.65
	CLB_3		2.5±2		4.59
DF	DF_1	1	5.8±1.9	****	7.67
	DF_2		4.7±1.3		7.70
	DF_3		3.5±1.1		7.59

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac; PT – Portuguese; SPN: – Spanish; FRC – French; POL – Polish; ITL – Italian: SS – Statistical significance.

For multiphase semisolid formulations, such as creams, the globule/particle size is an important quality attribute, since it can directly impact product bioavailability (Sinamora, 2017). Marked statistical differences in this parameter were observed for every batch, see Table 5.4 and Table 5.9.

During microscopic analysis, and as broadly referred to in the literature, acyclovir batches revealed the presence of rectangular drug crystals (Inoue *et al.*, 2012; Trottet *et al.*, 2005). In

order to infer on the thermal behaviour of these products, DSC studies were performed, see Fig.5.2.

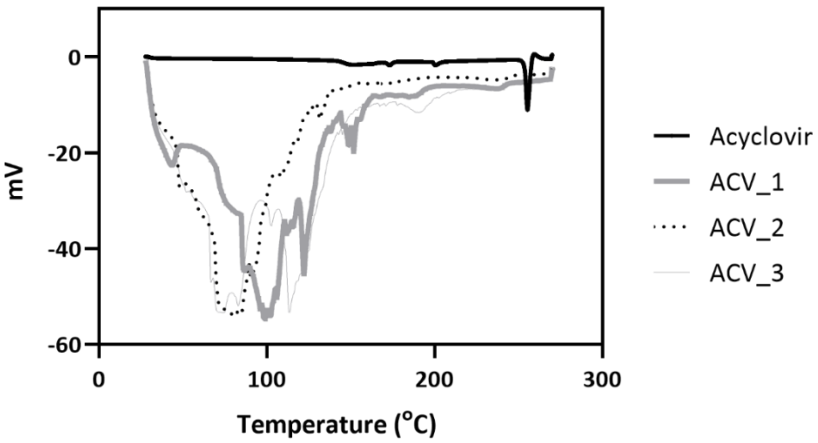


Fig.5.2 – DSC thermograms of acyclovir batches.

As depicted in Fig.5.2, acyclovir displayed an onset temperature of 254.46ºC, consistent with the reported values. Regarding the acyclovir batches, all products revealed different heating curves from those of the pure drug, where all the drug appears to be dispersed into the lipid matrix. Inter-batch pH differences were also observed.

5.3.2 Rheological studies

A comprehensive rheological assessment was then performed to investigate if there were batch-to-batch differences in this critical quality attribute. To achieve so, rotational studies were performed as well as oscillatory measurements. Regarding the first tests, the zero-shear viscosity, infinite-shear viscosity, relative thixotropic area and yield point were selected as endpoints. On the other hand, for oscillatory studies, the values of the linear viscoelastic region, storage and loss moduli and loss tangent, were addressed.

The viscosity curves are displayed in Fig.5.3- Fig.5.6. All formulations display a pseudoplastic flow and shear thinning behaviour. These properties, present in the majority of semisolid dosage forms, facilitate product application and by doing so, reinforce patient compliance (Binder *et al.*, 2019; Marto *et al.*, 2015).

The representative viscosity curves already display batch-to-batch differences, see Fig.5.3-Fig.5.6. Nevertheless, a parametric evaluation should be performed following the updated regulatory requirements. For that, several rheological endpoints were selected and

statistically compared, see Table 5.5 and Table 5.6. Please note that the 90% confidence intervals were also determined, as demanded by EMA (Table 5.9) (EMA, 2018b).

Regarding the rotational rheology measurements, hydrocortisone, clobetasol and acyclovir, batches revealed almost overlapping viscosity curves (Fig.5.3, Fig.5.4, Fig.5.6). Nevertheless, with the sole exception of acyclovir batches, marked statistical differences were observed in at least two rheological endpoints. For clobetasol batches, these differences report to the zero-shear viscosity, as well as for yield point. For hydrocortisone batches, differences in the infinite-shear viscosity, the relative thixotropic area and overall oscillatory profile were evidenced. Regarding diclofenac batches, differences in the infinite-shear viscosity and in the storage modulus were also perceptible.

All antifungal drug products as well as the etofenamate formulations revealed more pronounced rheological differences, which can be easily observed in the viscosity flow curves (Fig.5.4 and Fig.5.5). Tioconazole products revealed differences in all rheology endpoints, except for the linear viscoelastic region (LVR) plateau. Rheological batch variability concerning bifonazole products was mainly denoted in the rotational measurements (infinite-shear viscosity and yield point), wherein the oscillatory profile of these products proved to be similar. Regarding clotrimazole products, differences were observed in all selected rheological endpoints, with the exception of the loss tangent. Regarding etofenamate products, marked differences were registered in zero and infinite-shear viscosity, as well as in all oscillatory endpoints.

Table 5.5 – Rotational study results for all batch products. The results report to an n=3, mean ± SEM. The means were statistically compared by a one-way ANOVA using a Tukey multiple comparison test. The differences among the means were considered significant for values of * p<0.1, ** p<0.01 and **** p<0.0001.

Products	Batch	Zero-shear viscosity		Infinite-shear viscosity		Rotational yield point		Relative thixotropic area	
		Mean±SEM	SS	Mean±SEM	SS	Mean±SEM	SS	Mean±SEM	SS
HC	HC_1	13470 ± 577	n.s	10.0 ± 0.9	*	22.8 ± 0.8	n.s	19420	***
	HC_2	12770 ± 197		10.1 ± 0.2		20.6 ± 0.7		6400 ± 258	
	HC_3	12660 ± 181		13.0 ± 0.3		19.8 ± 0.5		8053 ± 421	
EF	ETF_1	24930 ± 3049	**	10394 ± 2902	****	24.3 ± 0.1	n.s	5272 ± 859	n.s.
	ETF_2	3687 ± 793		4 ± 2		24.0 ± 0.2		4930 ± 902	
	ETF_3	23197 ± 1095		4968 ± 492		24.4 ± 0.1		10017 ± 1622	
BZL	BFZ_1	15573 ± 1169	n.s	2.8 ± 0.7	****	25.1 ± 0.4	****	4492 ± 915	n.s
	BFZ_2	18933 ± 348		27 ± 2		56 ± 1		6290 ± 653	
	BFZ_3	17730 ± 96		11 ± 1		35.9 ± 0.2		3269 ± 126	
CLZ	CLT_1	17227 ± 44	**	14.5 ± 0.3	***	22.9 ± 0.5	****	6270 ± 349	***
	CLT_2	16957 ± 242		16.0 ± 0.5		24.3 ± 0.7		10284 ± 630	
	CLT_3	20630 ± 592		21.6 ± 0.7		46 ± 2		21607 ± 1564	
ACV	ACV_1	9660 ± 500	n.s	100 ± 7	n.s	51 ± 2	n.s	30270 ± 3723	n.s
	ACV_2	8982 ± 296		93 ± 4		51 ± 2		30273 ± 621	
	ACV_3	10118 ± 699		104 ± 7		51 ± 3		35930 ± 2074	
TZ	TCZ_1	20403 ± 1075	**	10 ± 2	*	13.3 ± 0.2	*	17728 ± 3904	*
	TCZ_2	12473 ± 751		4.3 ± 0.4		9.9 ± 0.9		4412 ± 266	
	TCZ_3	24060 ± 403		2.0 ± 0.7		12.8 ± 0.1		9647 ± 535	
CLB	CLB_1	71180 ± 1682	**	5 ± 3	n.s	351 ± 1	**	59057 ± 2538	n.s
	CLB_2	93067 ± 6986		6 ± 2		322 ± 5		65007 ± 7405	
	CLB_3	82123 ± 334		6 ± 3		336 ± 3		62031 ± 7086	
DF	DF_1	7497 ± 631	n.s	2.8 ± 0.3	**	19.4 ± 0.6	n.s	2119 ± 237	n.s
	DF_2	7925 ± 271		5.4 ± 0.3		19.2 ± 0.3		1619 ± 123	
	DF_3	9246 ± 1245		5.9 ± 0.5		19.6 ± 0.1		2352 ± 279	

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac; SS – Statistical significance.

Table 5.6 – Amplitude and frequency sweep tests. Frequency results report to a frequency of 10 Hz. Results are expressed as n=3 ± SEM. The means were statistically compared by a one-way ANOVA using a Tukey multiple comparison test. The differences among the means were considered significant for values of * p<0.1, ** p<0.01 and **** p<0.0001.

Products	Batches	Amplitude sweep		Frequency sweep					
		LVR plateau G´ (Pa)		G´ (Pa)		G´´ (Pa)		Loss tangent	
		Mean±SEM	SS	Mean±SEM	SS	Mean±SEM	SS	Mean±SEM	SS
HC	HC_1	771 ± 119	n.s	579 ± 20	***	179 ± 6	**	0.310 ± 0.002	****
	HC_2	421 ± 34		277 ± 8		128 ± 4		0.460 ± 0.004	
	HC_3	748 ± 126		417 ± 25		160 ± 8		0.38 ± 0.01	
EF	ETF_1	234 ± 10	****	274 ± 1	****	45.7 ± 0.3	****	0.170 ± 0.002	****
	ETF_2	100 ± 0		116 ± 0		34.2 ± 0.1		0.290 ± 0.002	
	ETF_3	209 ± 4		242 ± 1		35.1 ± 0.4		0.140 ± 0.002	
BZN	BFZ_1	874 ± 53	n.s	1013 ± 142	n.s	218 ± 42	n.s	0.21 ± 0.02	n.s
	BFZ_2	1112 ± 359		956 ± 10		155 ± 8		0.160 ± 0.006	
	BFZ_3	793 ± 38		1005 ± 7		191 ± 1		0.190 ± 0.002	
CLZ	CLT_1	1142 ± 66	**	1482 ± 9	****	301 ±4	****	0.200 ± 0.003	n.s
	CLT_2	1190 ± 31		1628 ± 17		342 ± 3		0.210 ± 0.004	
	CLT_3	924 ± 10		1167 ± 2		238 ± 5		0.200 ± 0.004	
ACV	ACV_1	1201 ± 38	n.s	3782 ± 97	n.s	2186 ± 47	n.s	0.580 ± 0.003	n.s
	ACV_2	1133 ± 27		3676 ± 65		2117 ± 33		0.580 ± 0.002	
	ACV_3	1269 ± 49		3888 ± 128		2254 ± 61		0.580 ± 0.002	
TZ	TCZ_1	1147 ± 112	n.s	1697 ± 38	****	411 ± 6	****	0.240 ± 0.009	****
	TCZ_2	1656 ± 184		2692 ± 84		828 ± 12		0.310 ± 0.006	
	TCZ_3	1249 ± 216		202 ± 5		177 ± 3		0.880 ± 0.009	
CLB	CLB_1	10899 ± 1162	n.s	31649 ± 434	n.s	13297 ± 632	n.s	0.42 ± 0.01	n.s
	CLB_2	13030 ± 154		32630 ± 849		18373 ± 138		0.43 ± 0.01	
	CLB_3	11964 ± 658		62801 ± 641		30941 ± 407		0.49 ± 0.01	
DF	DF_1	102 ± 3	n.s	122 ± 1	***	29.3 ± 0.4	n.s	0.240 ± 0.005	n.s
	DF_2	105 ± 2		123 ± 1		30.2 ± 0.4		0.250 ± 0.005	
	DF_3	112 ± 2		132 ± 1		30.6 ± 0.3		0.230 ± 0.003	

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac; SS – Statistical significance.

5.3.3 Performance attributes - IVRT

Due to the overgrowing importance of IVRT as a tool to document the products extended pharmaceutical equivalence, the recently published EMA guideline includes a specific annex that solely reports to IVRT. Accordingly, detailed validation procedures, as well as specific acceptance criteria are well defined (Flühmann *et al.*, 2018). To comply with the guideline, the IVRT experimental protocol included: maintenance of skin mimicking conditions throughout the study; number of replicates (12); establishment of a 24 h period aiming not only to

maximize drug release, but also to foresee an *in vivo* administration dosage schedule; consideration of at least 6 sampling points within the linear portion of the release profile to estimate the IVRR; the amount of formulation applied to each donor compartment was consistent and did not deviate by more than 5%. Table 5.7 and Table 5.8 summarize IVRT results.

Table 5.7 – IVRT parameters. The results are expressed as mean ± SEM. An n=12 was considered for each product.

Products	Batch	IVRR (µg/cm²/√h)		Q_f (µg/cm²) Mean ± SD	% Dose depletion Mean ± SD
		Mean ± SD	Coefficient variation (CV, %)		
HC	HC_1	78±9	11.4	280±7	5.8±0.2
	HC_2	81±5	5.71	290±7	6.3±0.2
	HC_3	73±8	10.8	269±7	5.9±0.2
EF	ETF_1	738±252	34.2	6837±1318	14±3
	ETF_2	712±272	38.1	6257±588	13±1
	ETF_3	695±265	38.2	6802±663	14±1
BFZ	BFZ_1	278±35	12.7	1194 ±145	46±6
	BFZ_2	279±18	6.54	1217±68	48±4
	BFZ_3	293±37	12.9	1253±184	48±14
CLT	CLT_1	98±31	31.5	509±72	10±1
	CLT_2	91±26	28.1	424±45	8.7±0.9
	CLT_3	99±35	34.9	437±40	8.7±0.8
ACV	ACV_1	386±55	14.1	1079±32	6.0±0.2
	ACV_2	413±51	12.3	1137±35	6.3±0.2
	ACV_3	476±26	5.57	1341±14	7.4±0.6
TCZ	TCZ_1	73±26	36.0	280±41	5.5±0.8
	TCZ_2	68±12	18.0	281±36	5.9±0.8
	TCZ_3	74±14	19.1	230±38	4.6±0.7
CLB	CLB_1	9±1	12.1	42±1	17.7±0.7
	CLB_2	9.0±1.7	19.0	42±2	16.0±0.7
	CLB_3	10.2±1.8	17.4	48±2	20.0±0.9
DF	DF_1	914±73	7.97	4375±109	47±2
	DF_2	1006±116	11.53	4633±190	46±2
	DF_3	744±52	6.95	3234±79	33±1

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac; Q_f – Cumulative amount of drug released in the end of IVRT; DD – Dose Depletion.

As expected, NSAIDs products exhibited a higher drug release (µg/cm²), which is consistent with their therapeutic action site. Acyclovir batches displayed a moderate drug release, whilst antifungals (except bifonazole) and corticoid products revealed an inferior cumulative drug release, as their pharmacotherapeutic action is directed towards the skin outermost layers.

Linearity ($r^2 > 0.9$) was observed in each diffusion cell. According to EMA "The duration of IVRT should be sufficient to characterize the release profile, ideally at least 70% of the active substance applied is released". Nevertheless, none of our results proved to be compliant with this criteria, since no more than a 47±2% release was observed. This is consistent with other literature reports (Al-Ghabeish *et al.*, 2015; Bao *et al.*, 2017; Fernández-Campos *et al.*, 2017; Goebel *et al.*, 2013; Khanolkar *et al.*, 2017; Krishnaiah *et al.*, 2014; Kriwet and Müller-Goymann, 1995; Lauterbach and Müller-Goymann, 2014; Nallagundla *et al.*, 2014; Petró *et al.*, 2013; Xu *et al.*, 2015a, 2015b).

Etofenamate, clotrimazole and tioconazole revealed high IVRT intra-variability, as displayed by the high IVRR CV results (Table 5.7). Such trend can be motivated by the previously described microstructure differences. Hydrocortisone exhibited adequate, but borderline, CV values considering EMA criteria (CV<10%) (EMA, 2018b). For bifonazole, acyclovir, clobetasol and diclofenac, the CV values were closer to the FDA standard (CV<15%), proving that even though this agency also requires strict criteria, these are more reasonable than those documented by EMA (FDA, 2016c).

To further compare the product batches, the IVRR were statistically compared according to EMA, as well as FDA guidelines. The obtained confidence intervals are presented in Table 5.8.

As can be seen in Table 5.8, globally all products did not fit EMA criteria (90-111%), even though some borderline results were registered (hydrocortisone and bifonazole batches). Nevertheless, if FDA criteria are to be applied (75-133%), the majority of the batches are able to document its pharmaceutical performance equivalence.

Table 5.8 – Statistical treatment of IVRR data according to EMA and FDA guidelines. For example calculations please refer to Appendix B.

Products		**Confidence intervals (%) EMA**		**Confidence intervals (%) FDA**	
HC	HC_1 *vs.* HC_2	84.27	95.46	84.86	100.36
	HC_1 vs HC_3	86.26	101.72	84.73	111.78
	HC_2 *vs.* HC_3	97.60	111.75	95.80	122.95
EF	ETF_2 *vs.* ETF_3	76.42	134.35	73.96	143.97
	ETF_1 *vs.* ETF_2	71.75	125.27	68.64	125.33
	ETF_1 *vs.* ETF_3	72.03	121.53	62.76	115.86
BFZ	BFZ_1 *vs.* BFZ_2	91.99	107.1	92.49	114.66
	BFZ_1 *vs.* BFZ_3	86.49	104.30	85.37	115.69
	BFZ_2 *vs.* BFZ_3	88.99	102.9	90.38	109.54
CLT	CLT_1 *vs.* CLT_2	84.25	133.22	78.88	128.50
	CLT_1 *vs.* CLT_3	77.50	127.39	72.64	126.42
	CLT_2 *vs.* CLT_3	74.51	118.05	75.85	114.73
ACV	ACV_1 *vs.* ACV_2	84.37	102.59	83.53	111.33
	ACV_1 *vs.* ACV_3	74.11	87.11	108.50	131.75
	ACV_2 *vs.* ACV_3	80.64	92.48	113.28	149.21
TCZ	TCZ_1 *vs.* TCZ_2	79.74	104.27	79.87	106.05
	TCZ_1 *vs.* TCZ_3	79.55	119.73	74.23	126.36
	TCZ_2 *vs.* TCZ_3	87.18	131.37	83.34	135.49
CLB	CLB_1 *vs.* CLB_2	78.53	97.85	79.69	99.42
	CLB_1 *vs.* CLB_3	88.13	110.73	85.43	113.12
	CLB_2 *vs.* CLB_3	99.07	128.19	96.80	128.48
DF	DF_1 *vs.* DF_2	85.24	98.38	87.13	103.02
	DF_1 *vs.* DF_3	116.27	129.56	115.75	129.39
	DF_2 *vs.* DF_3	125.12	143.57	118.73	140.63

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac.

Table 5.9 – 90% Confidence intervals of microscopic analysis and rheological parameters. Confidence intervals were calculated based on the pairwise difference between population means.

	ACV		BFZ		CLB		CLT		DF		ETF		HC		TCZ	
	Zero-shear viscosity															
1_2	-490	1625	-6545	-175	-40648	-3125	-373	913	-2222	1366	13018	29468	-634	2034	4507	11353
1_3	-2531	1395	-6352	2038	-19602	-2285	-5527	-1280	-5394	1895	-6725	10192	-509	2129	-6654	-659
2_3	-3118	846	260	2147	-8764	30651	-5344	-2003	-4648	2005	-23039	-15980	-589	809	-13811	-9363
	Yield point															
1_2	-7.55	8.33	-34.6	-27.4	17.0	1.43	-3.58	0.781	-1.44	7417	-0.380	0.953	-0.395	4.88	1.03	0.776
1_3	-9.87	9.09	-11.9	-9.71	7.48	1.76	-26.9	-18.4	-1.69	1.25	-0.382	0.262	0.812	5.17	-0.109	1.19
2_3	-9.61	8.05	15.3	5.08	-27.8	-1.42	-25.7	-16.8	-1.06	0.33	-0.967	0.274	-1.58	0.08	-5.21	-0.514
	Infinite shear															
1_2	-7.55	8.33	-34.6	-27.4	17.0	1.43	-3.58	0.781	-1.44	7417	-0.380	0.953	-0.395	4.88	1.03	0.776
1_3	-9.87	9.09	-11.9	-9.71	7.48	1.76	-26.9	-18.4	-1.69	1.25	-0.382	0.262	0.812	5.17	-0.109	1.19
2_3	-9.61	8.05	15.3	5.08	-27.8	-1.42	-25.7	-16.8	-1.06	0.327	-0.967	0.274	-1.58	0.082	-5.21	-0.514
	Relative thixotropic area															
1_2	-2223	2217	-4734	1137	-26389	14489	-5894	-2134	-197	1197	-2910	3594	12096	13943	-677	27309
1_3	-13077	1757	-2081	4527	-15546	96	-19521	-11153	-1188	723	-9537	47	9862	12872	-6010	22172
2_3	-11309	-4.51	1285	4759	-19114	25064	-15725	-6921	-1527	62	-9932	-242	-2942	-364	-6795	-3675
	LVR plateau															
1_2	-2223	2217	-4734	1137	-26389	14489	-5894	-2134	-197	1197	-2910	3594	12096	13943	-677	27309
1_3	-13077	1757	-2081	4527	-15546 95	96	-19521	-11153	-1188	723	-9537	47	9862	12872	-6010	22172
2_3	-11309	-4.51	1285	4759	-19114	25064	-15725	-6921	-1527	62	-9932	-242	-2942	-364	-6795	-3675
	G´															
1_2	-198	410	-453	566	-3291	329	-196	-95.7	-3.01	2.18	155	60.82	265	37.7	-1222	-767
1_3	-525	312	-500	517	-1577	596	290	40.0	-12.0	-7.56	28.60	4.96	89.81	4.08	1436	554
2_3	-587	162	-80	-17	-1889	2870	400	22.2	-12.1	6.57	-128	-125	-208	-70.73	2187	792

	ACV		BFZ		CLB		CLT		DF		ETF		HC		TCZ	
	G´´															
1_2	-198	410	-453	566	-3291	1329	-196	-95.7	-3.01	2.18	155	161	265	338	-1222	-767
1_3	-525	312	-500	517	-1577	596	290	340	-12.0	-7.56	28.6	35.0	89.8	34.1	1436	1554
2_3	-587	162	-79.6	-17.0	-1889	2870	400	522	-12.1	-6.57	-128	-125	-208	-70.7	2187	2792
	Loss Tangent															
1_2	-0.010	0.010	0.004	0.091	-0.046	0.033	-0.021	0.008	-0.019	0.013	-0.137	-0.117	-0.167	-0.133	-0.085	-0.048
1_3	-0.017	0.003	-0.037	0.075	-0.039	0.032	-0.010	0.010	-4.96	0.020	0.013	0.033	-0.128	-0.025	-0.665	-0.608
2_3	-0.017	0.003	-0.046	-0.011	-0.037	0.044	-0.008	0.021	-0.003	0.03	0.140	0.160	0.034	0.113	-0.599	-0.541
	Particle / Globule size															
1_2	-0.151	0.151	0.125	1.675	1.62	2.58	0.4421	0.5779	0.842	1.36	1.42	2.98	6.43	7.17	0.424	1.98
1_3	1.21	1.99	0.491	1.909	2.835	3.765	0.0124	0.0876	2.05	2.55	-0.391	0.791	1.85	2.35	1.81	2.59
2_3	1.21	1.99	-0.321	0.921	0.893	1.507	0.3978	0.5222	1.02	1.38	1.18	2.82	4.27	5.13	0.215	1.79

5.4 Discussion

Batch-to-batch variability can be motivated essentially by two main factors: complexity of excipient source and differences in manufacturing procedures. Regarding the first factor, it is important to note that most excipients are naturally derived or semisynthetic compounds. A clear example is cetostearyl alcohol, an emulsifier present in the majority of the products in this study, see Table 5.2. Cetostearyl alcohol is prepared by the reduction of fatty acids, that can be obtained from vegetable/animal or other hydrocarbon sources. The proportion of stearyl to cetyl alcholol usually varies between 50-70% to stearyl and 20-35% for cetyl alcohol. Moreover, small quantities of other alcohols, such as myristyl alcohol, are frequently added thus potentially broadening the intrinsic variability of this specific excipient (Rowe *et al.*, 2012). Even though semisolid product manufacturers are expected to have controlled/fixed suppliers, there are several reports on excipient intra-supplier variability that can lead to differences in the final product (Zarmpi *et al.*, 2017). Other variability source relates to the manufacturing process. Critical method variables applied to semisolid product manufacture can include: (i) order and rate of component addition; (ii) temperature cycles; (iii) mixing methods; (iv) air trapping and (v) packaging procedures (Raghavan *et al.*, 2019; Simões *et al.*, 2019).

Differences regarding the manufacturing process are not estimated to be significant when dealing with the product batches, since manufacturers are expected to work with a well-established process window, that should accommodate minor differences arising from environmental conditions, as well as differences in materials (excipients/active substance) (van Heugten *et al.*, 2017). Nevertheless, with the imposition of strict acceptance criteria by regulators, these differences may result in significant variability results.

As depicted in Table 5.4, marked statistical differences in globule/particle size were observed for every batch. These, however, did not always directly correlate with differences in the rheology or release profile.

A closer inspection of Fig.5.3 reveals that for both hydrocortisone and clobetasol formulations, the differences in globule size did not influence the rotational and IVRT profiles, with all batches revealing similar trends. Despite all batches are compliant with the FDA equivalence requirements, solely HC_2 and HC_3 batches presented borderline results as per European guidelines. For clobetasol batches, despite the release profiles being quite similar, it could be observed that the batch with highest release, CLB_3, was also the one with lowest globule size. Regarding IVRT confidence intervals, solely FDA criteria were met.

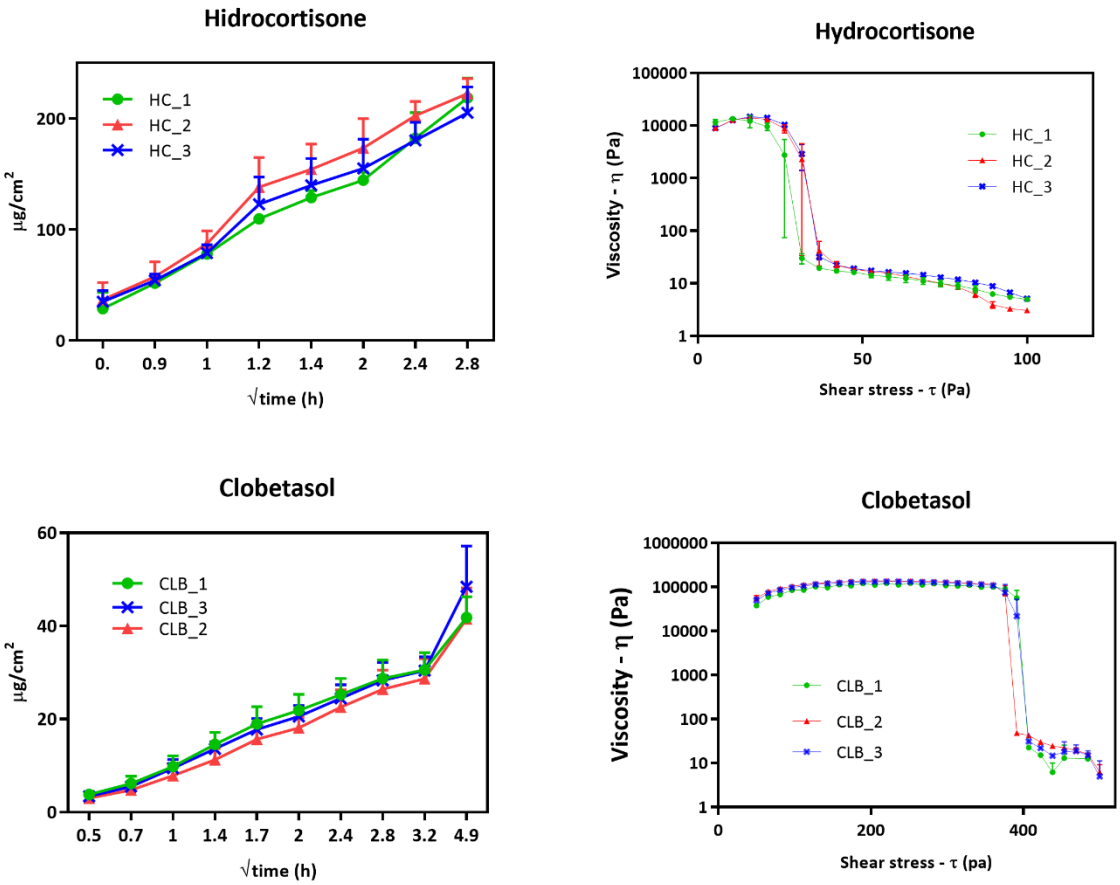


Fig.5.3 – IVRT profile (n=12± SEM) and viscosity curves (n=3± SEM) for hydrocortisone and clobetasol formulations.

A different scenario was registered with tioconazole formulations, where globule size, rheology and release differences were found for all batches. The formulation with lowest particle size, displayed the highest release (TCZ_3), however, no significant differences between TCZ_1 and TCZ_3 were found in the rotational profile (Fig.5.4). A different trend was observed for the diclofenac formulations, where DF_3, the batch with lowest globule size, displayed an inferior release when compared to the other batches. Nevertheless, for this emulgel formulation, the batch with highest particle size (DF_1) was the one with lowest viscosity, exhibiting a higher release (see Fig.5.4) This phenomenon may be attributed to particle coalescence prompted by differences in process parameters, such as the homogenization speed (Simões *et al.*, 2020b). IVRR confidence intervals for diclofenac and tioconazole did not meet EMA requirements. Moreover, for some pairwise comparisons in both formulations, even FDA requirements were not meet (Table 5.8).

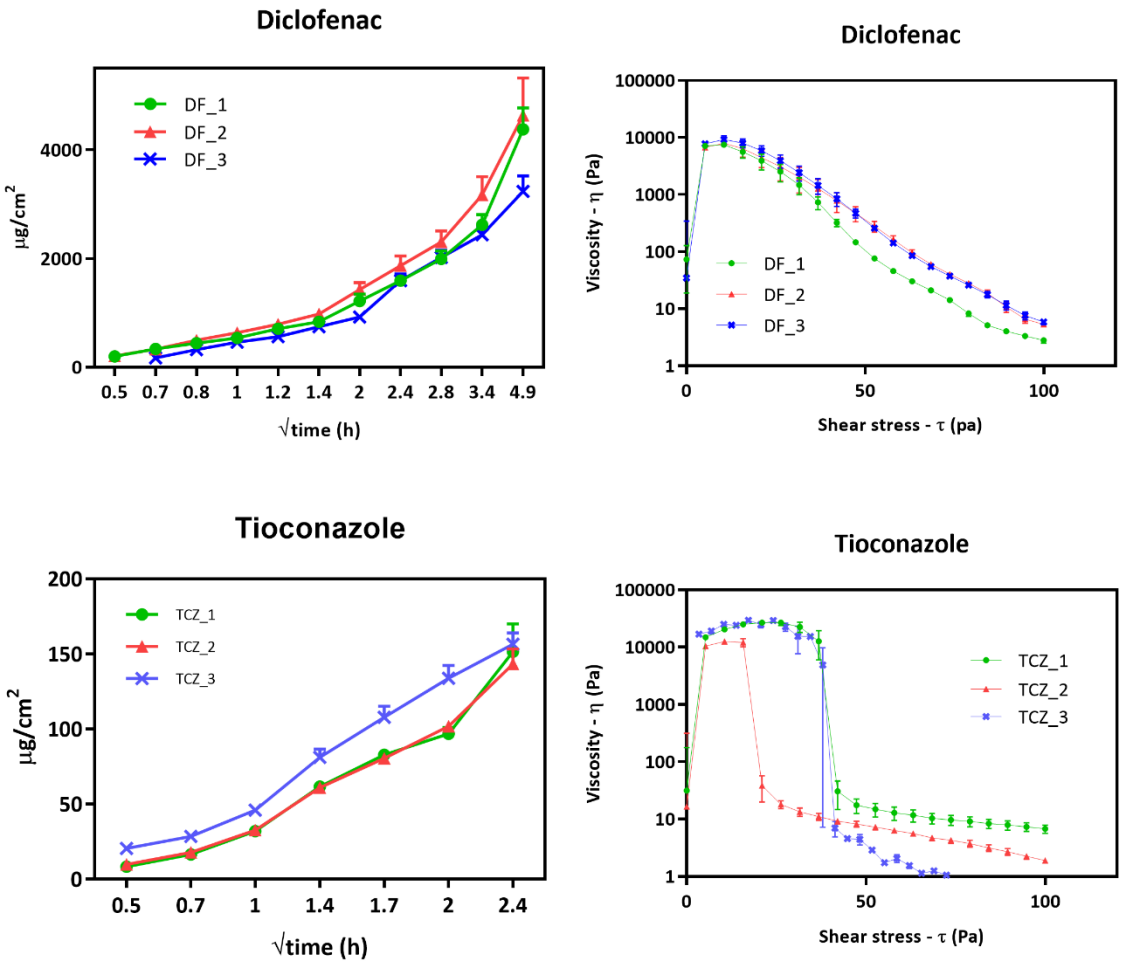


Fig.5.4 – IVRT profile (n=12 ± SEM) and viscosity curves (n=3 ± SEM) for diclofenac and tioconazole formulations.

Etofenamate, clotrimazole and bifonazole formulations presented similar tendencies, all sharing marked inter-batch differences between globule and rotational profile, see Fig.5.5. However, these did not have a direct repercussion in the release behaviour. For clotrimazole and bifonazole, the batches with lowest globule size, CLT_1 and BFZ_3, respectively, displayed lower viscosities. Bifonazole and clotrimazole batches overall revealed compliant IVRR confidence intervals results regarding FDA requirements; nevertheless, solely BFZ_1 and BFZ_2 comparison proved to be acceptable by EMA criteria. The variability observed in etofenamate formulations was highly evidenced, with none of the IVRR confidence intervals meeting nor EMA or FDA requirements (see Table 5.8).

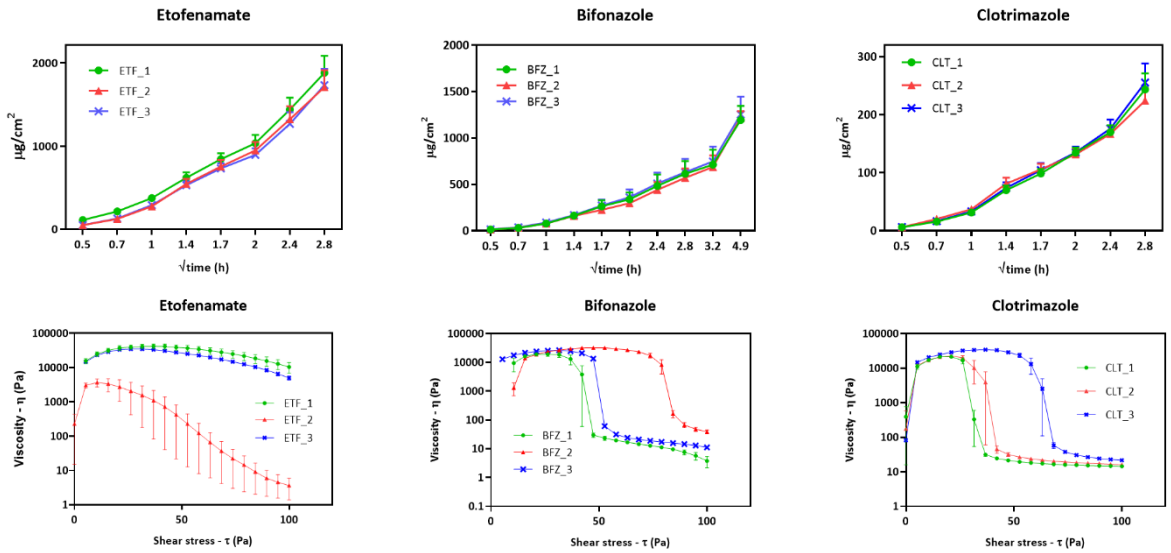


Fig.5.5 – IVRT profile (n=12± SEM) and viscosity curves (n=3± SEM) for etofenamate, bifonazole and clotrimazole formulations.

On the other hand, acyclovir batches revealed the inverse trend, with similar rotational profiles, but distinct release behaviour, see Fig.5.6. For this product, the batch that presented the highest release rate, ACV_3, was the one with superior particle size and a lower pH value. Note that, for this product, DSC thermograms (Fig.5.2) suggested that the majority of the drug is incorporated in the formulations lipid phase, despite all batches revealed different endothermic patterns (Ethier *et al.*, 2019). Similarly to the other products, confidence intervals regarding IVRR solely meet FDA requirements.

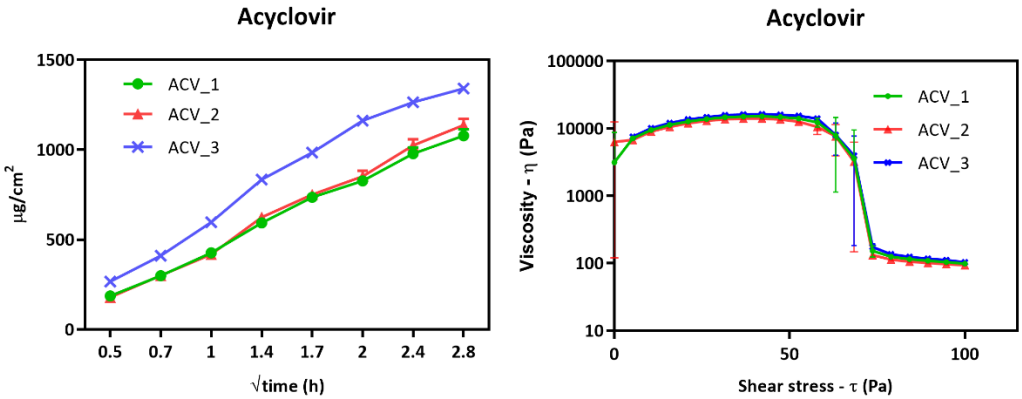


Fig.5.6 – IVRT profile (n=12 ± SEM) and viscosity curves (n=3 ± SEM) for the acyclovir formulation.

As mentioned in Table 5.6 and Table 5.7, high rheological variability is evidenced for all products. A similar trend was observed in a recent study by Victor Mangas-Sanjuán and collaborators. In this work, the rheological profile of 10 batches of a reference ointment

containing calcipotriol and betamethasone (Daivobet® 50 µg/0.5 mg/g) was assessed. Such findings were based on the determination of a wide range of rheological parameters, including the relative thixotropic area, yield stress, zero-shear viscosity, loss tangent at 1 Hz, G´, G´´, among others. After performing parametric as well as non-parametric analyses, inter-batch microstructure equivalence, following the new EMA criteria, could not be demonstrated for the majority of the parameters. This study concludes that an acceptance range of 10% is too demanding, given the high inter-batch differences observed among the same reference product (Mangas-Sanjuán *et al.*, 2019).

As previously mentioned, the basis of the abridged process for TGP bioequivalence documentation following the EMA guideline, relies on an extensive characterization of the RP. All generic products acceptance criteria are, therefore, ultimately dependent on reference product results. According to EMA requirements, generic products IVRR should be within a 90-111% confidence interval pertaining to the reference product. Inter-run variability should not surpass 10% and at least 12 replicates should be considered.

These criteria are different from those proposed by USA regulators, where only 6 replicates should be regarded, IVRR confidence intervals should be within 75-133% and IVRR inter-run variability should not exceed 15% (FDA, 2016b, 1997). Another specific requirement regards the percentage of drug release that should be at least 70% (EMA, 2018b). Note that to ensure sink conditions for an accurate characterization of release kinetics, cosolvents were used in the release medium. As previously mentioned, linearity (R^2 > 0.9) was observed in each diffusion cell, thus documenting the compliance with EMA guideline. Despite this fact, no more than 50% was attained (this corresponds to the diclofenac formulations). Once again, EMA should carefully review this specific requirement.

IVRT validation procedures, proposed by both agencies, are however quite similar. These report to: IVRT method development studies (receptor medium, membrane, experimental procedures); diffusion apparatus and laboratory qualification; linearity and range; precision and reproducibility; drug recovery; discriminatory ability and robustness.

IVRT results, displayed in Table 5.7 and Table 5.8 highlight that the criteria proposed by EMA do not account for semisolid dosage form and IVRT method intrinsic variability. If the equivalence criteria are not met while considering inter-batch products from the same manufacturer, generic companies continue to lack a real and viable alternative to CES.

For *in vitro* permeation studies, a wider CI between 69.84-143.9% may be accepted for complex products that exhibit high variability, low strengths and limited diffusion.

Nevertheless, for IVRT this extension does not apply. Moreover, a list of concrete examples is not provided by the authorities. According to the FDA, the 5% acyclovir cream (also used in this study) can be considered as a complex product since it is an oil-in-water emulsion and the drug is dispersed in both phases (Krishnaiah *et al.*, 2014; Murthy, 2017; Raghavan *et al.*, 2019). Nevertheless, this situation is also observed for other semisolid formulations, including the ones presented in this study. Can these products be also regarded as complex formulations and benefit from broader equivalence criteria?

A cross-functional analysis regarding microstructure and performance evaluation of topical products is now presented. The main objective was to inspect and characterize formulation physicochemical drivers that may account for batch-to-batch variability within RP semisolid dosage forms. Data concerning globule/particle size, pH, rheology and *in vitro* release profile were included in this analysis.

The resulting pool of data was further characterized using principal component analysis (PCA) and hierarchical cluster analysis (HCA) on principal components in order to rank batches, document and define meaningful groups of formulations according to the variability evaluated.

Specifically, PCA was applied aiming at (i) extracting key information from the pool of data; (ii) computing a compact and optimal description of the system; and finally (iii) performing an in-depth analysis of the relationships between product performance and formulation variables (Cova *et al.*, 2017; Rasmus Broa, 2014; Silva *et al.*, 2018).

The biplot representation in Fig.5.7 displays the scores corresponding to the different products and the loadings for each formulation variable on the first two principal components. The relative positioning of all batches considering the new orthonormal principal component system (PC1 *vs.* PC2) accounts for 67.9% of data variability.

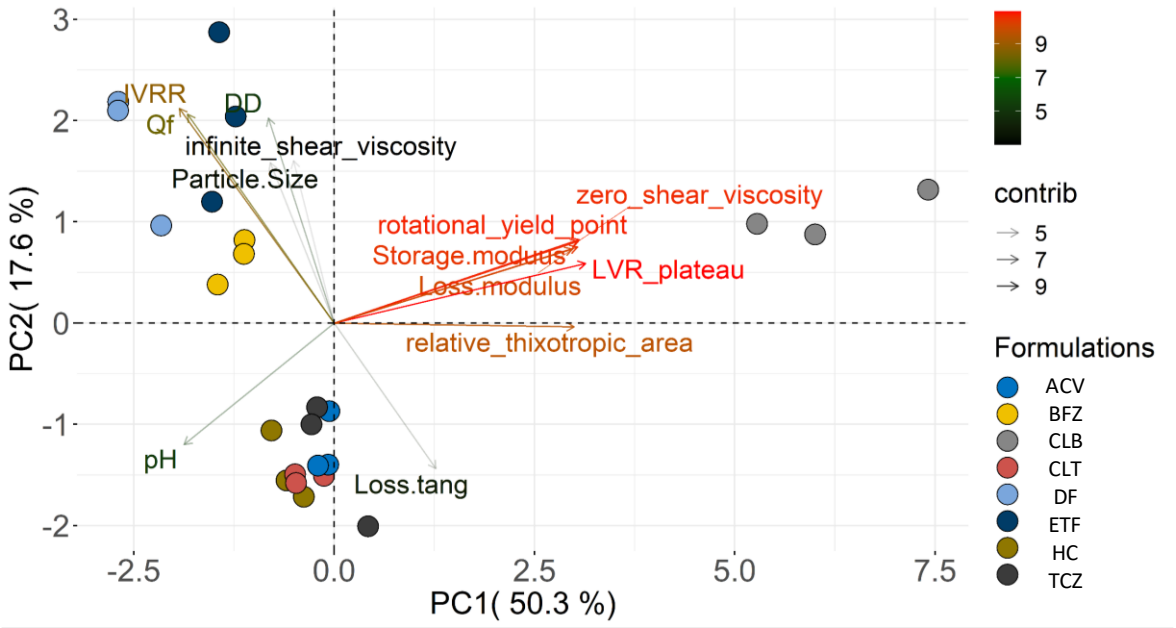


Fig.5.7 – Biplot representation of the 24 objects (8 RP x 3 batches) and the 13 corresponding variables on the first two components, retrieving a 67.9% of variance.

Key: HC – Hydrocortisone; ETF – Etofenamate; BFZ – Bifonazole; CLT – Clotrimazole; ACV – Acyclovir; TCZ – Tioconazole; CLB – Clobetasol; DF – Diclofenac. IVRR: – *In vitro* release rate; Q_t: Cumulative amount of drug release at 24 h; DD – Dose depletion. The most significant variables on PC1 and PC2 were selected using the procedure previously used in (Cova *et al.*, 2013).

To interpret the biplot, the relative batch positioning and the vectors length and direction should be carefully inspected. In this context, batches that share the same overall positioning exhibit a similar pharmaceutical profile. Our results indicate that the first principal component is mainly based on the formulation rheological profile (zero-shear viscosity, loss modulus, storage modulus, LVR plateau and relative thixotropic area, yield point) and the release profile, which determined the highest variability among the products. Table 5.10 summarizes the proportion of variance obtained for both PC1 and PC2, as well as the main responses linked with intra-product batch variability. Table 5.11 reports to the obtained PCA confidence intervals.

Table 5.10 – PCA analysis for each drug product.

	PC1	PC2	PS	IVRR	Q_f	DD	pH	η_0	τ_0	η_∞	S_R	LVR	G´	G´´	Loss tang
HC	65.68%	34.32%	X			X	X	X	X		X		X	X	X
ETF	69%	31%	X				X	X	X	X		X	X		X
BFZ	53.70%	46.20%		X	X						X	X	X	X	
CLT	70.67%	29.33%		X	X			X	X	X		X	X	X	
ACV	73.73%	26.27%	X	X	X	X	X			X		X	X	X	X
TCZ	62.29%	37.71%		X	X	X		X	X	X	X	X	X	X	
CLB	57.26%	42.74%	X	X	X		X						X	X	X
DF	82.26%	17.74%	X	X	X	X	X	X	X			X	X		X

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazole; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac. PS – Particle size; IVRR – *In vitro* release rate; Q_f – Cumulative amount of drug release at 24 h; DD – Dose depletion; Hyst. Area: Hysteresis area; η_0 – zero-shear viscosity; $\eta\infty$ – Infinite-shear viscosity; LVR – Linear viscoelastic region; G´ – Storage modulus; G´´ – Loss modulus. The most significant variables on PC1 and PC2 were selected using the procedure previously used in (Cova *et al.*, 2013).

Table 5.11 – Statistical analysis concerning the developed PCA model. The 90% CIs were calculated based on the absolute loading values. The bootstrap method was followed.

PC1		
Parameter	**90% CI**	**Standard Error**
LVR Plateau	[0.3420, 0.3920]	0.0162
Zero-shear viscosity	[0.3406, 0.3927]	0.0502
Rotational yield point	[0.3315, 0.3868]	0.036
Storage modulus	[0.3212, 0.3784]	0.0179
PC2		
Dose Depletion	[0.1133, 0.2673]	0.0464
Cumulative amount of drug released in the end of the study (Q_f)	[0.0677, 0.2478]	0.0661
In vitro release rate	[0.1451, 0.3105]	0.0527

HCA was then applied to identify batch similarity. Table 5.11 depicts the resulting dendrogram (Silva *et al.*, 2018).

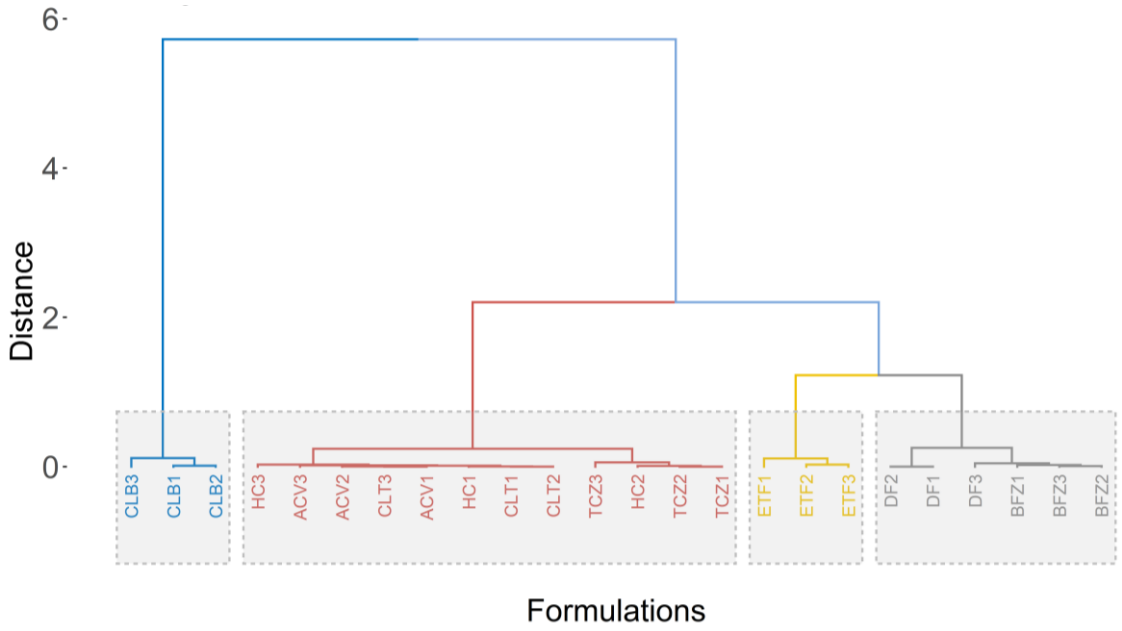


Fig.5.8 – Hierarchical cluster analysis (HCA), using the Euclidean distance and the Ward method, for all products taking into consideration their microstructure and performance attributes.

Key: HC – Hydrocortisone; ETF – Etofenamate; BFN – Bifonazol; CLT – Clotrimazole; ACV – Acyclovir; TCN – Tioconazole; CLB – Clobetasol; DF – Diclofenac.

For the majority of the products, as displayed in the dendrogram, a clear separation was achieved for all batches. Overall, two clusters are identified – one regards to clobetasol batch and the other one encompasses all the other products. Within the second cluster, the same trend repeats with two subgroups easily identifiable, one concerning diclofenac, bifonazole and etofenamate, and the other entailing all the remaining products.

Within this third cluster level, batch grouping is not carried out per product, again reflecting the variability previously observed.

In summary, rheological attributes promote the highest variability amongst the same product batches. However, responses related to product *in vitro* performance, such as Q_f and IVRR, also provide a considerable source of batch-to-batch differences.

As previously mentioned, microstructure inter-batch differences in semisolid drug products are mainly related with complexity of the excipient sources, and/or manufacturing procedures differences (Mangas-Sanjuán *et al.*, 2019; Pleguezuelos-Villa *et al.*, 2019). A recent study by Pleguezuelos-villa and collaborators stated the need of the regulatory agencies to properly define suitable acceptance criteria regarding microstructure equivalence. When defining the appropriate range of variability acceptance, the intrinsic variability of semisolid dosage forms should be accounted for. These authors have compared the *in vitro* release profile and the

rheological properties of two semi-solid formulations of 2% diclofenac diethylamine (manufactured by different laboratories). Even though both formulations shared the same qualitative and quantitative profile, microstructure equivalence, according to EMA requirements, failed to be supported. Both formulations were then clinically compared through pharmacokinetic studies. The results showed that no statistically significant differences were observed between both products (Pleguezuelos-Villa *et al.*, 2019). Taking into account this information and the fact that all studied products were evaluated through clinical endpoint studies, batch-to-batch microstructure differences in these products do not translate into a different efficacy and safety profile. Therefore, establishing reasonable microstructure sameness criteria, taking into account the intrinsic variability of the product being studied, is imperative in order to sustain a successful translation of the EMA draft guideline on quality and equivalence of topical products.

5.5 Concluding remarks

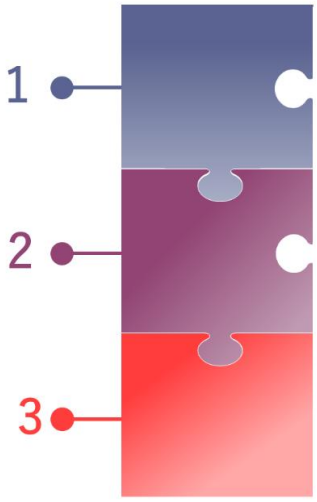
This chapter aimed at addressing critical requirements issued by the recent EMA draft guideline on topical products quality and equivalence. Even though this document represents a noteworthy regulatory advance concerning TGP bioequivalence demonstration, an attentive reading of the guideline clearly suggests that there are equivalence criteria that may prove to be extremely challenging to attain. As a proof of concept, we evaluated the microstructure and performance of 8 blockbuster reference topical products. A total of 3 batches was considered for each product.

Our results show that topical product microstructure varies from batch-to-batch. These variations, especially in what regards droplet/particle size and rheological behaviour, are particularly evident when considering batches retrieved from different manufacturing sites. Regarding product performance, evaluated through IVRT, if EMA requirements on CV, percentage of release and IVRR confidence intervals, are to be applied, none of batches is able to document its extended pharmaceutical equivalence. Nevertheless, if wider limits are accepted, such as the ones imposed by USA-FDA regulators, the majority of products document its equivalence.

In order to conduct a systematic evaluation on the parameters that play a major role on batch-to-batch variability, a multivariate analysis was performed. HCA proved that this analysis is able to differentiate same product batches. PCA analysis demonstrated that the rheological profile and IVRT attributes are the parameters which majorly provide the intrinsic variability of semisolid dosage forms.

According to these results, the selection of RP batches is not irrelevant, especially whenever involved in a TGP R&D program aiming at an abridged bioequivalence demonstration. In this context, generic manufacturers are encouraged to pay particular attention to this specific issue so that they can ensure both technical and regulatory success. However, the basis of this selection is mainly empirical, so further work and discussion with EMA is still necessary to specify more reasonable criteria. These should maintain rigorous quality standards, however, the intrinsic variability of topical semisolid dosage forms should be accounted for.

5.6 Highlights



According to EMA´s criteria, all RP addressed in this study revealed marked batch to batch differences in microstructure and performance attributes. Nevertheless, if FDA requirements are to be followed instead, equivalence can overall be inferred.

In light of the obtained results, RP batch selection is not irrelevant, especially whenever involved in a TGP R&D program aiming at an abridged bioequivalence demonstration. Generic manufacturers are encouraged to pay particular attention to this specific issue.

In IVPT studies, complex products exhibiting high variability, low strength and limited diffusion may benefit from wider CI, according to EMA requirements. For IVRT studies this extension is not applicable. Nevertheless, applying wider acceptance criteria in Q3/Q4 endpoints for complex formulations in which the RP displays significant intrinsic variability, is a key point to sustain a successful EMA guideline translation into practice.

6 TOPICAL BIOEQUIVALENCE: EXPERIMENTAL AND REGULATORY CONSIDERATIONS FOLLOWING FORMULATION COMPLEXITY

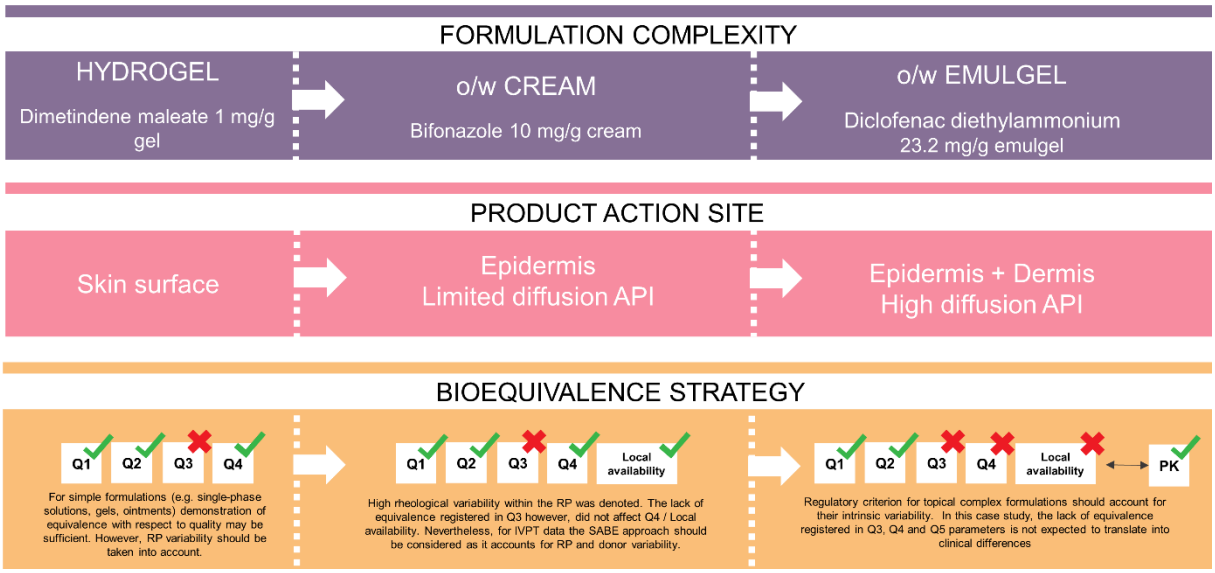


Fig.6.1 – Graphical abstract: Topical bioequivalence: experimental and regulatory considerations following formulation complexity.

This chapter has been adapted from the following publications:

Miranda, M., Veloso, C., Brown, M., Pais, A.A.C.C., Cardoso, C., Vitorino, C.,– Topical Bioequivalence: Experimental and regulatory considerations following formulation complexity (submitted manuscript).

Miranda, M., Brown, M., Pais, A.A.C.C., Cardoso, C., Vitorino, C.,– Tailoring bioequivalence – A topical antifungal case-study (manuscript in draft).

M.M and C. Vitorino conceived the presented idea and established the research program and implementation. A.A.C.C Pais assisted in the statistical analysis, more specifically in confidence interval calculations according to EMA/FDA guidelines. C. Veloso assisted in the dimetindene IVRT studies. Data curation, review and editing were provided by M.B, A.A.C.C Pais, as well as C. Vitorino. Supervision was provided by C. Vitorino and C.C. M.M did the experimental part of the work and wrote the first draft of the manuscript. All other authors substantially contributed to revisions. Funding acquisition was provided by C.C and C. Vitorino.

KEY CONCEPTS

- According to the EMA guideline, for simple formulations, BE may be demonstrated by documenting Q1/Q2/Q3 and Q4 equivalence. Nevertheless, when addressing complex semisolid dosage forms, equivalence regarding product efficacy should also be demonstrated.
- According to regulators, all methods used during product characterization studies should be discriminatory. To evaluate so, formulations with different CQA should be manufactured and collectively analyzed with the studied formulations. Suitable endpoints, retrieved from each method, should be defined in order to compare the formulations.
- As the *stratum corneum* is the main barrier for percutaneous absorption, IVPT experiments mimic *in vivo* drug permeation. This scientific rationale supports the usage of IVPT as a surrogate method to clinical endpoint studies, as these tests can likewise be used to infer on product efficacy. IVPT endpoints include the maximum flux and the cumulative amount of drug permeated in the end of the IVPT study. Similarly to the previous chapter, US and EMA criteria were cross compared. In accordance with US requirements, the use of a scaled average bioequivalence approach is recommended for IVPT data due to formulation complexity and donor variability.

6.1 Introduction

Encouraging the widespread availability of generic versions of medicines is an important public health priority with profound economic and social implications (Shin *et al.*, 2020; Vo *et al.*, 2020). By expanding the market-available generic portfolio, medication costs decrease, patient compliance with medication regimens is higher, and potential drug shortages can be minimized, due to greater product sourcing availability (Vo *et al.*, 2020).

Generic products affordability is linked with highly efficient scientific and regulatory mechanisms, used to develop and approve most of generic drug products (Shin *et al.*, 2018a). Pharmaceutical equivalence and BE of the TP towards the RP should be adequately documented to ensure therapeutic equivalence and interchangeability (Lukic *et al.*, 2020). Comparative pharmacokinetic studies represent the gold standard for BE assessment. Nevertheless, for the vast majority of topically applied and locally acting drugs, alternative approaches must be equated due to the absence of systemic drug absorption (Kamal *et al.*, 2020). Until recently, these have generally relied on clinical endpoint studies, which present multiple drawbacks, as reviewed in Chapter 1. However, with the increasingly broader range of *in vitro*/*ex vivo*/*in vivo* dermal pharmacokinetic approaches to assess topical absorption, the regulatory agencies have opened up to surrogate methods to document the bioequivalence of TGP, as thoughtfully addressed in Chapter 2 (Chang *et al.*, 2013a; Mohan and Wairkar, 2020; Quartier *et al.*, 2019; Raney *et al.*, 2015; Yacobi *et al.*, 2014). Clear examples of this “regulatory openness” include several FDA non-binding product-specific guidances for TGP development and the EMA draft guideline on quality and equivalence of

topical products (EMA, 2018b; FDA, 2016c). Although both agencies share some common points, their scope is completely different. The EMA recommends a "one-fits-all approach" with the only difference reporting to simple *vs.* complex formulations. On the other hand, the FDA proposes a specific guidance for each product. Despite the clear differences on the guidelines applicability, generally to grant a waiver of clinical endpoint studies, a modular framework for BE documentation is often accepted. First, the qualitative composition of the TP should be equivalent to the RP. Although this may seem unproblematic, difficulties may arise in selecting the excipient grade, as this information is not publicly available. This is to be followed by the quantitative equivalence (Q2) sameness. To achieve this, reverse engineering procedures may be required. Microstructure equivalence (Q3) should also be documented. Within this analysis, data on pH, droplet/particle size, product metamorphosis, rheological behavior analysis, stability profile, among other parameters, should be provided. Product performance equivalence (Q4), mainly supported by IVRT methods, should likewise be evidenced.

Finally, studies on local availability of the product should also be submitted. According to EMA, these can be further divided into two categories: permeation kinetic studies and pharmacodynamic studies. The first category includes (i) dermatopharmacokinetic studies for drugs that present limited diffusion and predominantly target the skin surface; (ii) IVPT studies for drugs that present a quantifiable permeation profile; and finally (iii) pharmacokinetic studies for drugs that are systemically bioavailable. In this context, the selection of permeation kinetic studies to be used depends mainly on the "site" where the drug can be quantified.

Local product availability equivalence can also be supported by pharmacodynamic methods. The most common methodology regards the vasoconstriction assay, which is solely applied to corticosteroids because of the respective skin bleaching properties. Antiseptic and *in vitro* skin infection and decolonization equivalence studies may also be applied, if satisfactorily validated (EMA, 2018b).

Even though this framework is shared by both agencies, there are also other singularities, namely the acceptance criteria and the overall statistical analysis.

Several recent publications have questioned the suitability of the EMA draft guideline criteria. Victor Mangas-Sanjuán and colleagues compared the rheological profile of 10 different batches of Daivobet® 50 µg/0.5 mg/g, an RP calcipotriol and betamethasone ointment. All rheological studies were performed between 6 months to one year after product manufacture. Although all batches were expected to present the same microstructure profile, no inter-batch equivalence was found based on the 10% acceptance range of EMA criteria,

thus reinforcing the unsuitability of this criterion for this particular formulation (Mangas-Sanjuán *et al.*, 2020, 2019). As the RP are expected to be clinically equivalent, the threshold of 10% regarding microstructure equivalence as per EMA requirements proved to be too strict and does not represent overall therapeutic equivalence. In another paper by the same group, a mathematical framework was proposed to define the minimum number of batches and units per batch and product to be compared. For this purpose, the authors considered a vast range of different scenarios of inter-batch and intra-batch variability (Xu *et al.*, 2020). Their results showed that for RP with low intra- and inter-batch variability, the minimum number of batches to be compared was 3, with 6 replicates. If the RP presented a difference of 2.5-5%, 6 batches with 12 replicates were required. Nevertheless, if the intra- or inter-batch variability exceeded 10%, the number of batches and replicates had to be further increased (Xu *et al.*, 2020). Furthermore, as discussed in Chapter 5, the selected pool of 8 RP presented marked intra-batch differences, with the rheological parameters as well as the IVRT indicators being the sources of the greatest discrepancies. Statistical analysis demonstrated that if EMA criteria are applied, none of the same product batches could be considered equivalent. Julie Quartier *et al.* developed a cutaneous biodistribution method, which provides insight into the spatial distribution of a drug in the epidermis/dermis (Quartier *et al.*, 2019). This methodology was then used to compare the biodistribution profile of econazole from an RP and two approved generic products under finite dose conditions using human skin. In this work, EMA requirements were not met; nevertheless, it should be taken into account that both generic products had comparable clinical endpoint studies (Quartier *et al.*, 2019).

Undoubtedly, the EMA draft guideline represents a noteworthy regulatory advance over clinical endpoint studies – the gold standard method for TGP BE demonstration. As extensively reviewed, due to the intricacies linked with dermal absorption, clinical studies require a complex structure (randomized, double-blind, placebo-controlled, parallel group), hundreds of patients (n > 500) and consequently high costs (Mohan and Wairkar, 2020; Narkar, 2010; Quartier *et al.*, 2019; Yacobi *et al.*, 2014). This scenario acts as a deterrent towards development of affordable topical generic products (Mohan and Wairkar, 2020). Although the modular framework (from Q1 to local availability) for BE assessment may be of extreme importance when addressing the market growth of TGP, the previously described reports shed light on the EMA regulatory criteria applicability, and consequently on the difficulties in translating the guideline into practice. Despite these limitations, the list of products for which surrogate methods can be used is growing year by year, as reviewed in Ili´c *et al.* (Ilić *et al.*, 2021). It is important to note that, unlike EMA, FDA primarily requires clinical endpoint studies to demonstrate topical bioequivalence.

Several research papers have addressed topical products BE evaluation following FDA regulatory requirements. Nevertheless, these usually report to acyclovir cream formulations, as these regard complex products where bioavailability is highly dependent on formulation characteristics (Krishnaiah *et al.*, 2014; Pensado *et al.*, 2019; Shin *et al.*, 2020; Xu *et al.*, 2015a, 2015c).

Considering the broad range of available topical products, the aim of the present chapter was to challenge the modular strategy recommended by regulators to address topical BE assessment applied to three case studies: a dimethindene maleate 1 mg/g gel, a bifonazole 10 mg/g cream and a diclofenac 20 mg/g emulgel. These formulations were selected in an attempt to address a wide range of technological features, as well as targeting sites.

In the dimethindene formulation, the API is included within a single-phase aqueous base (hydrogel). According to the EMA guideline, in these circumstances, BE may be demonstrated by supporting the extended pharmaceutical equivalence (Q1-Q4 sameness) (Kamal *et al.*, 2020). A test formulation with the same qualitative and quantitative composition was produced. Microstructure evaluation (Q3) was determined by evaluating the rheological behaviour of both TP and RP. IVRT tests were then conducted to compare product performance (Q4).

This scenario usually differs when more complex dosage forms are involved, such as creams or emulgels, where to document BE, Q1- local availability demonstration is required. The bifonazole cream is a biphasic semisolid system that acts on the skin surface, and the diclofenac emulgel not only exhibits a multiphasic nature, but also contains a penetration enhancer in its qualitative composition that contributes to the complexity of the product. Accordingly, this product targets deeper layers of the skin, such as the dermis. Therefore, this particular diclofenac formulation exerts its pharmacological action in the dermis and is one of the few topical products where comparative pharmacokinetic studies can be successfully used to assess and compare topical drug delivery. A recent study by Pleguezuelos-villa *et al.* was able to document BE of a generic diclofenac 20 mg/g emulgel (Pleguezuelos-Villa *et al.*, 2019). The same product was herein used and compared to the RP following the Q1- local availability strategy. Similar to the dimetindene formulation, the diclofenac TP was Q1 and Q2 equivalent to the RP. Q3 sameness was evaluated using rheological methods, and IVRT studies were likewise conducted to assess Q4 similarity. IVPT studies were performed to document the product efficacy and then cross compared with the pharmacokinetic results attained from the Pleguezuelos-villa *et al.* study. The same bioequivalence flowchart was used for the bifonazole cream formulation. However, in an attempt to “challenge” this strategy, a larger pool of products was used: the RP, a Q1/Q2 test formulation, a Q1 formulation (comparator product

A – CPA) together with a bifonazole cream formulation with Q1/Q2 differences (comparator product B – CPB).

For simplicity, this chapter was further divided into two parts. The first one addresses the two "extreme" case studies - the diclofenac and the dimetindene formulations. The dimetindene gel acts on the skin surface and has a monophasic structure, whereas the diclofenac products target the dermis and can be considered complex products. The second part solely addressed the bifonazole cream formulations, which, as mentioned above, are a biphasic semisolid system which predominately targets the skin superficial layers.

In this context, this work envisions to introduce a rationale for BE documentation according to the formulation complexity and intrinsic RP variability.

PART A

6.2A Materials and methods

6.2.1A Materials

Two topical products were included in the 1st part of the present chapter – a 0.1% (w/w) dimetindene gel and a 2% (w/w) diclofenac emulgel. For each product, a reference and a test product were studied. Whenever possible, 3 different batches were analyzed, but due to market availability, not all the batches had the same age.

The RP of the dimetindene maleate 1 mg/g gel formulation was Fenistil®. A Q1/Q2 equivalent formulation was manufactured (for confidential purposes this information could not be disclosed). Three batches were considered for each product. Table 6.1 provides information on the dimetindene products studied, as well as on their qualitative composition.

Table 6.1 – General information and qualitative composition of the dimetindene products used in the present study. The batch age is given in months (M). All studied products have an expiry date of 36 months.

Studied products		Reference Product: Fenistil®	Test product
	Used batches	RP1: Tested at M34 RP2: Tested at M35 RP3: Tested at M33	TP1: Tested at M8 TP2: Tested at M8 TP3: Tested at M7
Excipient	Function		
Benzalkonium chloride	Preservative	X	X
Disodium EDTA	Chelating agent	X	X
Propylene glycol	Solvent / humectant	X	X
Purified water	Solvent	X	X
Sodium hydroxide	Buffering agent	X	X

Key: RP – Reference Product; TP – Test Product.

For the diclofenac diethylammonium 23.2 mg/g emulgel, the RP considered was Voltaren® emulgelex (3 different batches) and the TP considered was a diclofenac Pharmakern® formulation. Moreover, for the evaluation of the IVRT discriminatory capacity, a different strength commercial formulation was likewise used – diclofenac emulgel 1% w/w (Voltaren Emulgel®). Information on the batches studied, as well as on the qualitative composition of the main products, is displayed in Table 6.2.

Propylene glycol was acquired from Merck, phosphate buffered saline (PBS) was purchased from Sigma. Water was purified using a Millipore MILLI-Q reagent water system and filtered through a 0.22 µm nylon filter before use. All other chemicals were of analytical grade or equivalent.

Table 6.2 – General information and qualitative composition of the products used in the present study. The batch age is given in months (M). All studied products have an expiry date of 36 months.

Studied products		Reference Product: Voltaren®	Test product: Pharmakern®
	Used batches	RP1: Tested at M16 RP2: Tested at M12 RP3: Tested at M12	TP Tested at M22
Excipient	Function		
Butylhydroxytoluene	Antioxidant	X	X
Carbomers	Gelling agents	X	X
Cocoyl caprylocaprate	Solubilizer	X	X
Diethylamine	Buffering agent	X	X
Isopropanol	Solvent	X	X
Mineral oil	Emollient / emulsifier	X	X
Oleyl alcohol	Permeation enhancer	X	X
Parfum	Organoleptic agent	X	X
Polyoxyethylene alkyl ethers	Emulsifier	X	X
Propylene glycol	Solvent / humectant	X	X
Purified water	Solvent	X	X

Key: RP – Reference Product; TP – Test Product.

6.2.2A Methods

6.2.2.1A Formulation production

To document the discriminatory power of the proposed rheology, IVRT and IVPT methods, different dimetindene and diclofenac formulations had to be prepared. Dimetindene test products, as well as altered formulations were prepared resorting to an Ultra-Turrax X 10/25 (Ystral GmbH, Dottingen, Germany), as well as a blending equipment. Laboratory scale batches (1 kg or 0.5 kg) were considered. The optimal settings pertaining to rate, duration and temperature of the manufacturing processes were carefully optimized during the formulation development studies (data not shown).

For the dimetindene TP formulations, the RP quantitative composition was replicated (data not shown). The formulations were conventionally prepared by hydrating the carbopol under continuous stirring. The humectant, preservative and drug previously dissolved in water were then added to the carbomer gel phase. This was followed by neutralization by adding a sodium hydroxide solution until the attainment of a smooth hydrogel texture. In order to manufacture a formulation with significantly different rheology profile, carbopol content was reduced by half and NaOH content was likewise reduced. The formulations were in all cases stored at 20-25^{o}C and protected from light.

Regarding the diclofenac formulations, only the diclofenac 5 mg/g emulgel had to be prepared. This formulation was obtained by diluting with water the commercial diclofenac 10 mg/g product. Despite the manufacturing procedures were not the same as those of the RP, this formulation enabled the assessment of the IVRT method discriminatory capacity.

6.2.2.2A Microstructure evaluation

Comparative microstructure studies constitute a key parameter to establish Q3 equivalence (EMA, 2018b; Simões *et al.*, 2020a). There are multiple tests that should be performed within this scope when addressing semisolid dosage forms, such as visual and microscopy appearance, particle/globule size, API polymorphic form, vehicle metamorphosis, pH, API distribution, among others (Ethier *et al.*, 2019). Despite the relevance of an overall and inclusive assessment of all microstructure parameters, the present work focuses exclusively on rheological properties due to their regulatory importance as per the EMA draft guideline requirements. According to this document, a complete rheological characterization, including rotational and oscillatory measurements, should be presented to infer on the rheological behaviour equivalence (EMA, 2018b; Simões *et al.*, 2020a). In this context, the rheological profile of all products was investigated using the same rheometer, as well as analysis software previously described in Chapters 3 and 5. All measurements were likewise performed in triplicate at 32°C, the physiological skin temperature. A sample hood was used to minimize sample volatilization and a positive displacement syringe was used to place the formulations in a lower TMP35 plate. A preset gap of 0.1 mm was considered for all samples.

Rotational tests were performed using a C35/2°/Ti cone geometry. Approximately 0.3 g of the formulation were placed on a lower plate. The main objective of the viscosity curves was to obtain a detailed viscosity profile with the zero-shear plateau, the shear thinning region and the infinite-shear plateau. In this context, for the dimetindene gel formulation, a linear CS flow ramp ranging from 0.01 to a final 250 Pa was measured for 400 s. To determine the apparent thixotropy (Pa/s), a shear rate from 0.01 to 300 s^{-1} and again down to 0.01 s^{-1}, during 150 s was used. On the other hand, to acquire the viscosity curve of the diclofenac emulgel formulation, the same procedures used in Chapter 5 were followed. These included a linear CS flow ramp measured from 0.01 to a final 100 Pa for 300 s. To assess emulgel thixotropy, the same procedure as described above was replicated, but the acquisition time was extended to 180 s.

Regarding oscillatory measurements, the same conditions were used for both formulations. A plate geometry (P35/Ti) was used and approximately 1 g of the formulations were placed in the *peltier* plate. First, an amplitude sweep was performed in the range of 0.01 and 500 Pa at

1 Hz to determine the linear viscoelastic region (LVR), as well as the flow point (τ_f). Frequency sweep analysis was then performed within the LVR range to determine the storage modulus (G´) and loss modulus (G´´) from 100 to 0.1 Hz. The results are presented for 1 Hz.

Rheology method validation

Rheology method validation was performed in terms of precision, selectivity and sensitivity.

The suitability and the discriminatory capacity of rheological methods should be adequately documented. As indicated in section 6.2.2.1A, formulations with different rheological profiles were manufactured. For dimetindene maleate 1 mg/g gel, both carbopol and NaOH concentrations were reduced. These excipients play a key role in the viscosity profile of the formulations. For diclofenac 20 mg/g emulgel, the strategy adopted in Chapter 4 was replicated, with the RP being diluted with ultrapure water (1:1) to obtain a formulation with distinct rheological characteristics. By tracing the rheological profile of the altered formulations, the sensitivity and selectivity of the proposed methods can be substantiated, as the differences in microstructure are highly dependent on excipient concentration (EMA, 2018b; Ethier *et al.*, 2019; Ili and Daniels, 2017; Mezger, 2010; Pleguezuelos-Villa *et al.*, 2019). The methods were considered sensitive when the rheological endpoints obtained with the RP were higher compared to the altered formulations. On the other hand, to assess method selectivity, the 90% CI were established. If the CI concerning the RP and the altered rheology formulation falls outside the 75-133%, selectivity of the purposed rheological analysis can be concluded. Overall, the same rationale applied to the clotrimazole case study (CS1) was herein transposed.

6.2.2.3A Product performance evaluation – IVRT studies

The *in vitro* release profile was determined for all products in the present study and its acquisition was done by the same diffusion system used in the previous chapters of this thesis. Qualification studies results were already presented in Chapter 4. Throughout IVRT method development and validation studies, the regulatory requirements pertaining to EMA draft guideline, FDA acyclovir guidance, as well as the USP Product Performance Tests, were closely considered (EMA, 2018b; FDA, 2016c; USP, 2009). An infinite dose – 300 mg – was evenly applied to the membrane surface (SUPOR 450 pore size 0.45 µm, Pall Corporation, USA), which separated the donor from the receptor compartments. Efforts were made to ensure a reproducible and consistent formulation application procedure (not deviating more than 5%) (EMA, 2018b). To select a suitable release medium, solubility studies were conducted, as described in Appendix A. For all diffusion experiments, the membrane was previously soaked

in purified water for 30 min. The receptor medium was continuously stirred at 600 rpm and maintained at a temperature of 37°C by means of a circulating water bath. Before the release experiments, the system was allowed to equilibrate at least for 30 min. Samples of the receptor phase (300 µL) were withdrawn at several sampling points and analysed through validated HPLC methods (see Appendix A). After each collection, the same volume of medium was replaced with pre-heated receptor solution. All release studies were performed under occlusive conditions.

The calculations pertaining to the cumulative amount and percentage of drug released, in addition to the *in vitro* release rate were already described in Chapters 4 and 5. According to the European regulatory requirements, n=12 replicates was considered for each product. Table 6.3 summarizes the specific IVRT conditions used for each product.

Table 6.3 – Receptor solution, sampling times and donor drug loading used for IVRT studies according to product. All results report to mean ± standard error of the mean (SEM) (4≤n≤7).

Formulation	Receptor phase	Sampling times (h)	Donor drug loading
Dimetindene gel 0.1% (w/w)	PBS-Ethanol (80:20, v/v) pH= 7.4 Solubility: 51.76 ± 0.02 mg/mL	0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 6 h.	300 mg
Diclofenac emulgel 2% (w/w)	PBS-Ethanol (80:20, v/v) pH= 7.4 Solubility: 14.5 ± 0.5 mg/mL	0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12 and 24 h.	300 mg

IVRT Method validation

According to regulatory requirements, membrane inertness, linearity, precision and robustness studies should be carried out to validate the IVRT method. Moreover, the IVRT discriminatory power should also be documented (EMA, 2018b; FDA, 2016c; USP, 2009). To evaluate if there were interactions between Tuffryn membranes and the molecules under study, membrane inertness studies were performed by incubating the membrane in a 35 µg/mL solution of the drug being studied, in the same experimental conditions as IVRT studies. A negative control (solution without membrane) was in all cases prepared. The membrane was considered to be inert, if at least, a 95% drug recovery was achieved (EMA, 2018b; FDA, 2016c; Tiffner *et al.*, 2018).

To test linearity, the amount of drug released per unit area should be linear with respect to the square root of time. In this context, a coefficient of determination (R^2) in excess of 0.9 was considered acceptable. Cells that did not display the adequate linearity were not considered.

For method precision and reproducibility studies, three IVRT runs were conducted, on three different days, by two different operators, each with a set of 12 vertical diffusion cells. For this analysis, the same batch was used. Intra- and inter-run variability were estimated for IVRR and Q_f. Because these studies were performed by two operators, this assessment also enabled the determination of operator variability. Even though a coefficient of variation (CV%) of 10% is required by EMA, in this work a CV% of less than 15%, was considered acceptable. The reasons that support this option, which were already disclosed in the previous chapters, essentially report to the intrinsic variability linked to IVRT, and the fact that this threshold is considered suitable by the FDA (FDA, 2016c; Tiffner *et al.*, 2018).

To evaluate whether the proposed methods have an adequate discriminatory power, sensitivity, specificity and selectivity were assessed. For that, IVRT endpoints – IVRR and Q_f were determined using formulations with different strength and rheology profiles, as previously described in section 6.2.2.1A. The IVRT method was considered to be sensitive if the IVRR/Q_f of the lower strength formulations were lower than the nominal formulation and the IVRR/Q_f of the higher strength formulations were higher than the nominal formulation. On the other hand, the method was considered specific if a linear relationship (R^2>0.9) was achieved between formulations with different drug concentration levels (EMA, 2018b; Mudyahoto *et al.*, 2020; Rath and Kanfer, 2020). Selectivity was demonstrated by assessing the ability of the IVRT method to discriminate IVRR/Q_f between a formulation with significant changes in critical inactive ingredients against the formulation under study. As noted in the rheology method validation studies, changing the concentration of thickening agents or diluting the formulation (diclofenac) is expected to lead to formulations with a distinctive rheological profile. Changing product rheology will influence product release behaviour. Therefore, the concentration of thickening agents can be considered as a critical material attribute (CMA), since it impacts a critical quality attribute (CQA) of the formulation – the IVRT related endpoints. In this context, the formulations prepared with a distinctive rheology profile were also examined during the IVRT studies to further document this relationship and to assess whether the purposed IVRT methodology was selective. Considering this information, the selectivity of the method could be stated if the confidence interval (CI) determined with the endpoints retrieved from all altered formulations (strength and rheology profile) falls outside the range of 90-111%. For IVRT discriminatory studies, 12 replicates of each formulation were also performed.

Finally, to investigate the robustness of the IVRT method, two IVRT runs of 12 replicates each were performed at small temperature differences, +2°C and −2°C, from the nominal temperature of 37°C specified by the IVRT. The method was considered robust if the IVRR and

Q_f did not deviate more than 15% from the mean release rate at nominal method parameter settings (EMA, 2018b; FDA, 2016c).

6.2.2.4A IVPT using human skin

Considering the regulatory requirements, only the diclofenac 20 mg/g emulgel was evaluated in IVPT studies. The same diffusional system of IVRT studies was used for the experiments. The experimental procedures developed were based on the EMA draft guideline and the FDA acyclovir draft guidance (EMA, 2018b; FDA, 2016c). First, pilot studies were performed, followed by pivotal studies that included a larger pool of donors. The same experimental procedures were used for both studies. Finite dose conditions (8-12 mg/cm^2) of the formulation were applied to the donor compartment. The receptor medium was continuously stirred at 600 rpm and all experiments were conducted in a temperature-controlled water bath to ensure a skin surface of 32±1^{o}C. All IVPT runs were performed under non-occlusive conditions to mimic the in-use condition (Kamal *et al.*, 2020). A PBS pH=7.4 solution was used as the receiver medium. Samples of the receptor phase (300 µL) were withdrawn at 2, 20, 22, 24, 26, 28, 30, 44, 46 and 48 h. After each collection, an equal volume of fresh temperature-equilibrated permeation medium was added to the receptor chamber.

According to the EMA draft guideline, the 90% confidence interval (CI) for the ratio of means between the test and reference products should be determined for the maximal flux (J_{MAX}) and the cumulative amount of drug permeated at the end of the IVPT study (A_{TOTAL}) (EMA, 2018b). J_{MAX} corresponds to the maximal rate of absorption and its analogous to the comparison of the C_{MAX} for test and RP products in the case of plasma pharmacokinetics. Similarly, A_{TOTAL} is calculated through equation (i) and can be compared to the area under the curve (AUC) of the incremental diclofenac permeation profile. Please note that IVPT methods should be adequately validated by testing a formulation at 50% of the proposed product strength to register non-equivalence with the product under study. The formulation diclofenac emulgel 1% w/w was used for this purpose. Franz cells containing non-dosed skin were also considered to infer the potential interference stemming from the biological matrix.

Biological membrane preparation

Human surgical waste skin pieces used for IVPT experiments were obtained from two different sources: (i) Centro Hospitalar de Lisboa Central, where the experimental protocol was approved by the Bioethics Committee. Written informed consent was obtained from the participants involved in this study (Process number 447/2017); (ii) Genoskin®. The tissue was obtained from plastic reduction surgeries. For both skin sources, after tissue excision, all

specimens were transported in saline solution (normal saline) under refrigeration (for less than 24 hours). After transport, the tissue was cleaned, subcutaneous fat was removed, and the outer layers of skin containing the *stratum corneum* (SC), viable epidermis, and some dermis were frozen at -20^{o}C. The day before the IVPT experiments, the epidermis was isolated by a thermal process (heat separated epidermis). Please note that the use of dermatomed skin could have been equated instead. Nevertheless, the choice of membrane depends largely on the solubility properties of the permeant. In the case of dermatomed skin, the relatively aqueous nature of the dermis will reduce the penetration of lipophilic compounds. Under these circumstances, the use of heat-separated epidermal (HSE) membranes is better suited to provide a quantifiable permeation profile (Benson and Watkinson, 2012). Briefly, the tissue was placed in a water bath at 60±2^{o}C for 60 seconds and then rested for 30 seconds at room temperature. With the aid of tweezers, the epidermis was separated, cut into 0.700 cm^2 and transferred to glass flasks filled with distilled water with the aid of a membrane support disk, used to keep the skin stretched. Special care was taken in order to maintain the *stratum* corneum side facing upwards. The skin sheets were then left overnight at 4^{o}C to stabilize.

On the day of the experiment, the skin was transferred to the diffusion cells. Afterwards, the barrier integrity of each skin piece was checked by measuring transepidermal water loss (TEWL) using a vapometer (Delfin Technology, Kuopio, Finland). Any skin piece with obvious signs of physical damage, stretch marks, or a TEWL value higher than 20.0 g/m^2/h was excluded from the experiment (Nagelreiter *et al.*, 2013; Shin *et al.*, 2020; Vitorino *et al.*, 2014). Table 6.4 summarizes the skin characteristics for each donor. In this study, the initial number of donors to be tested was 12 due to EMA draft guideline requirements; however, the lack of compliance with the skin integrity results led to the exclusion of 3 donors. Pilot studies were initially conducted with two donors, followed by pivotal studies performed with 7 donors. In all cases, the RP, TP and negative control formulations were tested in parallel. Moreover, two replicates per donor and formulation were always considered.

Table 6.4 – Human skin donors characteristics.

Study	Donor number	Gender	Skin type	Age	Anatomical region	Preparation method
Pilot study	1	Female	Type 3	43	Abdomen	HSE
	2	Female	Type 2	41	Abdomen	HSE
Pivotal study	3	Female	Type 3	57	Arm	HSE
	4	Female	Type 3	55	Gluteus	HSE
	5	Female	Type 2	29	Abdomen	HSE
	6	Female	Type 3	35	Thigh	HSE
	7	Male	Type 3	56	Abdomen	HSE
	8	Female	Type 2	41	Abdomen	HSE
	9	Female	Type 3	37	Abdomen	HSE

Key: HSE – Heat Separated Epidermis.

Mass Balance studies

According to OECD and EMA guidelines, mass balance studies should be conducted after the IVPT experiments to assess the amount of drug remaining on the donor compartment, on the skin and delivered into the skin (EMA, 2018b; Hossain *et al.*, 2019; OECD, 2010). For this purpose, the following procedure was adopted: the donor chamber was washed with 1 mL of methanol and the washing solutions were collected. The skin was then transferred into Eppendorf® tubes and the remaining diclofenac was also extracted with methanol. Both the donor compartment and skin samples were stirred overnight at 25ºC. Afterwards, all samples were sonicated (10 min), centrifuged at 11 740 x g for 10 min in a Minispin®(Eppendorf Ibérica S.L., Madrid, Spain) filtered by a 0.45 µm nylon membrane and transferred to HPLC vials for analysis.

The total recovery of drug at the end of the IVPT experiment was calculated by considering the mass of formulation initially applied to the donor chamber ($m_{applied}$) and the sum of the final cumulative amount of diclofenac that permeates the biological membrane into the receptor chamber(m permeated, which is equivalent to A_{TOTAL} in IVRT studies), the drug extracted from the formulation remaining in the donor chamber (m_{donor}) and the drug extracted from the biological membrane (m_{skin}) at the end of the experiments. The mass balance was then calculated according to the following equation:

$$mass\ balance\ = \frac{m\ donor + m\ permeated\ (receptor) + m\ skin}{m\ applied} \times 100 \qquad (6.1)$$

Procedure reliability was confirmed by the total drug recovery (%), which should be in the range of 100% ± 10% range (EMA, 2018b).

All IVPT as well as mass balance samples were analysed by regulatory compliant HPLC methods, please see Appendix A.

6.2.2.5A Data Analysis and Statistics

The procedures described in Appendix B were followed. Example calculations are provided in the same section.

For rheology data, the Shapiro-Wilk test (p=0.05) was first performed to evaluate if the data followed a normal distribution. Since several endpoints did not present a normal distribution, the 90% CI of the ratio between the TP/RP was calculated following EMA draft guideline, as previously described (Appendix B).

6.3A Results and discussion

6.3.1A Rheology and IVRT method validation

The rheology method validation studies were conducted to evaluate whether the proposed methodology was able to reflect microstructure differences between the reference products (all RP batches were included in this analysis) and the formulations prepared with distinctive rheological profile. This is of outmost importance as according to the EMA draft guideline, evidence should be provided on the test discriminatory capacity, as well as the suitability of the respective acceptance criteria (EMA, 2018b). To address this, a simplified approach of the framework provided in Chapter 3 was herein followed. The results are summarized in Table 6.5.

The validation of the rheological dimetindene method demonstrated adequate sensitivity for most endpoints, as well as selectivity, since statistically significant differences were registered between the RP and the altered formulation (Table 6.5). Nevertheless, it should be noted that although the RP viscosity profiles displayed overlapping curves (Fig.6.3), the CV% results pertaining to the RP, were high (2.5-80%). This may be ascribed to batch variability, or alternatively, to batch age, since all reference products were analysed at the end of their life cycle (33-35 months). Note that the yield point estimated by rotational methods could not be calculated for the dimetindene altered formulation due to the pronounced fluidity of the product, which instantaneously exhibited shear thinning behaviour.

For diclofenac rheology method validation, the altered formulation was simply achieved by diluting the commercial product with water (please see Chapter 4). This procedure may be

insufficient to clearly characterize rheological differences, since selectivity failed to register for $\tau_{0.ROT}$, G´ and G´´ endpoints. Nevertheless, the majority of the endpoints (6 out of 9) could successfully report an adequate discriminatory power, therefore the purposed methods were considered fit for the purpose of this study (Table 6.5). Please note that in this case study, the CV% values for the RP regarding the rheological endpoints were generally lower when compared to dimetindene formulations, suggesting a lower inter-batch variability. This could be motivated by the lower batch age of the RP when compared to the dimetindene gel formulation, as all diclofenac batches were tested 12-16 months after manufacture.

Table 6.5 – Rheology method validation results. Rheology endpoint results pertaining to the RP regard an n = 9. For the altered rheology formulations, an n=3 was considered. To assess method selectivity, the 90% CI of the ratio average (altered rheology/RP) is presented. If the CI of the considered rheological endpoints surpasses 75-133% interval, selectivity could be inferred.

Rheological endpoints	Dimetindene maleate 1 mg/g gel					Diclofenac 20 mg/g emulgel				
	RP		Altered rheology formulation			RP		Altered rheology formulation		
	Mean	CV%	Mean	CV%	90% CI	Mean	CV%	Mean	CV%	90% CI
η_0 (Pa.s)	1882	12	6.59	30.29	0.28 – 0.42	8098	20	7232	2	73.82 – 111.7
τ_{ROT} (Pa)	17	2.5	N.D		-		5.1	16.2	1.7	99.3 – 101.4
η_∞ (Pa.s)	1.45	53.05	0.07	6.87	2.47 – 12.67	30.6	41.8	4.9	24.3	9.71 – 30.88
S_R (Pa/s)	2023	80	120	20	3.39 -17.45	2030	26	120	98	36.5 – 65.0
LVR (Pa)	306	34	67.7	3.7	15.32 – 36.35	107	5	79	0	69.9 – 78.5
τ_f (Pa)	78.1	40.1	17.4	1.4	14.32 – 42.10	26.0	3.3	19.5	5.6	71.6 – 78.5
τ_{OSC} (Pa)	15.6	20.6	2.03	5.62	10.54 – 16.72	15.9	9.1	11.9	11.7	66.9 – 84.1
G´ – 1Hz (Pa)	280	30	56.9	7.0	14.68 – 30.98	106	3.93	111	1.72	100.5 – 109.6
G´´ – 1Hz (Pa)	27.9	40.4	11.0	5.3	26.39 – 69.86	13.3	4.4	13.8	4.5	98.6 – 109.4
Acceptance criteria | Sensitivity	RP > Altered rheology									
Status (Rot. Tests)	Compliant					Compliant except for $\tau_{0.ROT}$				
Status (Osc. Tests)	Compliant					Compliant				
Acceptance criteria | Selectivity	RP ≠ Alt. rheology									
Status (Rot. Tests)	Compliant					Compliant except for η_0 and $\tau_{0.ROT}$				
Status (Osc. Tests)	Compliant					Compliant except for G´ and G´´				

Key: 90% CI – 90% Confidence Interval; RP – Reference Product; η_0 (Pa.s) – Zero-shear viscosity; η_∞ (Pa.s) – Infinite-shear viscosity; $\tau_{0.ROT}$ (Pa) – Yield point obtained through rotational methods; S_R (Pa/s) – Relative thixotropic Area; $\tau_{0.OSC}$ (Pa) – Yield point obtained through oscillatory methods; LVR plateau (Pa) – Linear Viscoelastic Region plateau; τ_f (Pa) – Flow point; G´ – Storage modulus; G´´ – Loss modulus; Rot. Tests – Rotational tests; Osc. Tests – Oscillatory tests; N.D – Not-determined; Green label (C) – Compliant results; Red label (NC) – Non-compliant results.

Similarly, IVRT method validation studies were likewise performed (EMA, 2018b). The acceptance criteria and the results of the IVRT methods are summarized in Table 6.6 and in Fig.6.2. Please note that the DF IVRT method validation results were already addressed in Chapter 4. Nevertheless, to present a cross comparison with the dimetindene formulations, the DF results were once again displayed.

Regarding membrane inertness studies, since a recovery of 95.2% and 99.4% was obtained for both dimetindene and diclofenac, respectively, there was no evidence of significant drug binding to the membrane. Therefore, it can be inferred that the membrane did not present a rate limiting barrier for API diffusion (EMA, 2018b; Tiffner *et al.*, 2018). An interesting work by Mekjaruskul and colleagues investigated the effect of various membranes on the release performance of dexamethasone using a USP dissolution apparatus IV (Mekjaruskul *et al.*, 2021). The authors concluded that the materials and sources of the membranes affected drug dissolution profiles, by revealing membrane-drug binding effects. Similar to the present study, Mekjaruskul *et al.* also used polyethersulfone membranes. Even though these membranes provided acceptable recovery results (> 90%), there were significant differences between suppliers. Herein, the same membrane supplier was used for all IVRT studies. Nevertheless, when undertaking IVRT development studies attention should be paid to this parameter in order to define suitable acceptance criteria regarding the membrane type, as well as supplier (Mekjaruskul *et al.*, 2021).

Linearity was successfully registered in all diffusion cells (Dimetindene gel: $R^2 = 0.96 \pm 0.03$ and diclofenac emulgel: $R^2 = 0.98 \pm 0.01$). Therefore, steady-state kinetics conditions were achieved. Although both EMA and FDA guidelines recommend $R^2 \geq 0.90$ over the entire IVRT time range, the correlation coefficient is not a very discriminatory parameter, therefore a higher R^2 ($R^2 > 0.97$) should be registered to demonstrate adequate linearity of release. All these requirements have been extensively discussed in the recently held FDA and Center for Research on Complex Generics Co-Hosted Workshop: In *Vitro* Release Test (IVRT) and In *Vitro* Permeation Test (IVPT) Methods: Best Practices and Scientific Considerations for ANDA Submissions (Aug 18-20 2021). As evidenced in Table 6.6, this was successfully achieved.

Table 6.6 – Acceptance criteria and results for IVRT method validation studies based on regulatory requirements (EMA, 2018b; FDA, 2016c).

Parameter	Results	Acceptance criteria	Status
Membrane inertness	Dimetindene recovery: 95.2% Diclofenac recovery = 99.4%	Recovery ≥ 95%	Compliant
Linearity	Dimetindene gel: R^2 = 0.958 ± 0.025 (n=36) Diclofenac emulgel: R^2 = 0.980 ± 0.010 (n=36)	R^2 > 0.90	Compliant
Precision and reproducibility, including operator variability (IVRR data reports to µg/cm²/√h and Q_f to µg/cm²)	<u>Dimetindene gel</u> Run 1 (operator A): IVRR = 116 (9.5%) | Q_f=287 (6.3%) Run 2 (operator B): IVRR= 107 (14.8%) Q_f=270 (7.0%) Run 3 (operator B): IVRR= 109 (14.2%) | Q_f=280 (10.6%) Intra-run variability CV% Q_f = 8.0% | CV% IVRR = 12.7% Inter-run, including operator variability (n=36) CV% Q_f = 8.6%| CV% IVRR = 13.4%	Intra-run, inter-run, including operator variability: CV% Q_f ≤ 15% CV% IVRR ≤ 15%	Compliant
	<u>Diclofenac emulgel</u> Run 1 (operator A): IVRR = 708 (7.9%)| Q_f = 3070 (8.1%) Run 2 (operator B): IVRR = 676 (8.6%)| Q_f = 2933 (6.9%) Run 3 (operator A): IVRR = 784 (7.6%)| Q_f = 3418 (7.6%) Intra-run variability (n=36) CV% IVRR = 8.0% | CV% Q_f = 7.6% Inter-run and operator variability (n=36) CV% IVRR = 10.0% | CV% Q_f = 9.9%		Compliant
Selectivity [Data reports to IVRR (µg/cm²/√h)]	<u>Dimetindene gel</u> (n=12) 0.05% *vs.* 0.1% → CI = [58.49 – 72.93%] 0.1% *vs.* 0.2% → CI = [226.8 – 260.7%] 0.1 % *vs*. ≠ rheology formulation → CI = [104.6 – 129.72%] <u>Diclofenac emulgel</u> (n=12) 0.5% *vs*. 1% (simultaneous assessment of ≠ rheology formulation) → CI = [63.11 – 72.08%] 2% *vs.* 1% → CI = [127.33 – 143.42%]	CI between different strength products falls outside the limits [90-111] %	Compliant
Robustness [Data reports to IVRR (µg/cm²/√h)]	Dimetindene gel (n=12) Mean IVRR 37ºC = 116 (9.5%) Mean IVRR 35ºC = 99 (11.6%) Mean IVRR 39ºC = 105 (13.0%) Diclofenac emulgel (n=12) Mean IVRR 37ºC = 676 (8.6%) Mean IVRR 35ºC = 645 (8.6%) Mean IVRR 39ºC = 685 (7.1%)	Mean IVRR of runs with minor temperature fluctuations should not deviate more than 15% from the IVRR of the nominal method parameter settings	Compliant

Key: Green label (C) – Compliant results. Please note that there are subtle differences when comparing the selectivity of the Cis of the DF formulation in this chapter, and the one previously presented in Chapter 4. These are related with the fact, that this chapter used the EMA statistical approach, whilst in Chapter 4, the FDA was instead equated.

Regarding precision, reproducibility and operator variability studies, the results met the established criteria. The maximum CV% registered was attained when estimating the inter-run variability of the dimetindene formulation (13.38%). Even though 15% was the defined CV% acceptance threshold, it is important to consider that this value refers to the FDA acyclovir guidance, since EMA allows a maximum of 10% deviation (EMA, 2018b; FDA, 2016c). Similar for the rheology method validation results, higher CV% values were recorded for the dimetindene formulation. Although this may be motivated by batch age, it should be noted that EMA 10% CV criteria may be challenging to attain, since it does not account for the intrinsic variability associated with IVRT. Variability causes may be related to air entrapment, inability to uniformly spread the formulation upon the membrane and difficulty in reproducing the exact amount of formulation loaded in the system (Bao and Burgess, 2018) . Nevertheless, several papers report similar CV results in IVRT precision studies (<15%) (Mudyahoto *et al.*, 2020; Rath and Kanfer, 2020).

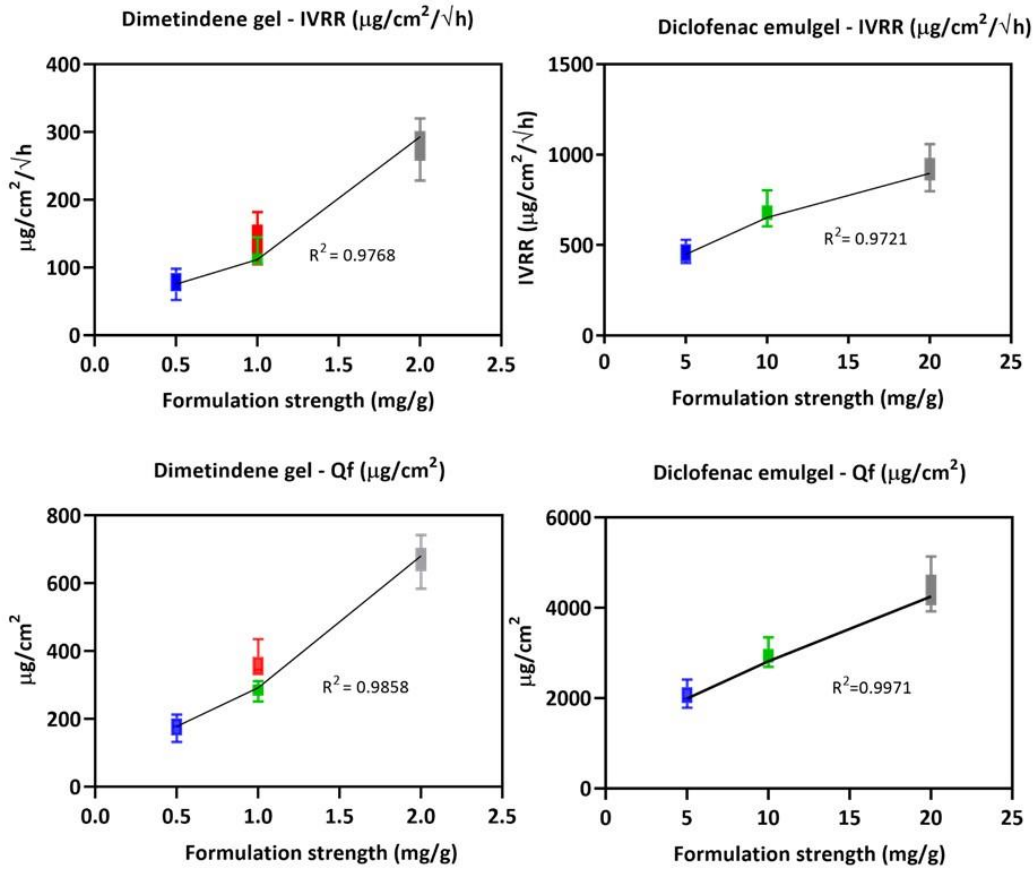


Fig.6.2 – Box and Whiskers plots of the measured release rates/ cumulative amount released for the different strength and altered rheology formulations. Please note that the altered rheology formulation for dimetindene is signalled in red, whilst for diclofenac it is overlapped with the lower strength product (signalled in blue).

IVRT method discriminatory capacity was successfully documented, as the requirements for sensitivity, specificity and selectivity were met. For the selectivity studies, the IVRR (n=12) of the different strength formulations and from the significantly different rheology profile were statistically compared. As displayed in Table 6.6, all CIs regarding the lower *vs*. intermediate strength, higher *vs*. intermediate strength formulations and also nominal formulation *vs*. rheology altered formulation were outside the range 90-111%; hence, the method was considered selective to establish differences in release rates. When the FDA criteria was used instead (75-133%), selectivity was still found (FDA, 2016c). Both IVRT methods also demonstrate suitable sensitivity and specificity because: (i) the IVRR of the lower strength formulations was lower than the nominal formulation and the IVRR of the higher strength formulations was higher than the nominal formulation (sensitivity); (ii) a linear relationship ($R^2 \geq 0.9$) was observed between the release rates of the products with different strengths Fig.6.2) (EMA, 2018b; Mudyahoto *et al.*, 2020; Rath and Kanfer, 2020).

The ability of the method to be unaffected by minor variations in the experimental conditions as also supported, as the mean IVRR did not deviate more than 15% from the IVRR of the nominal method parameter settings (Table 6.6). Therefore, the methods are considered to be robust.

6.3.2A Microstructure and product performance evaluation

Dimetindene maleate (DM) is a histamine H_1-receptor antagonist, which considerably reduces capillary hyperpermeability, commonly associated with immediate hypersensitivity reactions. Its antiallergic and antipruritic characteristics have been extensively confirmed for systemic administration through oral and intravenous dosage forms. Nevertheless, due to its mechanism of action, this API is also effective when administered topically as it relieves itching and skin irritation. Moreover, DM anaesthetic properties are also useful for the treatment of sunburns (Althaus and Berthet, 1992; PAR, 2015b).

Gels are monophasic systems where all vehicle ingredients are miscible with each other. In these semisolid dosage forms, the liquid phase is constrained within a 3-dimensional matrix which can be of natural or synthetic origin. Based on the liquid medium entrapped within the 3-dimensional matrix, gels can be classified as hydrogels or alternatively as organogels (Surber and Knie, 2018). The DM formulation used in the present study regards a hydrogel, since all of the therapeutic applications mentioned above call for vehicles with specific requirements such as non-greasiness and cooling effect (Surber and Knie, 2018). Technologically, these formulations are simple to manufacture since they contain few components compared to

more complex systems such as creams (Ethier *et al.*, 2019). As per the EMA draft guideline requirements, to document the BE of these systems, extended pharmaceutical equivalence (Q1/Q2/Q3 and Q4 sameness) needs to be demonstrated (EMA, 2018b). In terms of the qualitative and quantitative profile, the TP meets the sameness criteria when compared towards the RP. In order to shed light on microstructure equivalence, rheology studies were performed, see Fig.6.3.

First, the rotational profile was assessed by determining the flow curves and the thixotropic behaviour of all formulations. As displayed in Fig.6.3, all batches exhibit a non-Newtonian and shear thinning behaviour. The acquired viscosity profiles clearly indicate three distinct regions: i) a narrow shear viscosity plateau, from which the zero-shear viscosity (η_0) can be estimated; (ii) a shear-thinning range with a shear stress-dependent viscosity function $\eta = f(\tau)$. This region starts when plastic flow occurs at a given critical stress – the rotational yield point (τ_0); and finally (iii) a region stemming from the incremental shear stress over the microstructure that induces a drastically viscosity decrease, leading to a 2nd plateau. From this region, the infinite-shear viscosity (η_∞) can be estimated (Mezger, 2010; Simões *et al.*, 2020a).

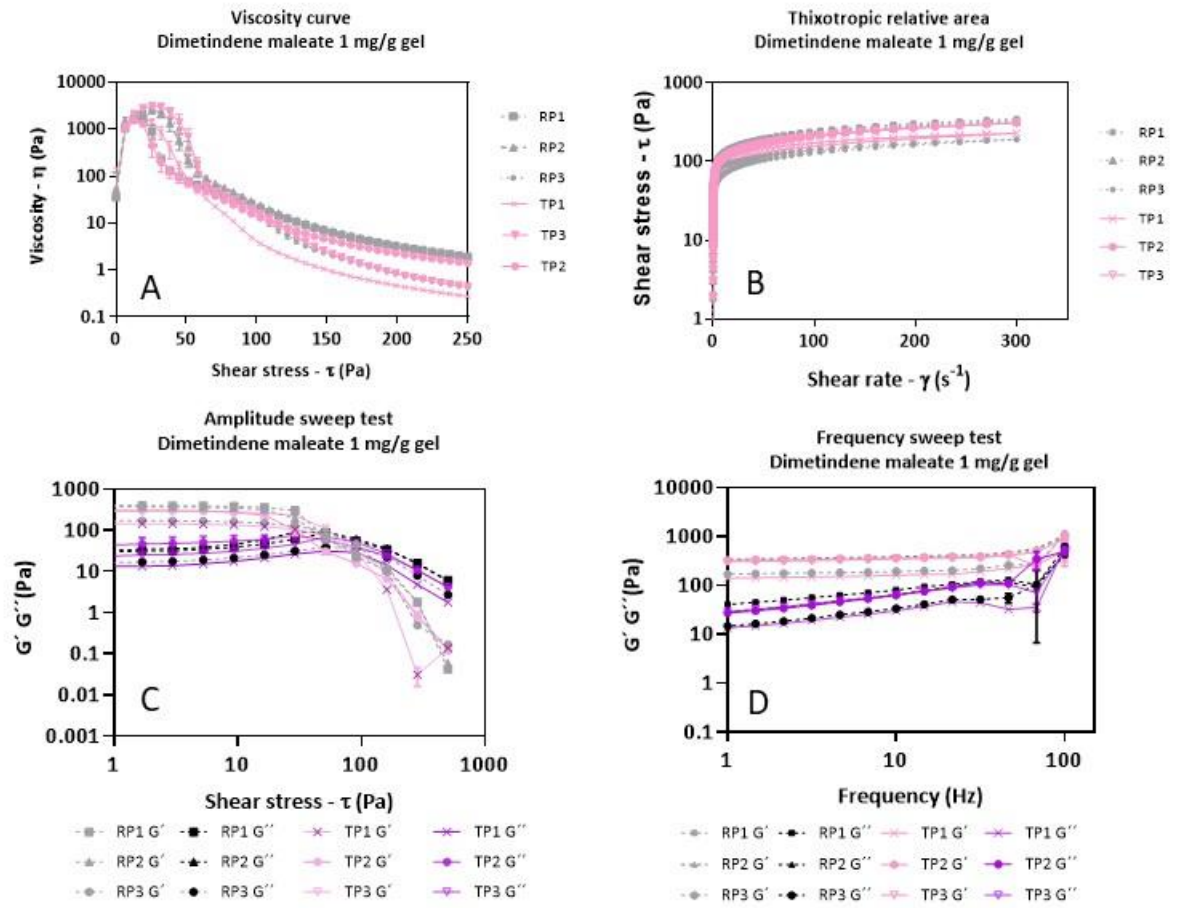


Fig.6.3 – Rheology profile of the dimetindene maleate 1 mg/g gel. All results report to mean ± SEM. Three replicates were used per batch formulation. A – Viscosity curve; B – Thixotropic behaviour; C – Amplitude sweep test; D – Frequency sweep test.

When representing viscosity *vs*. shear stress, the three batches of each product presented identical profiles (Fig.6.3A). Afterwards, the viscoelastic properties of all formulations were assessed by determining both amplitude and frequency sweep behaviour. Regarding the amplitude sweep test, the plots of the elastic (G´) and the viscous (G´´) moduli *vs.* the shear stress for each batch, revealed a linear viscoelastic region. Within this shear stress plateau, the microstructure was preserved (Fig.6.3C). The profiles of the frequency-dependent elastic and viscous moduli were similar for both products Fig.6.3D). Higher G´ than G´´ values were obtained in both amplitude and frequency sweep experiments, consistent with the predominantly elastic behaviour of semisolid dosage forms (see Fig.6.3C-D).

As previously mentioned, several rheological endpoints were considered, in order to statistically compare both formulations (see Table 6.7).

Table 6.7 – Rheological properties of dimetindene 1 mg/g gel test (TP) and reference (RP) formulations and the 90% CI of the ratio average (TP/RP). (RP: n=9 and TP: n=9).

Rheological endpoints	RP		TP		90% CI	Status
	Mean	RSD	Mean	RSD		
η_0 (Pa.s)	1882	RP1: 7 RP2: 6 RP3: 6 Overall: 11	1730	TP1: 4 TP2: 1 TP3: 2 Overall: 15	81.8 – 102.5	C
τ_{ROT} (Pa)	16.9	RP1: 2.3 RP2: 0.8 RP3: 0.5 Overall: 2.4	16.7	TP1: 1.0 TP2: 2.4 TP3: 2.0 Overall: 3.7	96.3 – 101.8	C
η_∞ (Pa.s)	1.4	RP1: 6.09 RP2: 6.51 RP3: 6.57 Overall: 50.02	0.7	TP1: 9.75 TP2: 7.15 TP3: 12.54 Overall: 71.31	25.3 – 86.6	NC
S_R (Pa/s)	2023	RP1: 15 RP2: 9 RP3: 7 Overall: 75	1369	TP1: 10 TP2: 13 TP3: 7 Overall: 71	38.4 – 127.2	NC
LVR (Pa)	306	RP1: 5 RP2: 6 RP3: 1 Overall: 32	254	TP1: 3 TP2: 0.3 TP3: 4 Overall: 32	59.9 – 115.3	NC
τ_f (Pa)	78.1	RP1: 1.7 RP2: 2.1 RP3: 1.7 Overall: 37.8	58.2	TP1: 2.7 TP2: 6.9 TP3: 2.0 Overall: 37.3	50.3 – 112.0	NC
τ_{OSC} (Pa)	15.6	RP1: 9.51 RP2: 16.46 RP3: 11.88 Overall: 19.42	14.6	TP1: 9.22 TP2: 14.32 TP3: 15.08 Overall: 16.11	80.7 – 110.2	C
G´ – 1Hz (Pa)	280	RP1: 3 RP2: 1 RP3: 2 Overall: 28	254	TP1: 0 TP2: 2 TP3: 1 Overall: 32.01	65.7 – 121.5	NC
G´´ – 1Hz (Pa)	27.9	RP1: 3.1 RP2: 5.8 RP3: 4.8 Overall: 38.12	23.1	TP1: 4.3 TP2: 7.2 TP3: 3.7 Overall: 30.29	60.6 – 119.5	NC

Key: CI – Confidence Interval; η_0 – Zero-shear viscosity; τ_{ROT} – Rotational yield point; η_∞ - Infinite-shear viscosity; S_R – Relative thixotropic area; LVR – Linear Viscoelastic Region; τ_f – flow point; τ_{OSC} – Oscillatory Yield point; G´- Storage modulus; G´´ – Loss modulus; Green label (C) – Compliant results; Red label (NC) – Non-compliant results.

The overall RSD (%) of both formulations were highly similar. The variability of the considered parameters ranged from 2.4% (τ_{ROT}), to 75% (S_R). All rheological endpoints retrieved from the RP were slightly higher than those registered in the TP, suggesting a firmer consistency. The differences found in both products can be ascribed to differences either in the (i) scale of

manufacturing; (ii) batch age – as the TP and the RP were studied at different stages of the product life cycle; and (iii) source of raw materials (EMA, 2018c).

It is important to note that a direct application of EMA criteria – "the 90% confidence interval for the difference of means of the test and comparator products should be contained within the acceptance criteria of +/-10% of the comparator product mean, assuming normal distribution of data (EMA, 2018b)" – solely applies to the yield point (τ_{ROT}). All remaining parameters did not meet the EMA draft guideline requirements because (i) the data was not normally distributed; (ii) there was more than 10% difference between the rheological endpoints of TP and RP, or due to (iii) the lack of compliance with the 90-111% confidence interval. Based on these results, and similarly to the published work by Maria Pleguezuelos-Villa, a larger criterion [75-133%] was selected to assess the TP rheological equivalence towards the RP. With this acceptance range, for the endpoints that displayed the lowest inter-batch RSD% – η_0, $\tau_{0.ROT}$ and the $\tau_{0.OSC}$ – equivalence could be sustained.

These endpoints are highly important from a technological, patient compliance as well as from a clinical perspective. The yield point refers to the critical stress at which the formulation starts to plastically deform. While increasing the shear stress, the degree of strain exerted in the microstructure increases accordingly, which in turn delays the complete relaxation of the structure in a given time frame of a respective stress point. Therefore, above a critical value of stress, extreme shear thinning conditions lead to an irreversible change in the microstructure of the product, which is reflected in a drastic reduction in viscosity (Dabbaghi *et al.*, 2021). In this context, and as described in detail by Dabbagthi *et al.*, the initial endpoints of the viscosity curve (η_0 and τ_{ROT}) describe the rheological stages that a product goes through, ranging from a static state that mimics the product in a container to the initial shear corresponding to the high-flow state that resembles the application of the product on the skin (Dabbaghi *et al.*, 2021).

However, according to the draft guideline on quality and equivalence of topical products, an effort should be made to support equivalence based on a comprehensive rheological characterization. In this context, the 90% CI should be compliant with the remaining endpoints.

A close inspection of Table 6.7 reveals that intra-batch results display low variability, but when the inter-batch results are compared, the variability increases dramatically. As previously mentioned, this can be ascribed to the aging process that increases the variability of the rheological parameters to a great extent.

The next parameter to be evaluated concerned the pharmaceutical performance of the formulation. According to the EMA guideline for simple formulations, such as gels, to establish bioequivalence, the extended pharmaceutical equivalence needs to be documented, and IVRT tests are required for this purpose. Fig.6.4 displays the obtained IVRT profiles for all dimetindene products.

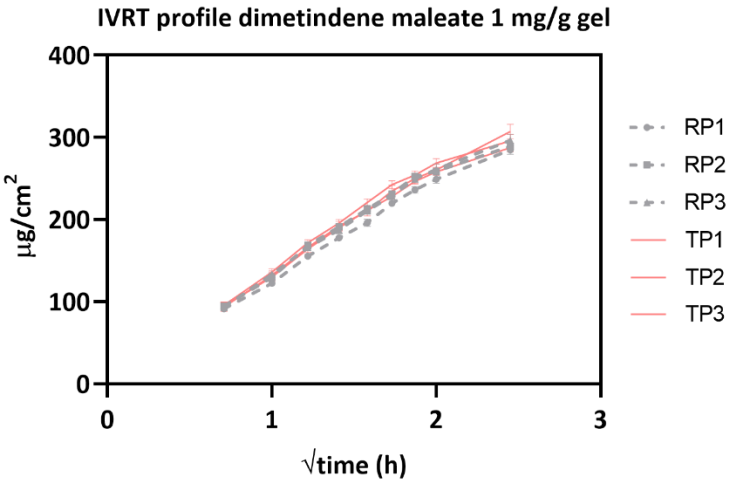


Fig.6.4 – IVRT profile of all dimetindene maleate 1 mg/g gel products. Results report to n=12 mean ± SEM.

Both formulations revealed overlapping release profiles, which were in line with their equivalence in what concerns Q1, Q2. When performing the statistical analysis of the IVRT endpoint, the 90% CI met the EMA criterion of 90-111% (Table 6.8).

Table 6.8 – 90% confidence interval calculated regarding the IVRR and the total cumulative amount of drug released at the end of the IVRT study (6h).

IVRR TP/RP ($\mu g/cm^2/\sqrt{t}$) 90% CI	**Total cumulative amount TP/RP ($\mu g/cm^2$) 90% CI**	**Acceptance criteria**	**Status**
97.75 – 105.8%	98.76 – 104.8%	EMA: 90-111%	C
		FDA: 75-133%	C

Key: Green label (C) – Compliant results.

Considering the results, it is possible to conclude that the rheological differences found in η_∞, S_R, LVR, τ_f, G´ and G´´ endpoints are not translatable into a different formulation performance. This information seems to suggest that the yield point estimated through rotational or oscillatory methods ($\tau_{0.ROT}$ /$\tau_{0.OSC}$) in conjunction with the η_0, prevail in what concerns the pharmaceutical performance of the product (similar release profiles).

Notwithstanding these observations, based on the European regulatory requirements, despite there is sufficient evidence to demonstrate equivalence of Q1, Q2 and Q4, equivalence of Q3 could not be supported. Therefore, the extended pharmaceutical equivalence of this TP towards the RP failed to be documented. It should be noted that the batch age might have played a crucial role in the rheological results obtained. In this context, generic manufacturers should be encouraged to pay special attention to this specific aspect, as there is a myriad of interdependencies in semisolid microstructure even in simpler technological systems.

The second case study reported herein regards a diclofenac diethylammonium 23.2 mg/g o/w emulgel formulation. This is a complex formulation as it is not only biphasic, but also contains permeation enhancer excipients, namely oleyl alcohol. These excipients directly influence the bioavailability of the API. Under these circumstances, additional permeation kinetic or, if possible, pharmacodynamic equivalence tests are required to submit a generic application (EMA, 2018b; FDA, 2016c). Interestingly, this topical product constitutes one of the few exceptions where a pharmacokinetic evaluation can be used to establish bioequivalence, as diclofenac, when topically administered, targets the skin layers, namely the dermis (Drago *et al.*, 2017; Holt *et al.*, 2015; Maroo *et al.*, 2013). The TP selected in the present study displays Q1 and Q2 sameness towards the RP. Furthermore, the bioequivalence of this specific TP towards the RP (which was also used in this study) was adequately documented by pharmacokinetic studies, please refer to the work of Mangas-Sanjuán *et al.* (Mangas-Sanjuán *et al.*, 2020, 2019). Although pharmacokinetic studies can be used to establish bioequivalence, in this work the aim was to assess whether IVPT tests can be successfully used for the same objective.

Following the strategy previously described for dimetindene, rheology studies were likewise performed for the diclofenac formulations. This was followed by IVRT and IVPT experiments. In contrast to the dimetindene products, since diclofenac displays a higher formulation complexity, an attempt was made to select products with a closer batch age (RP = 12-16 months and TP = 22 months). The rheology profile results are displayed in Fig.6.5 and Table 6.9.

The rotational profile of the diclofenac formulations reflected a non-Newtonian, shear thinning and thixotropic behaviour, see Fig.6.5. Amplitude sweep tests revealed a linear viscoelastic region for all the products studied, with higher values of G´ than G´´, which further consubstantiates the elastic behaviour of semisolid dosage forms. This trend was also evident in the frequency sweep experiments. Contrary to the dimetindene formulations, clear differences in all endpoints were immediately apparent after inspection of the rheograms, with the TP consistently displaying higher values when compared to the RP batches (Fig.6.5).

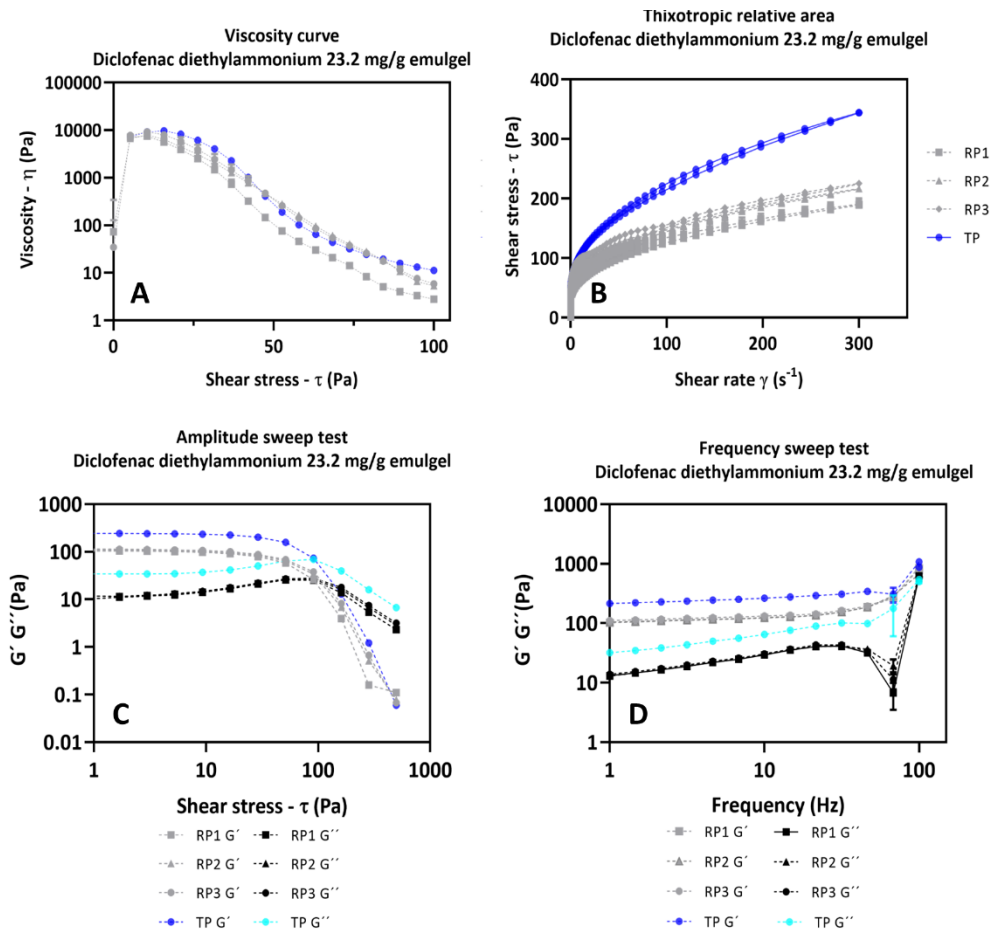


Fig.6.5 – Rheology profile of the diclofenac diethylammonium 23.2 mg/g emulgel. All results report to mean ± SEM. Three replicates were used per batch formulation. A – Viscosity curve; B – Thixotropic behaviour; C – Amplitude sweep test; D – Frequency sweep test.

When statistically comparing the rheological endpoints, a normal distribution failed to be registered for the majority of the rheological parameters. In this context, similar to the dimetindene formulations, the 75-133% CI was set as the acceptance interval. The endpoints and statistical analysis retrieved from the rheological studies are shown in Table 6.9.

Table 6.9 – Rheological properties of diclofenac diethylammonium 23.2 mg/g emulgel generic (TP) and reference (RP) formulations and the 90% CI of the ratio average (TP/RP) (RP: n=9 and TP: n=3).

Rheology endpoints	RP		TP		90% CI	Status
	Mean	CV	Mean	CV		
η_0 (Pa.s)	8098	RP1: 1 RP2: 6 RP3: 23 Overall: 20	9203	75-133%	93.7 – 142.3	NC
τ_{ROT} (Pa)	16.1	RP1: 4.21 RP2: 2.94 RP3: 6.06 Overall: 5.1	18.3	75-133%	106.5 – 120.27	C
η_∞ (Pa.s)	30.6	RP1: 11.3 RP2: 8.5 RP3: 8.8 Overall:41.8	32.1	14.8	65.8 – 203.0	NC
S_R (Pa/s)	2030	RP1: 19 RP2: 13 RP3: 21 Overall: 26	1743	7	66.5 – 117.4	NC
LVR (Pa)	107	RP1: 3 RP2: 4 RP3: 2 Overall: 5	237	12	203.4 – 242.2	NC
τ_f (Pa)	26.0	RP1: 0.5 RP2: 0.5 RP3: 0.6 Overall: 3.3	68.4	2.9	252.7 – 272.9	NC
τ_{OSC} (Pa)	15.9	RP1: 2.9 RP2: 11.0 RP3: 4.1 Overall: 9.08	23.1	15.9	127.4 – 164.1	NC
G´ – 1Hz (Pa)	106	RP1: 1 RP2: 1 RP3: 1 Overall: 4	214	3	193.5 – 211.9	NC
G´´ – 1Hz (Pa)	13.3	RP1: 3.6 RP2: 1.1 RP3: 3.6 Overall: 4.3	31.9	14.4	2018.2 – 260.4	NC

Key: η_0 – Zero-shear viscosity; τ_{ROT} – Rotational yield point; η_∞ - Infinite-shear viscosity; S_R – Relative thixotropic area; LVR – Linear Viscoelastic Region; τ_f C flow point; τ_{OSC} – Oscillatory Yield point; G´- Storage modulus; G´´ – Loss modulus; Green label (C) – Compliant results; Red label (NC) – Non-compliant results.

Overall, equivalence was found only for the yield point estimated by rotational experiments. For all other endpoints, the ratio of means between the test and reference formulations was not within the EMA or FDA limits. Similar results with the same formulations were observed by Pleguezuelos-Villa *et al.* (Pleguezuelos-Villa *et al.*, 2019). Contrary to the dimetindene formulations, when considering diclofenac RP rheological endpoints the inter-batch variability was consistently low.

Taking these results into account, and similarly to what was previously observed for the dimetindene formulation, no equivalence was found with respect to rheology parameters. IVRT studies were then performed to evaluate if these differences affected product performance. Fig.6.6 portraits the release profile of all diclofenac products, whilst Table 6.10 presents the confidence intervals for the IVRR, as well as for the total cumulative amount of diclofenac released.

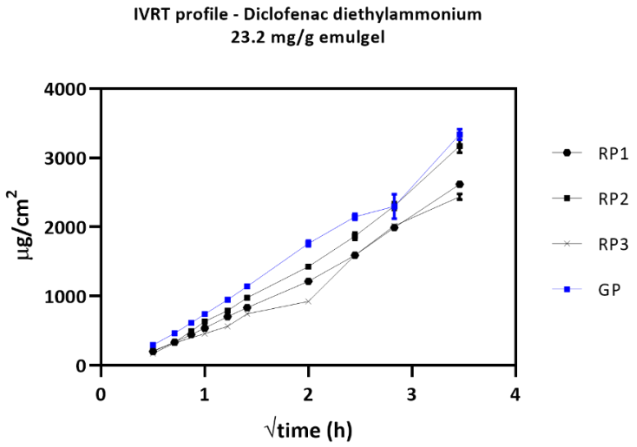


Fig.6.6 – IVRT profile of all diclofenac diethylammonium 23.2 mg/g emulgel products. Results report to n=12; mean ± SEM.

Even though the release profiles of both products were similar, overall, the product with higher viscosity – the TP – exhibited higher release. Not surprisingly, compliance with the EMA requirements failed to be registered. Nevertheless, if the FDA criteria were applied, some TP-RP batch comparisons would yield compliant results. More specifically, if RP3 had not been included in this analysis, the results would have been compliant according to FDA requirements. This highlights that the selection of the RP batches is highly important.

Taking the above into account, for this specific formulation, contrarily to what was previously established for the dimetindene maleate formulation, the rheological differences highly impacted product performance. This highlights that the selection of the RP batches is highly important, as concluded in Chapter 5.

Table 6.10 – 90% confidence interval calculated regarding the IVRR and the total cumulative amount of diclofenac released at the end of the IVRT study (6h).

	IVRR TP/RP (µg/cm²/√t) 90% CI			**Q_f TP/RP (µg/cm²) 90% CI**			
	Results	Status		Results	Status		Acceptance criteria
		EMA	FDA		EMA	FDA	
Overall comparison							
RP *vs.* TP	116.1 – 135.7	NC	NC	118.9 – 144.5	NC	NC	EMA: 90-111% FDA: 75-133%
Batch to batch comparison							
RP1 *vs.* TP	114.2 – 127.6	NC	C	113.2 – 128.5	NC	C	
RP2 *vs.* TP	103.1 – 118.5	NC	C	105.0 – 125.1	NC	C	
RP3 *vs.* TP	140.7 – 156.0	NC	NC	153.1 – 173.64	NC	NC	

Key: Green label (C) – Compliant results; Red label (NC) – Non-compliant results.

As mentioned above, local availability studies need to be performed to support BE in highly complex semisolids. In accordance with EMA guideline, these studies can be performed with solely one RP batch *vs.* one TP batch. RP2 and RP3 share the same batch age (12 months), while RP1 was studied at 16 months. As RP1, RP2 and TP (the latter studied at 22 months) revealed similar release profiles, batch age does not seem to influence product performance for this specific product.

Considering all data, RP2 batch was selected for IVPT studies due to its closer release profile as well as similar viscosity attributes to TP.

6.3.3A Product efficacy profile – IVPT kinetic studies

IVPT studies using human skin were then performed to compare the permeation profile of diclofenac emulgel RP and TP. Although IVPT studies can be performed in the same equipment as IVRT experiments, their scope is entirely different, as IVPT tests measure the non-steady state rate of skin permeation, whereas IVRT aims to determine the steady-state rate of drug release (Ethier *et al.*, 2019). Other IVPT key features regard: (i) usage of a biological membrane, typically excised human skin. The integrity of membrane barrier should be qualified by a suitable test before and after IVPT experiments; (ii) usage of a physiological buffer receptor solution. The receiver medium should be compatible with the skin, assure the maintenance of sink conditions throughout the study, and promote adequate stability of the IVPT samples; (iii) finite dose conditions should be replicated in order to resemble an *in vivo* application. Furthermore, (iv) mass balance studies should be performed in order to assess the amount of drug remaining on the donor compartment, on the skin and delivered into the skin (Ethier *et al.*, 2019; Lehman and Franz, 2014; Thomas *et al.*, 2020).

The first step to be considered while developing an IVPT method is the performance of a pilot study. In this study, skin from different donors should be considered, with several replicates per donor. Three parallel treatments should be studied, the RP, the TP and a product with a differentiated flux profile, e.g., a 50% strength formulation. Please note that all product samples should be blinded to minimize the risk of bias. Along with the development of a fit for purpose/validated method, all of these procedures aim to determine the permeation profile range as well as to demonstrate the precision, reproducibility and selectivity of the intended method. Additionally, experimental conditions such as apparatus suitability, dosing amount and sample application procedures, sampling times, mass balance and membrane integrity assessment protocols should be calibrated and/or optimized.

The pilot study was conducted with 2 different donors and two replicates per donor were considered. The cumulative amount permeated and the flux profiles of the two diclofenac emulgel formulations, as well as negative control formulation, in individual skin sections are shown in Fig.6.7. Furthermore, the results from the mass balance studies are portrayed in Table 6.11.

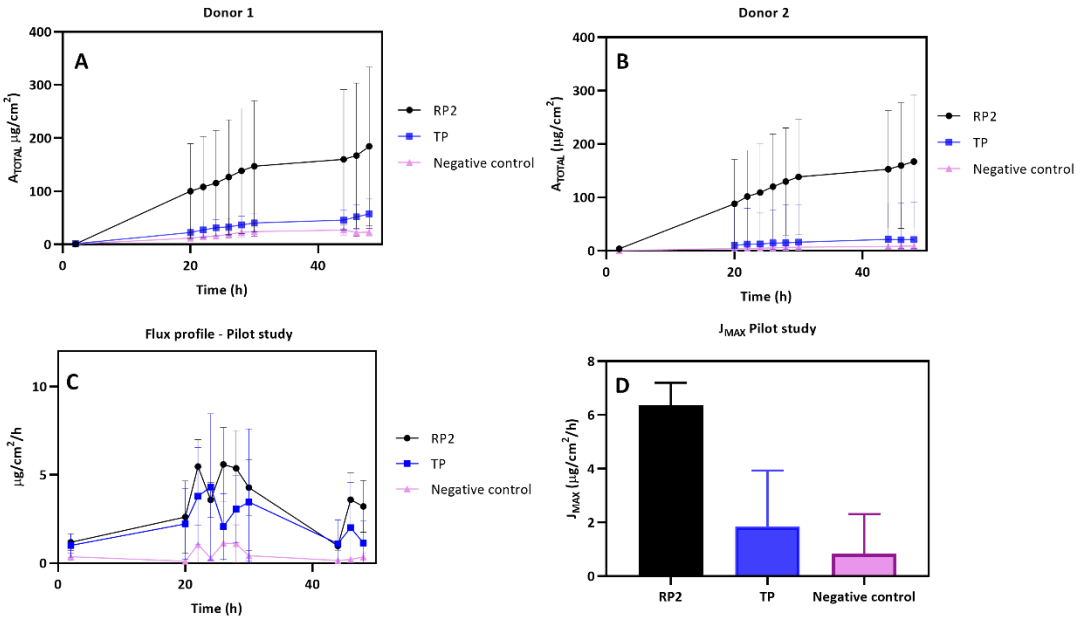


Fig.6.7 – A – Permeation profiles for diclofenac products attained for donor 1 in pilot IVPT studies. B – Permeation profiles for diclofenac products attained for donor 2 in pilot IVPT studies. C – Overall diclofenac flux profiles attained during IVPT pilot studies. D – Overall diclofenac J_{MAX} attained during IVPT pilot studies. All results report to mean ± SEM (n=2 from 2 donors).

When the three diclofenac emulgel products were initially screened and compared by conducting pilot IVPT studies, the diclofenac permeation into and through the skin was much higher from the RP2 compared to the generic, as well as negative control formulation (Fig.6.7A/B). Nevertheless, when plotting the flux profiles of all formulations, these differences were attenuated, with the TP and RP2 displaying a different rate profile when compared to the negative control formulation (Fig.6.7C). For RP2 the mean maximum rate (5.595 μg/cm^2/h) occurred at 26h, whilst for the TP it occurred at 24 h with a mean value of 4.136 μg/cm^2/h. Conversely, the negative control formulation exhibits a maximum peak in the rate profile at 26h with a mean value of 1.1325 μg/cm^2/h.

Based on the differential permeation and flux profiles displayed in Fig.6.7, the following conclusions can be drawn:

- IVPT pilot study results demonstrate that the selected experimental parameters adequately characterize the cutaneous pharmacokinetics of diclofenac across the study timeframe, since the maximal rate of absorption (flux) and the decline in flux were suitably identified (see Fig.6.7C).
- IVPT method sensitivity is demonstrated since according to the obtained flux profiles, indicating that the method was able to detect changes in the permeation profile between formulations of different strengths (Fig.6.7C/D).
- IVPT mass balance results, presented in Table 6.11, are overall within the EMA draft guideline criteria. Even though non-compliant results were attained for replicate 1 of donor 1 in both RP2 and TP formulations, these were borderline (112% and 88%, respectively). Regarding the negative control formulation, the same scenario was registered with replicate 2 of the 1st donor (111% drug recovery). Nevertheless, when considering the 1st replicate of donor 1 (12% drug recovery), the extraction procedures were not to the level prescribed. Reasons for this be related to the lower drug concentration of this product, along with the low amount of formulation placed in the membrane, due to the need to perform the IVPT study under finite dose conditions. Hence, for such conditions, the EMA 90-110% drug recovery criteria are extremely challenging to attain and ultimately may be too tight.
- Finally, skin integrity measurements performed before and after IVPT experiments, revealed that overall barrier function and integrity were adequately maintained throughout the study, as suggested by the observed TEWL values. Please note that besides TEWL measurements, after IVPT experiments, all skin segments were visually inspected for leaks and no leaks were observed in these skin sections.

Since the number of replicates and donors considered in this preliminary assessment was relatively low and since high inter and intra-donor variability was observed, especially when considering the RP2 formulation, no statistical analysis of the results was performed.

Other reasons which may ground this high variability regard the difficulty in attaining human skin and furthermore, the lack of procedure standardization in what concerns the harvest of the skin. Please note that in this study, in order to enlarge the pool of donors, different skin sources were used.

Despite that some non-compliant results were attained, overall these conditions serve the purpose to establish the conditions for the pivotal study.

Table 6.11 – Pilot study skin integrity and mass balance results. Two donors and two replicates were considered for pilot study.

Formulation	**RP2**				**TP**				**Negative control formulation**			
Donor	1		2		1		2		1		2	
Initial TEWL ($g/m^2/h$)	8.90	0.00	0.00	0.00	6.50	0.00	11.8	0.00	0.00	5.10	0.00	0.00
Final TEWL ($g/m^2/h$)	18	10.6	27.7	18.4	22.2	16.4	23.3	0.00	11.4	17.3	10.3	10.7
Acceptance criteria	TEWL < 20.0 $g/m^2/h$											
Donor compartment (µg)	111	2.50	20.5	49.1	62.9	154	22.7	162	0.150	107	105	77.7
Skin (µg)	110	2.08	19.0	98.0	74.7	24.3	112	18.2	2.43	5.42	5.07	8.60
Mass balance (%)	112	109	99	110	88	102	95	96	12	111	107	98
Acceptance criteria	90-110%											

Key: Green label – Compliant results; Red label – Non-compliant results.

For pivotal studies, 7 donors were used to assess the permeation profile of the RP, TP, as well as the negative control formulation (Thomas *et al.*, 2020). Even though a larger pool of donors was initially considered, the lack of compliance with TEWL requirements led to the exclusion of some donors. Nevertheless, two replicates per donor were considered in all cases, as specified in the pilot study protocol. Permeation and flux profiles obtained for IVPT pivotal studies are shown in Fig.6.8.

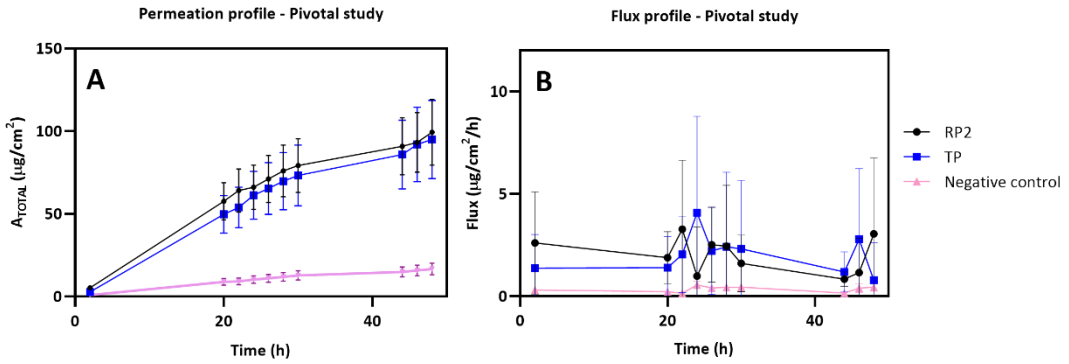


Fig.6.8 – A: Permeation profiles for all tested formulations in pivotal IVPT studies. B: Flux profiles attained during IVPT pivotal studies. Results report to the mean ± SEM calculated from duplicate sites from the same donor (7 donors; 2 replicates per donor).

As confirmed during the pilot studies, the developed method proved to have an adequate selectivity and sensitivity, as the method is able to reflect changes in the permeation as a function of differences in drug delivery. Overall, the mass balance and skin integrity results displayed in Table 6.12, generally met the established acceptance criteria. However, it should be denoted that some borderline results concerning TEWL values were registered after IVPT experiments.

Table 6.12 – Skin integrity and mass balance results pivotal study results. Seven donors and two replicates were considered for the pivotal study.

Donor	D3		D4		D5		D6		D7		D8		D9	
	RP2													
Initial TEWL ($g/m^2/h$)	0	0	6.9	5.8	5.1	8.1	2.5	8.9	0	14.8	11.6	5	12.5	5.6
Final TEWL ($g/m^2/h$)	18	104	23.5	21.0	12.3	8.90	22.0	19.0	20.0	10.2	21.0	13.0	22.0	8.5
Donor compartment (µg)	134	12.67	54.67	54.7	91.0	92.9	4.14	82.8	88.7	43.2	27.8	31.3	37.4	16.9
Skin (µg)	57.3	6.71	157	138	109	122	10.8	48.6	31.8	50.2	53.1	45.0	28.8	123
Mass balance (%)	110	110	109	107	112	106	112	93	101	85.0	91	80.0	76.0	98.0
	TP													
Initial TEWL ($g/m^2/h$)	1.70	6.30	11.2	15.1	19.3	15.3	7.90	0.00	0.00	16.7	17	9.60	7.30	0.00
Final TEWL ($g/m^2/h$)	10.7	1.90	20.0	12.8	11.5	13.3	25.0	15.8	0.80	9.6	17.3	13.6	9.3	11.2
Donor compartment (µg)	9.65	75.6	44.6	48.2	33.0	82.1	8.73	85.7	9.64	8.56	65.0	96.8	157	93.3
Skin (µg)	16.5	59.3	7.83	97.3	69.3	11.3	10.8	17.1	184	140	43.6	74.6	21.5	48.4
Mass balance (%)	106	94.0	107	103	94.0	106	91.0	88	105	85	93.0	94.0	106	100
	Negative control formulation													
Initial TEWL ($g/m^2/h$)	11.0	15.7	4.90	14.5	12.3	8.50	6.30	16.3	12.5	9.80	17.0	19.0	16.2	7.60
Final TEWL ($g/m^2/h$)	10.1	11.9	8.60	8.50	18.2	8.90	22.3	19.5	22.3	14.8	22.9	17.2	12.4	6.60
Donor compartment (µg)	32.9	61.0	93.2	54.6	6.64	36.1	11.9	15.7	15.9	13.3	82.1	46.0	13.5	28.7
Skin (µg)	68.5	38.5	14.3	45.8	3.24	41.6	42.2	58.1	16.5	19.6	34.1	21.5	39.9	13.3
Mass balance (%)	99.0	99.0	112	100	16.1	99.0	61.0	87.0	34.0	39.0	153	78.0	85.0	77.0

Key: Green label – Compliant results; Red label – Non-compliant results.

A closer inspection of the results showed that the increase of TEWL was not correlated with an enhanced permeation rate. Moreover, no leaks were observed in the excised human membranes. Taking these observations into account, the results retrieved from these diffusion cells were considered. Borderline/non-compliant mass balance results were also obtained from some donors. Increased drug losses were observed in some donors, especially when testing the negative control formulation.

Another parameter that must be evaluated during IVPT validation studies concerns method selectivity. IVPT method selectivity can be defined as the ability of the method to discriminate the cutaneous pharmacokinetics of a drug between products that exhibit differences in drug delivery (FDA, 2016c). Even though the FDA requires that the demonstration of method sensitivity should only be performed during pilot studies, the EMA requests that it should be performed in both pilot as well as pivotal studies. For IVPT method selectivity to register, the CI must fall completely outside the 80-125% range. According to Table 6.13, this was successfully achieved.

In the present work, two statistical approaches were used to compare IVPT data – EMA and FDA. On a side note, it is important to further clarify the assumptions in which these statistical methods are based. First of all, both of them regard a paired comparison, where the difference between the TP and RP is calculated considering each individual donor. It is important to note that since IVPT data does not follow a normal distribution, it should be natural log transformed prior to any calculation. In the EMA approach, the arithmetic mean of all individual T-R differences should be calculated. On the other hand, in the FDA approach, a similar rationale to that presented in the EMA guideline on investigation equivalence for highly variable drugs is used. By other words, there is an attempt to standardize the difference due to the observed variability in the reference product. Under this paradigm, the within-subject standard deviation (S_{WR}) should be evaluated for each IVPT endpoint attained with the RP formulation. If S_{WR} >0.294, the product is considered highly variable, and the scaled average bioequivalence (SABE) methodology can be used (FDA, 2016c; Pensado *et al.*, 2019).

IVPT method selectivity results, calculated by both approaches, are next summarized (Table 6.13).

Table 6.13 –90% Confidence interval calculated for J_{MAX} (µg/cm^2/h) and A_{TOTAL} (µg/cm^2) at the end of the permeation experiment (48 h) for RP and negative control formulations following FDA and EMA approaches.

		J_{MAX} (µg/cm^2/h)	Status	A_{TOTAL} (µg/cm^2)	Status	Acceptance criteria
Pairwise comparison	Approach used					
RP *vs.* Negative control formulation	FDA	SCI_{UB} = 4.3675 GMR = 0.2437	C	SCI_{UB} = 6.43 GMR = 0.15	C	SCI_{UB} > 0 GMR ≠ [0.8-1.25]
	EMA	18.73 – 31.71	C	11.57 – 18.91	C	90% CI falls outside [80-125]
TP *vs.* Negative control formulation	FDA	SCI_{UB} = 4.92 GMR = 0.25	C	SCI_{UB} = 7.06 GMR = 0.15	C	SCI_{UB} > 0 GMR ≠ [0.8-1.25]
	EMA	1.71 – 7.65	C	10.80 – 20.23	C	90% CI fall outside [80-125]

Key: J_{MAX} – Maximal flux; A_{TOTAL} – Cumulative amount permeated at the end of the IVPT study; In the EMA approach: J_{MAX} and A_{TOTAL} 90% CI were calculated based on the geometric mean of the duplicate values obtained per donor. In the FDA approach: SCI_{UB} – upper bound of the 90% confidence interval; A_{TOTAL} and J_{MAX} are reported as the anti-logarithm of the arithmetic mean (lower-upper 90% confidence interval) of the natural log-transformed values; Negative control formulation *vs.* RP2 (n=7 donors); Green label (C) – Compliant results.

The IVPT method proved to have discriminatory capacity towards formulations with different strengths. For both the J_{MAX} and A_{TOTAL} endpoints, the CIs fell completely outside the 80-125% interval, as per the EMA and FDA guideline criteria.

Regarding the pivotal results, both RP2 and TP displayed similar permeation as well as flux profiles, contrary to what was expected from the pilot study. This may be ascribed to the larger pool of donors. IVRT and rheological differences between both products did not seem to have a significant impact in the product permeation profile. Nevertheless, it should be noted that high variability was registered within each formulation. The statistical analysis of the results is presented in Table 6.14.

Table 6.14 –90% confidence interval calculated for J_{MAX} (µg/cm^2/h) and A_{TOTAL} (µg/cm^2) at the end of the permeation experiment (48 h) for diclofenac emulgel formulations RP2 *vs.* TP following EMA and FDA approach.

	J_{MAX} (µg/cm^2/h)	Status	A_{TOTAL} (µg/cm^2)	Status	Acceptance criteria
Approach used					
EMA	68.42 – 141.22	NC	69.60 – 143.89	NC	90% CI = [80-125%] A wider 90% confidence interval limit to a maximum of [69.84 – 143.19] may be accepted (EMA, 2018b)
FDA	SCI_{UB} = 0.8772 GMR = 0.9830	NC	SCI_{UB} = 0.7710 GMR = 0.8584	NC	$SCI_{UB} < 0$ GMR $\in [0.8 - 1.25]$

Key: J_{MAX} – Maximal flux; A_{TOTAL} – cumulative amount permeated at the end of the IVPT study; All formulations were tested in 7 donors and 2 replicates per donor were considered. In the EMA approach: J_{MAX} and A_{TOTAL} 90% CI were calculated based on the geometric mean of the duplicate values obtained per donor. In the FDA approach: SCI_{UB} – upper bound of the 90% confidence interval; *A_{TOTAL} and J_{MAX} are reported as the anti-logarithm of the arithmetic mean (lower-upper 90% confidence interval) of the natural log-transformed values; Green label (C) – Compliant results; Red label (NC) – Non-compliant results.

Even though borderline results were attained with the EMA approach, none of the products could be considered equivalent in accordance with EMA requirements.

Following the FDA approach, for the A_{TOTAL} and J_{MAX} measurements, the within-subject standard deviation (S_{WR}) was evaluated. For both endpoints S_{WR}>0.294, therefore the SABE methodology was followed (Pensado *et al.*, 2019).

In the SABE approach, bioequivalence can be inferred if the geometric mean ratio (GMR) falls within the range [0.8, 1.25] for the selected bioequivalence margin and if the upper bound of the 90% confidence interval (SCI_{UB}) for the quantity, $(\mu_T - \mu_R)^2 - \sigma^2_{WR}(\ln(1.25)/0.25)^2$, is less than or equal to zero. μ_T and μ_R regard the population means of the test and reference products, respectively, and σ^2_{WR} refers to the reference population variance (Pensado *et al.*, 2019). Example calculations of this statistical method are provided in Appendix B. Even though the GMR was within the confidence interval of 0.80-1.25, the products could not be considered as bioequivalent due to the lack of compliance with SCI_{UB} requirement in either A_{TOTAL}, as well as J_{MAX} endpoints.

When inspecting the results, a pronounced variability in IVPT data is readily observed. The sources of IVPT variability may be attributed to the (i) drug product itself, (ii) skin-drug product interactions and also to the (iii) intrinsic variability associated with IVPT studies (Pensado *et al.*, 2019).

Increasing the number of batches to assess the permeation kinetic profile is pointed out as a possible solution to overcome this issue. This strategy was already considered for demonstration of equivalence of a 2% (w/w) diclofenac emulgel, as detailed in the European Public Assessment reports (PAR, 2020) Furthermore, due to the observed variability, the donor sample size should have been increased. According to a study by Tothfalusi *et al.* for highly variable drugs, the sample size to use should be established based on the within-subject variability. Since larger absolute differences between the two logarithmic means are expected to occur, it is recommended that a 10% deviation between the means (e.g. GMR = 1.10) should be considered, which clearly yields a higher donor sample size for IVPT (Tothfalusi and Endrenyi, 2011). As previously mentioned, the TP herein used presented equivalent pharmacokinetic profile towards the RP in the work carried out by Pleguezuelos-Villa and colleagues (Pleguezuelos-Villa *et al.*, 2019). The authors performed microstructure, IVRT and pharmacokinetic studies. Although IVPT studies were not performed, similar to our results, equivalence was not demonstrated with respect to microstructure parameters. However, these differences did not translate into meaningful bioavailability divergence. Based on the results, the authors were able to conclude that in this case study, microstructure tests tend to overestimate the impact of formulation differences (Pleguezuelos-Villa *et al.*, 2019).

Another interesting paper described a dermal physiologically-based pharmacokinetic model aimed at predicting the skin permeation and disposition, of a diclofenac sodium gel. This virtual bioequivalence approach (VBE) correlated product quality attributes and API physicochemical properties with the skin (patho)physiology. By doing so, this model was able to provide a direct relationship between systemic and local (skin and synovial fluid) exposure to diclofenac. The verification and validation procedures of this approach were based on the principles of fit-for-purpose modeling, which included an assessing of the observed data of diclofenac concentrations in skin tissues/plasma and a correlation of this information with the performance of the modeling platform. The VBE method described was accepted by the FDA to document the bioequivalence of the diclofenac gel product. This case study highlights the potential of these quantitative tools to support alternative bioequivalence approaches (Tsakalozou *et al.*, 2021b, 2021a).

Altogether, the data herein presented does not question that this diclofenac TP is bioequivalent to the RP, as this assessment has already been thoroughly documented through pharmacokinetic studies and approved by the regulatory authorities. Nevertheless, they show that quantifiable, statistically significant differences in dermal bioavailability of the drug do not necessarily translate into clinically significant differences according to EMA criteria.

6.4A Concluding Remarks

In this chapter, we have examined the experimental procedures as well as the regulatory mechanisms underlying the BE assessment using two extreme case studies that differed in the formulation technological features, as well as therapeutic site of action.

For simple formulations that predominantly target the skin surface, such as the dimetindene maleate 1 mg/g gel formulation, bioequivalence should be sustained by establishing Q1, Q2, Q3 and Q4. Regarding more complex formulations, such as emulgels, which target more profound skin layers, Q1-Q4 equivalence should be established. Furthermore, local availability studies should be performed in order to sustain equivalence regarding product efficacy. For these purpose, IVPT tests or other surrogate tests for clinical endpoint studies can be considered.

In all cases, the variability of the donors as well as the RP has to be statistically considered.

Overall, these results highlight that when considering a waiver from clinical endpoint studies for topical generic products, further work and discussion with the regulatory agencies is required. The statistical criteria in several cases may be too demanding for some products as a result of their batch-to-batch and shelf life intrinsic variability in microstructure and performance.

PART B

6.2B Materials and methods

6.2.1B Materials

Bifonazole cream products were acquired from the European Market. Four different products were considered: (i) The bifonazole 10 mg/g cream RP (Bayer) – Canesten Unidia® (Portuguese market), CanesMycospor® (Spanish market) and Canesten Extra® (German market). Five batches of this product were considered. Please note that the different commercial names are due to the market source; (ii) A qualitative and quantitative formulation was replicated – TP. Three batches were considered; (iii) A Q1 formulation was also studied - comparator product A (CPA) – Amycor® 1% (Merck Serono), and finally (iv) a Q1/Q2 different formulation – comparator product B (CPB) – Levelina® crema (Ern Laboratories) – was also studied. Due to market availability, only one batch was considered for CPA and CPB formulations. The qualitative composition of all products is shown in Table 6.15.

Propylene glycol was acquired from Merck, and phosphate buffered saline (PBS) was purchased from Sigma. Water was purified using a Millipore MILLI-Q reagent water system and filtered through a 0.22 μm nylon filter before use. All other chemicals were of analytical grade or equivalent.

Table 6.15 – General information and qualitative composition of the products used in the present study. Batch age is given in months (M). The RP has an expiry date of 5 years, CPA of 3 years and CPB of 4 years. [a] – Batch age during IVRT and rheology studies; [b] – Batch age during IVPT studies.

Studied products		RP	TP	CPA	CPB
	Used batches	RP1: 32M[a]+37M[b] RP2: 32M[a] RP3: 26M[a] RP4: 14M[a]+19M[b] RP5: 12M[a]	TP1: 12M[a] + 17M[b] TP2: 17M[a] TP3: 17M[a]	CPA: 11M[a]	CPB: 11M[a]
Excipient	Function				
Benzyl alcohol	Preservative	X	X	X	
Cetostearyl alcohol	Emulsifier	X	X	X	X
Cetyl palmitate	Thickener	X	X	X	
Disodium EDTA	Chelating agent				X
Methyl parahydroxybenzoate	Preservative				X
Mineral oil	Emollient / emulsifier				X
Octyldodecanol	Emulsifier	X	X	X	
Polyoxyethylene stearate 40	Emulsifier				X
Polysorbate 60	Emulsifier	X	X	X	
Propyl parahydroxybenzoate	Preservative				X
Polyethylenglycol 400	Co-solvent				X
Purified water	Solvent	X	X	X	X
Sodium Hydroxide	Buffering agent				X
Sorbitan monostearate	Emulsifier	X	X	X	
Vaseline	Thickener				X

Key: RP – Reference Product; TP – Test Product; CPA – Comparator Product A, and CPB – Comparator Product B. [a] – IVRT and Rheology studies; [b] – IVPT studies.

6.2.2B Methods

6.2.2.1B Formulation production

To evaluate the discriminatory ability of the methods used to assess microstructure, performance, and local availability, different bifonazole cream formulations were manufactured. These included a 5 mg/g, a 20 mg/g, and a placebo cream formulation.

Furthermore, a different rheology formulation was manufactured. Based on formulation development studies, it was determined that a critical excipient responsible for the viscosity profile was cetostearyl alcohol, therefore its content was reduced by half to obtain a different microstructure formulation. For the TP, the Q1/Q2 composition of the RP was replicated (data not shown). In the manufacturing process, both the aqueous and lipophilic phases were prepared separately and heated to 68 ± 2°C. Afterward, bifonazole was added to the dispersed phase. Both phases were then combined and cooled to 20-25^{o}C. All formulations were prepared conventionally resorting to an Ultra-Turrax X 10/25 (Ystral GmbH, Dottingen, Germany) as blending equipment. Laboratory scale batches (1 kg or 0.5 kg) were considered. The optimal experimental settings in terms of speed, duration and temperature of the manufacturing processes were carefully optimized during the formulation studies (data not shown).

Similarly to Part A of the present chapter, to document the discriminatory power of the proposed rheology, IVRT and IVPT methods, different bifonazole formulations had to be prepared. These differences regarded product strength (placebo, 50% and 200% formulations) and different rheology profile formulations. To manufacture the previously mentioned formulation, the cetostearyl alcohol content has reduced to half. This excipient was herein selected due to its impact on the product viscosity profile.

6.2.2.2B Microstructure evaluation

Comparative microstructure studies were performed in line with Part A. The conditions developed in chapter 5 were herein applied. Briefly, rotational tests were performed with a C35/2^{o}/Ti cone geometry. For the flow curve [$\eta = f(\tau)$], a linear CS flow ramp ranging from 0.01 to a final 100 Pa was measured for 300 s. On the other hand, to assess the thixotropic behaviour (Pa/s), a shear rate from 0.01 to 300 s^{-1} and again down to 0.01 s^{-1}, during 180 s was used. In all tests, approximately 0.3 g of each formulation were used. Regarding oscillatory measurements, a plate geometry (P35/Ti) was used and approximately 1 g of the formulations were placed in the *peltier* plate. An amplitude sweep ranging from 0.01 and 500 Pa at 1 Hz was firstly conducted to estimate the linear viscoelastic region (LVR), as well as the flow point (τ_f). Afterwards, a frequency sweep analysis was performed within the LVR range to determine the storage modulus (G´) and loss modulus (G´´) from 100 to 0.1 Hz. Results are presented for 1 Hz.

Rheology method validation

As previously mentioned in Part A, the suitability and discriminatory capacity of the rheological method was documented by analysing a formulation with different rheological profile. One of the excipients which plays a central role on the bifonazole cream viscosity profile is cetostearyl alcohol. Therefore the concentration of this excipient was reduced by half to obtain a formulation with significantly different viscosity. The methods were considered sensitive if the rheological endpoints obtained with the RP were higher compared to altered formulations. On the other hand, to assess method selectivity, the 90% CI was determined. If the CI between the RP and the altered rheology formulation was outside the range of 75-133%, the selectivity of the method can be concluded.

6.2.2.3B Product performance evaluation – IVRT studies

The *in vitro* release profile was determined for all products in the present study and the respective acquisition was done by the same diffusion system used in the previous chapters of this thesis. Diffusion system qualification studies results were already presented in Chapter 4 and bifonazole IVRT conditions were already described in Chapter 5. IVRT conditions are briefly summarized in Table 6.16.

Table 6.16 – Receptor solution, sampling times, and donor drug loading used for IVRT studies according to product. All results report to mean ± standard error of the mean (SEM) (4≤n≤7).

Receptor phase	PBS-Ethanol (50:50, v/v) pH= 7.4
Donor drug loading technique	Positive displacement syringe
Applied formulation	150 mg, evenly placed over the membrane. Efforts were made to ensure a reproducible and consistent formulation application procedure (not deviating more than 5%)
Sampling times (h)	0.25, 0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h
Temperature	37 ^{o}C, to assure 32 ^{o}C at the membrane surface
Membrane	SUPOR 450 pore size 0.45 µm, Pall Corporation, USA The membrane was previously soaked in purified water for 30 min
Agitation	600 rpm
Equilibration period	30 min
Sampling and replacement volume	300 µL
Occlusion	Performed with Parafilm® in the donor compartment, as well as in the sampling arm

The samples were subsequently analysed using validated HPLC methods, please see Appendix A.

The calculations reporting to the cumulative amount and percentage of drug released, in addition to the *in vitro* release rate were already described in Chapters 4 and 5. According to the European regulatory requirements, a n=12 was considered for each product.

IVRT Method validation

The methodology used for bifonazole cream IVRT validation studies was in line with the one used in Part A of the present chapter.

6.2.2.4B IVPT using human skin

For IVPT studies, the same diffusional system was used. The experimental procedures followed EMA draft guideline and the FDA acyclovir draft guidance (EMA, 2018b; FDA, 2016c). According to method development studies, the following parameters were set for the IVPT experiments. Finite dose conditions (8-12 mg/cm^2) of the formulation were applied in the donor compartment. The receptor medium was continuously stirred at 600 rpm and all experiments were conducted in a temperature-controlled water bath to ensure a skin surface of 32±1^{o}C. All IVPT runs were performed under non-occlusive conditions to mimic the in-use setting (Kamal *et al.*, 2020). Due to the limited solubility of bifonazole, a PBS-PEG (60:40, v/v, pH= 7.4) solution was used as the receiver medium. Samples of the receptor phase (300 µL) were withdrawn at 2, 20, 22, 24, 26, 28, 30, 44, 46 and 48 h. After each collection, an equal volume of fresh-temperature-equilibrated permeation medium was added to the receptor chamber. In a first stage, a pilot study was performed to infer on the suitability of the purposed method conditions. This was then followed by a pivotal study, where a larger pool of skin donors was employed.

According to the EMA draft guideline, the 90% confidence interval (CI) for the ratio of means between the test and reference products should be determined for the maximal flux (J_{MAX}) and the cumulative amount of drug permeated at the end of the IVPT study (A_{TOTAL}) (EMA, 2018b). J_{MAX} corresponds to the maximal rate of absorption and its analogous to the comparison of the C_{max} for test and RP products in the case of plasma pharmacokinetics. Similarly, A_{TOTAL} is calculated through equation (i) and can be compared to the area under the curve (AUC) of the incremental bifonazole permeation profile. IVPT methods should be adequately validated by testing a formulation at 50% of the proposed product strength, in order to register non-equivalence with the RP/TP. Furthermore, to infer on the potential interference deeming from the biological matrix or dosage form, Franz cells containing non-dosed skin and a placebo formulation should likewise be considered.

Biological membrane preparation

The same skin sources and membrane preparation techniques described in Part A were herein used. Nevertheless, a larger pool of donors was considered in bifonazole IVPT studies, please see Table 6.17.

Table 6.17 – Human skin donor characteristics.

Study	Donor number	Gender	Skin type	Age	Anatomical region	Preparation method
Pilot study	1	Female	Type 3	43	Abdomen	HSE
	2	Female	Type 2	41	Abdomen	HSE
	3	Female	Type 2	29	Abdomen	HSE
Pivotal study	4	Female	Type 3	57	Arm	HSE
	5	Female	Type 3	55	Glute	HSE
	6	Female	Type 2	29	Abdomen	HSE
	7	Female	Type 2	29	Abdomen	HSE
	8	Female	Type 3	35	Thigh	HSE
	9	Male	Type 3	56	Abdomen	HSE
	10	Female	Type 2	41	Abdomen	HSE
	11	Female	Type 3	37	Abdomen	HSE
	12	Female	Type 3	39	Thigh	HSE

Key: HSE – Heat Separated Epidermis.

Mass balance studies

Mass balance studies were also performed to assess the amount of drug remaining on the donor compartment, on the skin and delivered through the skin (EMA, 2018b; Hossain *et al.*, 2019; OECD, 2010). At the end of the IVPT runs, the donor compartments were washed with 1 mL of acetonitrile and the respective washing solutions were collected. Afterwards, the skin was transferred into Eppendorf® tubes and the remaining bifonazole was likewise extracted with acetonitrile. All samples were sonicated (10 min), centrifuged at 11 740 x g for 10 min in a Minispin®(Eppendorf Ibérica S.L., Madrid, Spain), filtered by a 0.45 µm nylon membrane and transferred to HPLC vials for analysis.

The total recovery of drug at the end of the IVPT experiment was calculated as previously referred in Part A. Procedure reliability was confirmed by the total active ingredient recovery (%), which should be within the range of 100% ±10% (EMA, 2018b). All IVPT as well as mass balance samples were analysed by regulatory compliant HPLC methods, please see Appendix A.

6.2.2.5B Data Analysis and Statistics

The procedures described in Appendix B were followed. Example calculations are provided in the same section.

For rheology data, the Shapiro-Wilk test (p=0.05) was first performed to evaluate if the data followed a normal distribution. Since several endpoints did not present a normal distribution, the 90% CI of the ratio between the TP/RP was calculated following EMA draft guideline, as previously described (Appendix B).

6.3B Results and discussion

6.3.1B Rheology and IVRT method validation

According to the EMA guideline, evidence on the discriminatory power of the product characterization methods should be properly justified. To this end, both rheology and IVRT methods validation studies were carried out. Even though American and European directives are clear on the level of validation required for IVRT studies, the same still does not occur for rheology method validation. In an attempt to present a validation protocol directed towards this technique, the same strategy proposed in Part A of the present chapter was herein adapted. Method validation was performed in terms of precision, selectivity and sensitivity. All RP formulations were considered in this analysis, as well as the negative control formulation.

In what concerns rheological method precision, or in other words, the closeness of the repeated individual measures, it should be noted that extremely high CV were registered in the RP (11-512%) (EMA, 2009). However, if the individual RP batches CV% values are considered instead, method precision can overall be supported, please see Table 6.18.

Table 6.18 – Rheology method validation results. Rheological endpoints results pertaining to the RP regard a n=15 sample size. For Altered rheology formulations, n=3 was considered. To assess method selectivity, the 90% CI of the ratio (altered rheology/RP) is presented. If the CI of the considered rheological endpoints surpasses 75-133% interval, selectivity can be inferred.

	Reference Product							**Negative control formulation**		
Rheological endpoints	**Mean**	**CV% overall**	**CV% RP1**	**CV% RP2**	**CV% RP3**	**CV% RP4**	**CV% RP5**	**Mean**	**CV%**	**90% CI**
η_0 (Pa.s)	18905	13.0	13.0	3.19	0.93	0.65	7.24	9779	1.0	45.12 – 60.35
τ_{ROT} (Pa)	43.3	27.3	2.44	4.15	1.02	6.61	4.47	24.2	2.7	42.46 – 79.85
η_∞ (Pa.s)	16.2	59.1	45.3	11.6	14.8	17.6	4.23	0.041	2.868	0.13 – 0.89
S_R (Pa/s)	8473	63.0	35.3	18.0	6.68	16.7	16.7	8556	19	61.24 – 240.64
LVR (Pa)	1037	512	6.88	4.04	8.14	4.47	13.2	420	3.0	31.47 – 56.46
τ_f (Pa)	307	53.0	11.2	0.00	13.2	4.77	4.66	103	32	21.97 – 59.07
τ_{OSC} (Pa)	25.6	24.8	10.0	7.87	23.62	10.7	5.06	27.7	19.8	81.72 – 147.87
G´ – 1Hz (Pa)	828	11.0	4.00	1.00	1.00	1.00	0.13	449	2.0	48.71 – 60.84
G´´ – 1Hz (Pa)	233	23.0	10.0	10.0	2.00	9.0	9.0	115	9.0	40.17 – 63.17
Validation parameter	Acceptance criteria									
Sensitivity	Acceptance criteria: RP > Altered Rheology formulation Rotational endpoints: Compliant except for S_R Oscillatory endpoints: Compliant except for τ_{OSC}									
Selectivity	RP ≠ Altered Rheology formulation Rotational endpoints: Compliant for all endpoints Oscillatory endpoints: Compliant for all endpoints									

Key: η_0 (Pa.s) – Zero-shear viscosity; η_∞ (Pa.s) – Infinite shear viscosity; $\tau_{0.ROT}$ (Pa) – Yield point obtained through rotational methods; S_R (Pa/s – Relative thixotropic Area; $\tau_{0.OSC}$ (Pa) – Yield point obtained through oscillatory methods; LVR plateau (Pa) – Linear Viscoelastic Region plateau; τ_f (Pa) – Flow point; G´ – Storage modulus; G´´ – Loss modulus; Sensi – Sensitivity; Green label – Compliant results.

To document the sensitivity of the method, the rheological endpoints otained with the negative control formulation should be lower when compared to the RP. For 7 out of 9 endpoints this condition was registered, nevertheless, lack of compliance was observed for the relative thixotropic area (S_R) and for the yield point estimated through oscillatory endpoints (τ_{OSC}).

The relative thixotropic area (S_R) derived from the thixotropic rheograms provides information on the formulation breakdown and recovery and after the shearing process, respectively. The S_R, also referred to as the hysteresis loop area, is generally regarded as the measure of the thixotropy in the formulation (Ethier *et al.*, 2019; Mezger, 2010).

As shown in Fig.6.11, all RP batches revealed a full thixotropic recovery, which is regarded as a good stability indicator, since it reflects the capacity of the cream microstructure to fully recover after the shear termination. The high variability herein denoted may be associated with batch age, since the batches with more prolonged shelf life (RP1, RP2 and RP3) displayed an inferior S_R (4492, 6290, 3269 P/s, respectively) when compared with the batches at an early

life cycle stage (RP4 + RP5 = 12750, 15563 P/s, respectively). The thixotropic properties in this case study appear to reflect a time dependent structure degradation, as batch age increases, the S_R decreases, which is indicative of a weaker internal structure and a lower tolerance to stress when compared to the "younger" batches (Ethier *et al.*, 2019; Mezger, 2010).

The yield point estimated through oscillatory measurements regards the minimum shear stress that must be applied to induce material flow. In this work, this parameter was retrieved from amplitude sweep measurements and corresponded to shear stress value in which the LVR *plateau* ceased. No obvious correlation between batch age and yield point was observed. Despite the lack of sensitivity reporting to the τ_{OSC}, the remaining amplitude sweep indicators (τ_F and LVR plateau) adequately documented compliance with the established criteria.

In what concerns method selectivity, it should be noted that a direct application of EMA criteria is not possible, since (i) several rheological parameters did not follow a normal distribution and (ii) a higher than 10% CV is registered between the different RP batches. In this context, and similarly to the published work by Maria Pleguezuelos-Villa, a larger criterion [75-133%] was selected for selectivity studies, as well as for equivalence studies (EMA, 2018b; Pleguezuelos-Villa *et al.*, 2019). To support method selectivity, statistical inequivalence between the RP and the negative control formulation should be observed. Even though, the calculated 90% CI were outside the 75-133% criteria (Table 6.18) the CI of the τ_{OSC} endpoint was partially inside this range. This occurrence is in line with the previously discussed sensitivity results.

Taking all the information into account, the rheological methods were considered fit for the purpose of this study since an adequate precision and discriminatory power were overall demonstrated.

IVRT method validation studies were likewise performed according to American and European guidelines, the results are summarized in Table 6.19 (EMA, 2018b; FDA, 2016c).

Table 6.19 – Acceptance criteria for bifonazole 10 mg/g cream IVRT method validation studies based on regulatory requirements (EMA, 2018b; FDA, 2016c). The same RP batch was used during IVRT validation studies.

Parameter	Results	Acceptance criteria	Status
Membrane inertness	Bifonazole recovery: 99.63%	Recovery ≥ 95%	Compliant
Linearity	R^2 = 0.979 ± 0.017 (n=36)	R^2 > 0.90	Compliant
Precision and reproducibility, including operator variability (IVRR data reports to µg/cm²/√h and Qf to µg/cm²)	Run 1 (operator A): IVRR = 310 (6%) | Qf = 1258 (8%) Run 2 (operator B): IVRR = 243 (8%) | Qf = 967 (7%) Run 3 (operator B): IVRR = 326 (6%) | Qf = 1277 (6%) Intra-run variability (n=36) RSD IVRR = 6.75% |RSD Qf = 7.07% Inter-run variability (n=36) RSD IVRR = 14.04% | RSD Qf = 14.10%	Intra-run and inter-run variability: RSD Qf ≤ 15% RSD IVRR ≤ 15%	Compliant
Selectivity [Data reports to IVRR (µg/cm²/√h)]	n=12 0.5% *vs.* 1% → CI = [14.05 – 17.11%] 1% *vs.* 2% → CI = [273.29 – 329.53%] 1% *vs.* ≠ CQA → CI = [39.60 – 49.97%]	CI between different strength products falls outside the limits [90-111] %	Compliant
Robustness [Data reports to IVRR (µg/cm²/√h)]	n=12 Mean IVRR 37ºC = 310 (6%) Mean IVRR 35ºC = 305 (8%) Mean IVRR 39ºC = 317 (6%)	Mean IVRR of runs with minor temperature fluctuations should not deviate more than 15% from the IVRR of the nominal method parameter settings	Compliant

The release medium provided to be suitable according to regulatory requirements, as sink conditions were registered. As addressed in Chapter 4, historically, ethanol based solutions are commonly employed as a release medium in IVRT experiments due to its miscibility profile with aqueous solutions, and overall suitability for analytical processing (Raney, 2021a). The selection of a diffusion membrane is also a key parameter of the IVRT method. A suitable IVRT membrane should provide an inert holding surface, but not constitute a barrier for drug release. The membrane must display chemical compatibility with both formulation, as well as release medium, and should not contain leachables. Furthermore, a reduced back diffusion should be observed, in order to avoid product transformation. Membrane inertness studies revealed that the selected SUPOR membrane fulfils these requirements.

The selection of the release medium, membrane and overall experimental setup (sampling points, speed, temperature, amount of formulation applied) should enable the acquisition of a linear release profile, reflecting Higuchi kinetics (Higuchi, 1961). As linearity was observed in

every diffusion cell, it was demonstrated that steady-state kinetics conditions were achieved with these experimental conditions (Table 6.19). Even though the guidelines recommend $R^2 \geq 0.90$ over the entire IVRT time range, the correlation coefficient is not a very discriminatory parameter, therefore a higher R^2 ($R^2 > 0.97$) should be registered to demonstrate adequate linearity of release (EMA, 2018b; FDA, 2016c; Raney, 2021a).

Precision, reproducibility and operator variability studies results met the established acceptance criteria. The maximum CV% was attained when estimating the inter-run variability (14.10%). Although this value is still compliant with the 15% CV acceptance threshold defined by the FDA, it should be noted that EMA criteria is far stricter only allowing a maximum of 10% deviation (EMA, 2018b; FDA, 2016c). Variability in IVRT results may be related to air entrapment, inability to uniformly spread the formulation upon the membrane and difficulty in reproducing the exact amount of formulation loaded in the system (Bao and Burgess, 2018). Nevertheless, several papers report similar CV results in IVRT precision studies (<15%) (Mudyahoto *et al.*, 2020). Therefore, setting a broader acceptance criterion is warranted from both a scientific and experimental perspective.

From a regulatory point of view, it should be noted that there are slight differences in what concerns method precision documentation, according to EMA draft guideline, ICH guidelines and the FDA acyclovir guidance (EMA, 2018b; FDA, 2016c; ICH, 2005). By EMA guideline, IVRT methods should present an adequate intermediate precision. For this, studies should be conducted with the same batch product, by different operators, on different days. In this work, these procedures were considered for validation purposes. On the other hand, according to ICH guidelines, the method intermediate precision is a part of the precision assessment, which also includes the documentation of the repeatability and reproducibility (ICH, 2005). According to this guideline, intermediate precision documentation can be sustained by submitting the method to specific variations that might occur during routine analysis, such as days, analysts (similarly to what was previously exposed for ICH requirements), but also including equipment variations. This rationale is also supported by the acyclovir FDA guidance (FDA, 2016c).

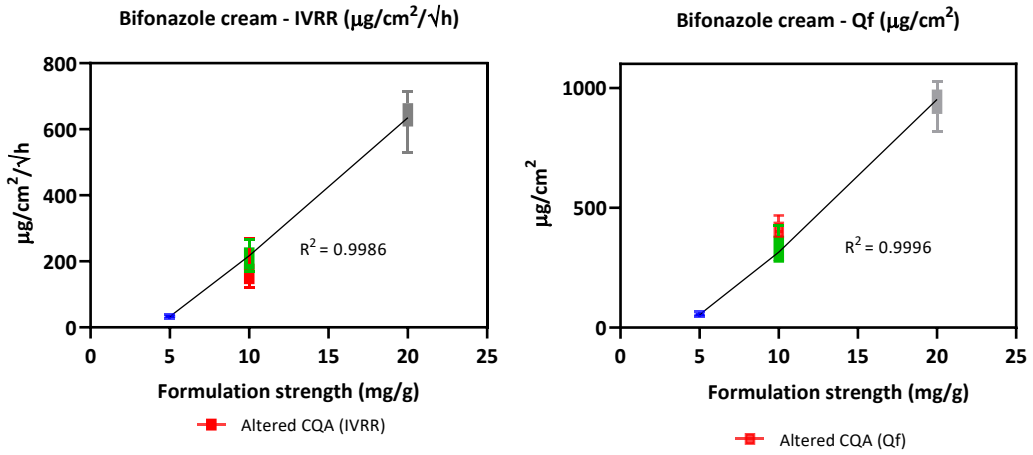


Fig.6.9 – IVRT sensitivity and specificity studies. Box and Whiskers plots of the measured release rates/ cumulative amount released for the different strength and altered rheology formulations. The altered rheology formulation is signaled in red.

The IVRT method discriminatory capacity was successfully documented, please see Fig.6.9 and Table 6.19. The IVRT method sensitivity was established since the following occurrence was registered: IVRR/Qf 0.5% bifonazole cream < IVRR/Qf 1% bifonazole cream < IVRR/Qf 2% bifonazole cream. Furthermore, both IVRT endpoints retrieved from the different strength formulations, presented a linear relationship ($R^2 \geq 0.9$) (Fig.6.9). In what concerns method selectivity, the CI reporting to the IVRR of the lower *vs*. nominal strength and higher *vs*. nominal strength formulations were outside the range 90-111%; hence, the method was considered selective to establish differences in release rates. Moreover, the supplemental selectivity was also adequately demonstrated, as statistical inequivalence between the RP and the altered rheology formulation was registered. These assumptions would also maintain if the FDA criteria was used instead (75-133%) (FDA, 2016c).

The ability of the method to be unaffected by minor variations in the experimental conditions are also supported, as the mean IVRR did not deviate more than 15% from the IVRR of the nominal method parameter settings (Table 6.19). Therefore, the method is considered to be robust.

6.3.2B Microstructure and product performance evaluation

After establishing suitable methodologies for both rheology/IVRT experiments, comparative studies between the different bifonazole cream formulations were carried out. As previously denoted in Chapter 5, this specific RP (RP1 to RP3), displayed statistically significant rheological variability between batches. Reasons which may contribute to this variability

might be related with the observed differences in the formulations pH, as well as globule size. Other aspects, such as batch age may also be relevant in what concerns variability, nevertheless, it should be noted that in this specific case, batch age was similar. In an attempt to pursue an enhanced RP characterization, a larger pool of batches was considered, with two extra batches being added to the analysis. An effort was made to select batches in an early life cycle stage, when compared to the previous ones, please see Fig.6.10.

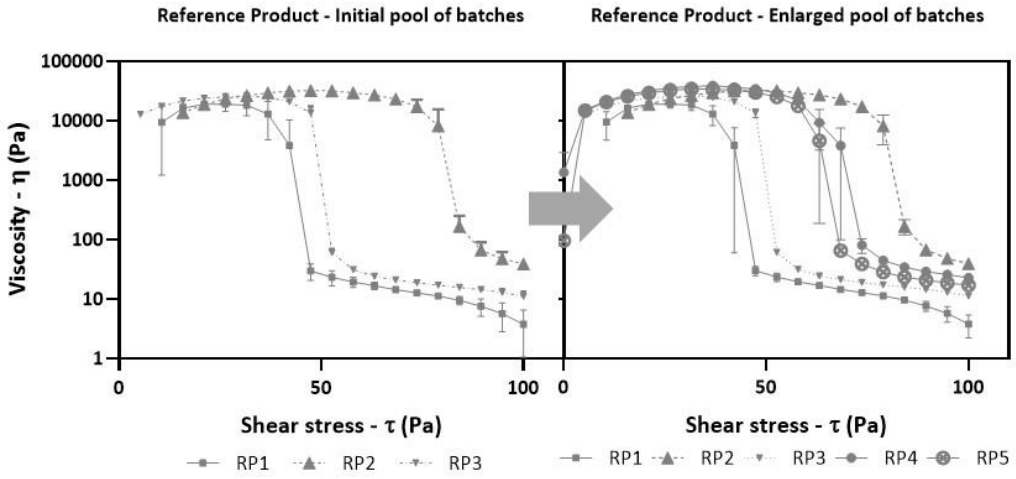


Fig.6.10 – Viscosity curves of bifonazole 10 mg/g cream reference products. All results report to mean ± SEM. Three replicates were used per batch formulation.

The added batches (RP4 and RP5) proved to have an intermediate viscosity profile when compared to the initial sample of batches. Moreover, both formulations presented closer viscosity behaviour, when compared to the initial products.

As described in the materials section, in this study, five RP batches, three TP batches and a single batch of CPA and CPB were considered. Please note that CPB presented Q1 differences (Table 6.15), whilst TP and CPA are Q1 equivalent towards the RP, moreover, TP also displays a quantitative equivalent (Q2) composition.

In what concerns the comparative rheological analysis, the results are summarized in Fig.6.11, and Table 6.20.

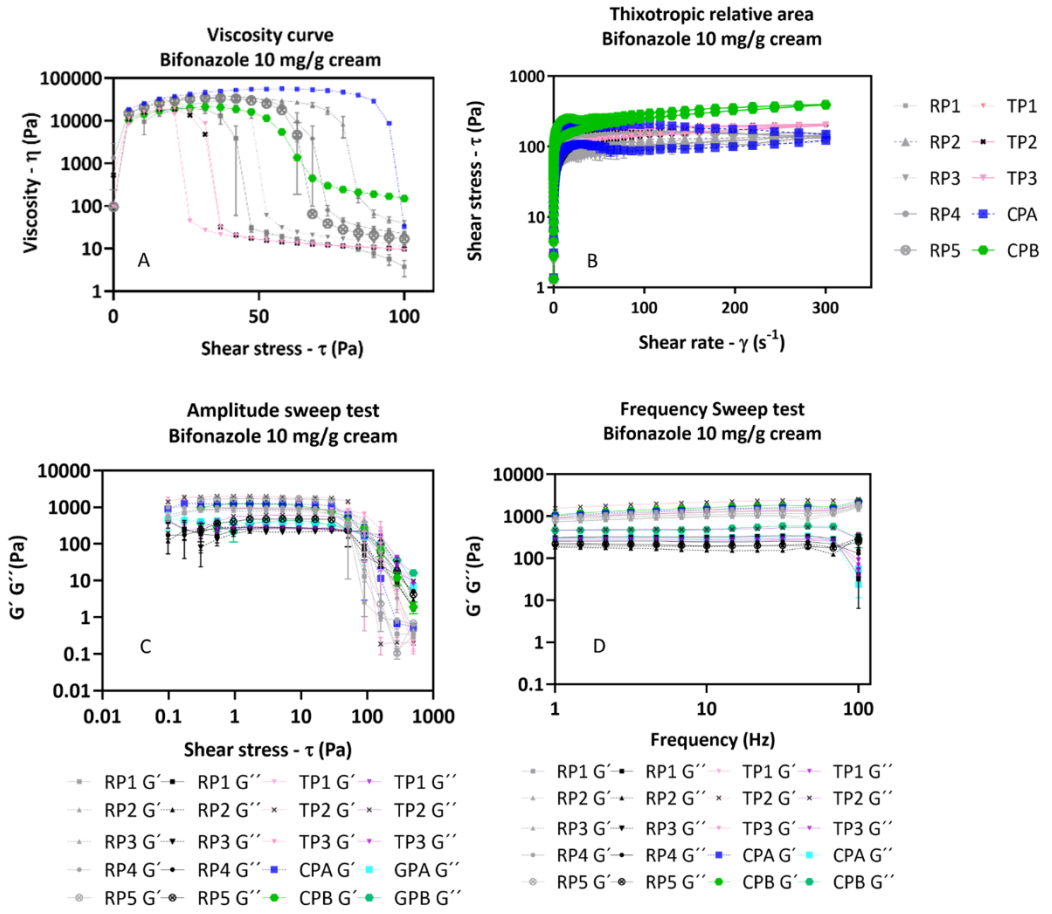


Fig.6.11 – Rheology profile of the bifonazole 10 mg/g cream products. All results report to mean ± SEM. Three replicates were used per batch formulation. A – Viscosity curve; B – Thixotropic behaviour; C – Amplitude sweep test; D – Frequency sweep test.

As previously denoted during rheology validation studies the viscosity curves of all formulations displayed a zero-shear *plateau* followed by a shear thinning region and an infinite shear *plateau* (Fig.6.11A). A variable shear stress application has been observed when dispensing doses from a container and applying them into the skin. It should be denoted that the container itself may cause variable shear stress on the formulation, as well as the patient may also induce a wide range of stress upon product application. In this context, the zero- shear viscosity is related with the ease of formulation dispensing from the container. On the other hand, the infinite-shear viscosity pertains to the spreadability of the product to the application site.

The overall RSD attained with the RP were higher when compared to the remaining formulations. This was expected due to the pronounced RP inter-batch variability. The rotational endpoints retrieved from the CPA, the qualitative equivalent formulation, were the highest, suggesting a firmer consistency. The opposite scenario was overall observed with the

TP, which presented the lowest rotational endpoints, thus highlighting a more fluid consistency (Fig.6.11A). This, however, was not registered when addressing the TP oscillatory profile, where higher values were denoted when comparing to the remaining formulations, please see Table 6.20. The RP, on the other hand, presented intermediate rheology endpoints.

Table 6.20 – Rheological properties of all bifonazole 10 mg/g cream formulations considered in this study. The 90% CI for the ratio average are presented for: (i) TP/RP; (ii) CPA/RP and; (iii) CPB/RP. (RP: n=15; TP: n=9; CPA: n=3; CPB: n=3).

Rheological endpoints	η_0 (Pa.s)	τ_{ROT} (Pa)	η_∞ (Pa.s)	S_R (Pa/s)	LVR (Pa)	τ_f (Pa)	τ_{OSC} (Pa)	G´ – 1Hz (Pa)	G´´ – 1Hz (Pa)
RP1 Mean (RSD%)	15573 (13.0%)	25.1 (2.44%)	2.83 (45.3%)	4492 (35.3%)	870 (6.88%)	233 (11.2%)	19.0 (10.0%)	955 (4.00%)	312 (10.0%)
RP2 Mean (RSD%)	18933 (3.19%)	56.1 (4.15%)	27.2 (11.6%)	6290 (18.0%)	878 (4.04%)	169 (0.00%)	31.7 (7.87%)	729 (1.00%)	184 (10.0%)
RP (overall) Mean (RSD%)	18905 (13.0%)	43.3 (27.3%)	16.2 (59.1%)	8473 (63.0%)	1037 (512%)	307 (53.0%)	25.6 (24.8%)	828 (11.0%)	233 (23%)
TP Mean (RSD%)	15470 (13.0%)	18.8 (5.5%)	9.6 (5.2%)	10573 (32.6%)	1238 (42.0%)	371 (54.0%)	41.7 (26.5%)	1121 (29.0%)	335 (31.0%)
CPA Mean (RSD%)	25779 (2.00%)	78.3 (0.60%)	32.6 (65.8%)	24877 (8.00%)	1228 (4.00%)	307 (26.0%)	25.4 (6.20%)	1034 (1.00%)	301 (12.0%)
CPB Mean (RSD%)	14187 (14.0%)	44.7 (0.10%)	150 (12.0%)	12433 (17.0%)	1189 (2.00%)	160 (10.0%)	13.1 (23.5%)	1081 (2.00%)	466 (2.00%)
90% CI Calculation									
RP1 *vs.* RP2	100.6-149.2	207.5 – 240.3	436 – 2846.7	80.50 – 265.2	89.32 – 114.1	61.23 – 87.01	138.2 – 202.7	71.89 – 81.34	48.02 – 72.73
RP *vs.* TP	74.02 – 90.60	37.75 – 53.92	45.08 – 134.56	103.0 – 221.5	92.19 – 146.5	85.86 – 168.8	135.3 – 198.1	115.1 – 150.27	117.82 – 169.0
RP *vs.* CPA	118.9 – 159.1	137.4 – 258.3	85.1 – 635.9	180.4-703.82	92.1 – 165.3	67.0 – 176.6	76.7 – 135.9	112.3 – 140.2	104.7 – 165.2
RP *vs.* CPB	73.9-76.4	103.9-111.4	1124.7-1312.2	163.5-190.8	115.7-123.4	54.8-61.0	49.8-53.8	129.6-132.9	199.4-209.6

Key: CI – Confidence Interval; η_0 – Zero-shear viscosity; τ_{ROT} – Rotational yield point; η_∞ - Infinite-shear viscosity; S_R – Relative thixotropic area; LVR – Linear Viscoelastic Region; τf – flow point; τ_{OSC} – Oscillatory Yield point; G´- Storage modulus; G´´ – Loss modulus; Green label – Compliant results; Red label – Non-compliant results.

In light of the RP variability, firstly, a statistical comparison between the batches with rather opposite rheology behaviour (RP1 *vs.* RP2) was carried out, see Table 6.20. The main purpose of this analysis was to investigate whether the rheological equivalence between the RP batches can be supported even when a more permissive acceptance criterion is considered. Table 6.20 summarizes the results. It is important to note that a direct application of EMA criteria – "the 90% confidence interval for the difference of means of the test and comparator products should be contained within the acceptance criteria of +/-10% of the comparator product mean, assuming a normal distribution of data (EMA, 2018b)" – does not apply to any of the considered endpoints. There was a difference of more than 10% between the rheological endpoints attained with the RP batches, and there was a lack of compliance with the 90-111% confidence interval. Based on these results, and similarly to the published work by Maria Pleguezuelos-Villa, a larger criterion [75-133%] was selected to assess rheological equivalence. Nevertheless, even when considering this broader criterion, solely equivalence

pertaining to the LVR was sustained (Table 6.20). Although borderline results were obtained for the η_0, τ_F and G´ endpoints, the statistical analysis is consistent with the obtained rheograms. Taking this information into account, equivalence pertaining to the rheology profile between the RP itself cannot be inferred.

When comparing the RP *vs*. the TP, equivalence is not registered for any of endpoints. The same scenario is observed with the RP *vs*. CPA formulation. On the other hand, for the CPB formulation, 3 endpoints proved to be equivalent towards the RP - τ_{ROT}, LVR and G. Although this formulation is not qualitative equivalent, the rheological profile is within the RP range, therefore it is with no surprise that some endpoints fit the 75-133% criteria.

Taking all the results into account, the comprehensive rheological analysis herein described suggests that rheologically, none of the formulations can be categorically considered as equivalent.

Facing the rheological variability herein registered, product performance (IVRT) was evaluated for all products, see Fig.6.12. A careful analysis of the release profiles suggests that the impact of these rheological differences is not perceptible, except when addressing the CPB formulation, which corresponds to the bifonazole product with distinct qualitative composition.

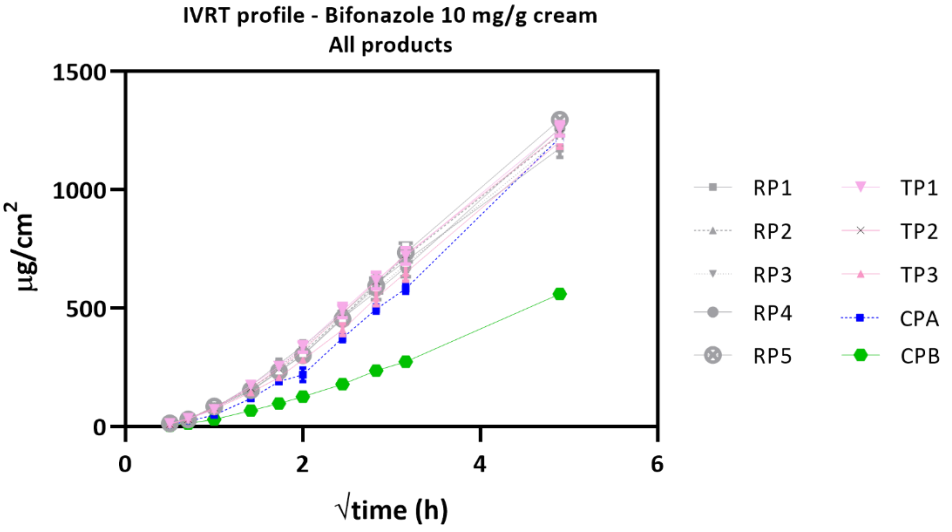


Fig.6.12 – IVRT profiles of all bifonazole products. Results report to n=12 mean ± SEM. For the RP 5 batches were considered, for TP 3 batches, and for the comparator formulations (CPA and CPB) solely one batch was considered.

The majority of the products exhibits overlapping release profiles, with the sole exception of CPB, which displays lower drug release. Nevertheless, this formulation in rheological studies

revealed an intermediate viscosity profile of those presented by the RP, please see Fig.6.12. This fact sustains that rheological differences for bifonazole cream 10 mg/g are not indicative of significant differences in product performance. On the other hand, differences in the qualitative composition, especially when contemplating thickening agents, highly influenced the release profile, thus proving the discriminatory capacity the IVRT method.

Correlation between qualitative composition and IVRT outputs was already described by Goebel and collaborators, who investigated diclofenac diethylamine *in vitro* release from gel formulations (Goebel *et al.*, 2013). In their research, four approved generic products, with Q1/Q2 differences and probably different manufacturing methods, were compared towards the RP. Their results demonstrated that solely one CP, with closer Q1/Q2 profile to the RP, revealed a similar release profile. The formulation with an additional co-emulsifier, cetostearyl alcohol, resulted in lower drug release compared to the reference formulation (Ethier *et al.*, 2019; Goebel *et al.*, 2013).

In the present study, all bifonazole products have cetostearyl alcohol as an emulsifier agent, nevertheless CPB presented several excipients with an emollient function, namely white vaseline and mineral oil. Moreover, this product also presented an additional emulsifier agent – polyoxyethylene stearate 40. This excipient by contributing to an increase of the formulation viscosity may lead to a reduced API release, and consequently to a lower IVRR (Ethier *et al.*, 2019; Rowe *et al.*, 2012).

Despite these observations, compliance with the 90-111% requirement, is what is key as *per* EMA draft guideline criteria.

Following the rationale presented during rheology studies, the 90% CI pertaining to the IVRR were calculated for each RP combination, please see Table 6.21. The results clearly present that if EMA criteria to IVRT endpoints has to be applied, several batch to batch pairwise comparisons would fail to meet the confidence interval requirements [90-111%].

Table 6.21 – 90% confidence interval calculated regarding the IVRR of bifonazole for all RP batch combinations (24 h).

IVRR (µg/cm²/√t) – 90% CI (%)					
	RP1	RP2	RP3	RP4	RP5
RP1		97.9-112.1	93.2-112.3	97.0-113.1	102.0-117.2
RP2	89.2-102.2		90.4-105.5	94.5-105.8	99.6-109.2
RP3	89.0-107.3	94.7-110.6		93.9-111.5	98.6-115.6
RP4	88.4-103.1	94.6-105.8	89.7-106.4		98.35-110.6
RP5	85.4-98.2	91.5-100.4	86.5-101.4	90.4-101.7	

Key: Green label – Compliant results; Red label – Non-compliant results.

Nevertheless, when pooling all 5 batches of the RP and comparing them with the CPA and TP product performance equivalence is registered. This however, as expected, is not registered for CPB see Table 6.22.

Table 6.22 – 90% confidence interval calculated regarding the IVRR and the total cumulative amount of drug released µg/cm² at the end of the IVRT study (24 h).

	IVRR T/R (µg/cm²/√t) 90% CI	Total cumulative amount T/R (µg/cm²) 90% CI	Acceptance criteria	Status
RP *vs.* TP	96.3 – 103.2	96.6 – 103.4	Within 90-111%	C
RP *vs.* CPA	90.7 – 100.3	93.7 – 104.2		C
RP *vs.* CPB	41.0 – 45.8	42.5 – 47.8		NC

Key: Green label (C) – Compliant results; Red label (NC) – Non-compliant results.

Since creams are complex dosage forms that require product efficacy equivalence demonstration, a batch of the RP as well as a batch of the TP should be selected to pursue to IVPT studies. Even though IVRT results were satisfactory, rheological differences are known to affect the IVPT profile. In this context, two different RP batches were selected and solely one TP batch was selected, as TP batches presented similar rheological profiles, as well as *in vitro* profiles. The RP batches selected were RP4 (high, but not "extreme" viscosity) and RP1 (low viscosity). TP1 was selected since low RSD (%) values were attained with this batch during IVRT/rheology studies (data not shown).

6.3.3B Product efficacy profile – IVPT kinetic studies

Following EMA guideline as well as FDA acyclovir draft guidance, product efficacy equivalence should be sustained for topical products with a complex microstructure, such as creams. IVPT studies are required for this purpose. The *in vitro* permeation profile of a formulation can be of value in change control during product life cycle management, but its importance as a kinetic test to demonstrate equivalence is irrefutable (Abd *et al.*, 2016; EMA, 2018b; FDA,

2016c; Ilić *et al.*, 2021). As the *stratum corneum* is the primary limiting barrier to dermal absorption, the determination of the IVPT profile using human skin closely resembles *in vivo* conditions (Abd *et al.*, 2016; Franz, 1975; Leal *et al.*, 2017).

As previously mentioned in Part A, the timeline concerning the development of a discriminatory IVPT method should include the performance of a pilot study to validate the experimental conditions, followed by the pivotal IVPT experiment. The following aspects should be closely considered when developing a suitable IVPT test: (i) human membrane characteristics, membrane preparation techniques, skin integrity evaluation methods, and respective acceptance criteria; (ii) choice of receptor medium, which should comply with sink conditions. Although the use of cosolvents is discouraged by the FDA, according to the EMA, their use may be justified given that skin integrity is not compromised. In this work due to limited permeability of bifonazole, a PBS-PEG (60:40, v/v, pH= 7.4) solution was used as a permeation medium. The solubility of bifonazole in this medium is 3.62 mg/mL, and the highest concentration of the API did not exceed 1/10 of this value; (iii) selection of suitable sampling points regimen, capable of presenting a meaningful permeation profile; (iv) selection and description of formulation dosing techniques. IVPT studies should be performed under finite dose conditions and a homogeneous spreading of the product over the skin should be ensured and finally; (v) other parameters should also be verified such as the absence of contamination and/or interferences, randomization and blinding procedures following ICH E8 criterion, validation of suitable analytical procedures for drug quantification, documentation of API stability over the IVPT study timeframe, as well as mass balance studies. Following IVPT method development studies, a pilot study should be performed to further confirm the suitability of method parameters (see Fig.6.13 and Table 6.23).

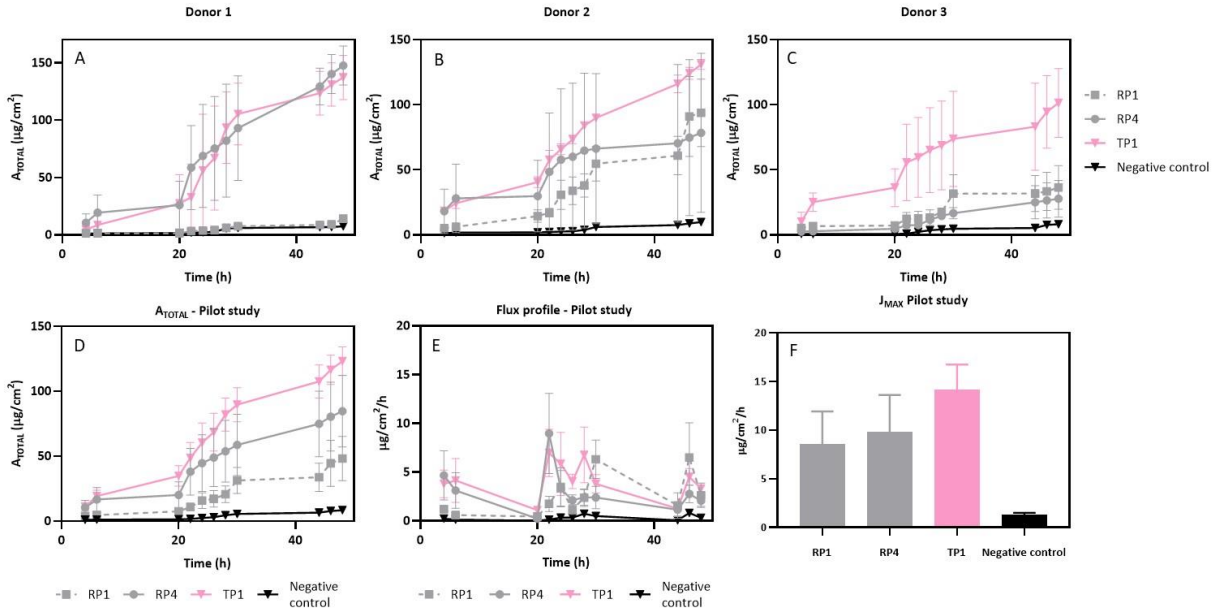


Fig.6.13 – A: Permeation profiles for donor 1 in pilot IVPT studies. B: Permeation profiles for donor 2 in pilot IVPT studies. C: Permeation profiles for donor 3 in pilot IVPT studies. D: Overall permeation profiles for bifonazole in pilot IVPT studies. E: Overall bifonazole flux profiles attained during IVPT pilot studies. F: Overall diclofenac J_{MAX} attained during IVPT pilot studies. All results report to mean ± SEM (n=2, meaning 2 replicates per donors).

Table 6.23 – Pilot study skin integrity and mass balance results. Three donors and two replicates were considered for pilot study.

	RP1						RP4						TP1						Negative control formulation					
Donor	**1**	**1**	**2**	**2**	**3**	**3**	**1**	**1**	**2**	**2**	**3**	**3**	**1**	**1**	**2**	**2**	**3**	**3**	**1**	**1**	**2**	**2**	**3**	**3**
Initial TEWL ($g/m^2/h$)	0.00	0.00	5.20	0.00	0.00	0.00	13.7	14.3	11.5	12.3	14.9	12	12	12.9	11.5	14	7.10	16.0	17.3	17.6	5.9	11.8	5.5	11.9
Final TEWL ($g/m^2/h$)	15.8	8.50	21.5	12.8	12.1	0.00	23.5	15.2	12.3	21.7	15.3	15.3	12	21	22	15.4	10.0	14.9	15.2	18.6	9.6	15.3	19.2	15.3
Acceptance criteria	TEWL < 20.0 $g/m^2/h$																							
Donor compartment (µg)	15.6	13.8	2.38	3.97	51.7	14.2	1.80	17.9	34.2	3.1	14.9	16.3	5.47	0.28	4.43	1.39	19.7	1.5	9.77	11.6	10.7	6.71	18.7	19.0
Skin (µg)	59.6	18.7	2.34	20.8	6.96	62.1	5.00	2.60	30.8	3.7	32.2	25.3	2.47	4.00	13.4	5.64	8.27	8.82	12.8	16.6	15.7	31.3	13.4	24.7
Mass balance (%)	93.0	41.8	51.1	109	98.6	99.3	117	111	92	115	94	110	91.4	115	103	105	97.5	108	91.0	105	90.4	99.4	108	109
Acceptance criterion	90-110%																							

Key: Green label – Compliant results; Red label – Non-compliant results.

In this preliminary assessment, skin from 3 donors was used. Two replicates were always considered for each donor. High intra and inter donor variability was registered, as depicted in Fig.6.13A-C. In donor 1, RP4 and TP1 showed similar permeation profiles, whilst RP1 and the negative control formulation presented a lower bifonazole permeation into and through the skin. In donor 2 and 3, however, the RP batches revealed a closer permeation profile, while TP1 and the negative control formulation displayed a superior and inferior permeation, respectively. Overall, the results of the IVPT pilot study revealed that TP1 exhibited superior permeation, followed by the RP4, RP1 and then the negative control formulation (Fig.6.13E-F).

Based on the pilot studies results, the following conclusions can be drawn:

- The experimental procedures adequately described the cutaneous pharmacokinetics of bifonazole since the maximal rate of absorption is achieved, followed by a decrease.
- IVPT method sensitivity is demonstrated since according to the obtained flux profiles, indicating that the method was able to detect changes in the permeation profile between formulations of different strengths.
- Overall, the mass balance results were compliant with the established criterion (Table 6.23). Nevertheless, it should be noted that meeting the 90-110% EMA acceptance criterion can be extremely difficult due to the need to perform IVPT under finite conditions requiring very small amounts of the formulation. Non-compliance with this requirement was observed for donor 1 (1 replicate of RP1 and TP1, and both replicates for RP4), as well as for donor 2 (a single replicate of RP1 and RP4).
- Regarding the skin integrity results after IVPT experiments, all membranes were checked for leakage and none was observed. Although values of more than 20 $g/m^2/h$ were obtained in some diffusion cells, these results were borderline and did not correspond to higher fluxes of A_{TOTAL} (numerical data not shown). In this context, the usage of PEG as a cosolvent did not affect the integrity of skin barrier throughout the study timeframe.

Since the number of replicates and donors considered in this preliminary assessment was relatively low and give the high inter and intra-donor variability observed, no statistical analysis of the results was performed. Taking all data into account, overall the proposed conditions serve the purpose to stablish pivotal study conditions.

For the pivotal studies, skin from 9 donors was used to comply with EMA guideline criteria (3 donors in pilot studies + 9 donors in pivotal studies = 12 donors). Similarly to the pilot study design, 2 replicates were always considered. Permeation and flux profiles are presented in Fig.6.14. Furthermore, mass balance study results are presented in Table 6.24.

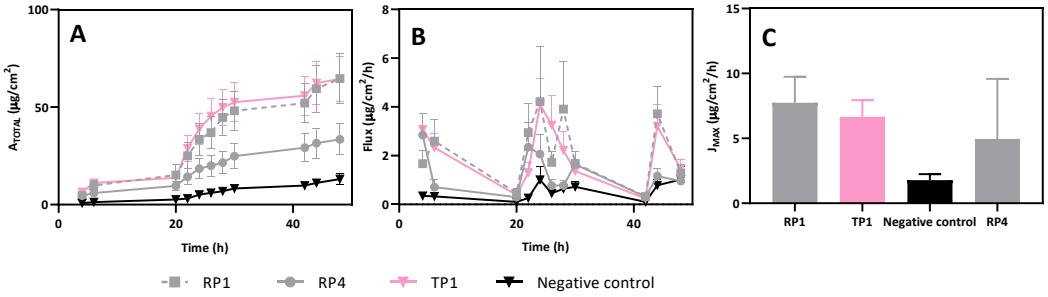


Fig.6.14 – A: Permeation profiles for all tested formulations in pivotal IVPT studies. B: Flux profiles attained during IVPT pivotal studies. C: Maximum flux attained during IVPT pivotal studies. Results report to the mean ± SEM calculated from duplicate sites from the same donor. RP1 = 8 donors; RP4 = 9 donors; TP = 9 donors; Negative control formulation = 4 donors. 2 replicates per donor were always considered.

Table 6.24 – Skin integrity and mass balance results for bifonazole 10 mg/g cream pivotal study results.

Donors	4	4	5	5	6	6	7	7	8	8	9	9	10	10	11	11	12	12
RP1																		
Initial TEWL (g/m^2/h)	0.00	6.70	0.00	6.90	2.50	6.90	9.90	4.20	0.00	0.00	N.D	N.D	0.00	0.80	2.90	2.30	0.00	6.50
Final TEWL (g/m^2/h)	10.4	13.4	15.2	12.0	15.8	6.60	21.0	14.3	15.9	14.3	N.D	N.D	4.30	5.60	8.60	15.2	21.0	14.3
Donor compartment (µg)	31.7	30.5	23.7	8.6	0.94	76.7	15.0	55.3	77.9	2.25	N.D	N.D	15.5	0.24	22.3	32.7	12.6	62.6
Skin (µg)	30.7	45.7	44.8	7.9	0.52	7.75	19.1	7.71	9.46	5.59	N.D	N.D	35.7	0.38	25.7	47.0	3.36	25.3
Mass Balance (%)	103	103	87.3	62.9	113	111	93.5	106	103	105	N.D	N.D	100	111	111	93.4	83.9	108
RP4																		
Initial TEWL (g/m^2/h)	7.00	7.00	8.6	17.4	8.90	8.60	9.90	2.60	2.50	14.2	12.3	8.8	4.40	5.10	2.60	9.20	4.20	4.70
Final TEWL (g/m^2/h)	12.9	11.5	0.00	9.00	11.8	15.6	21	20.6	13.6	24.6	22.7	15.4	4.10	8.66	4.30	5.00	0.00	9.30
Donor compartment (µg)	41.15	63.77	16.9	12.8	12.4	18.3	15.0	64.2	7.72	23.0	21.3	8.28	2.13	61.8	6.38	23.0	23.6	48.4
Skin (µg)	5.81	12.33	53.3	67.0	51.2	31.0	19.1	11.6	11.8	41.3	39.9	59.4	7.31	16.4	5.08	16.4	33.5	13.1
Mass Balance (%)	112	110	90.0	90.2	91.7	68.7	93.5	110	111	103	91.6	73.8	96.2	95.6	52.7	102	73.3	110
TP1																		
Initial TEWL (g/m^2/h)	2.80	2.30	6.00	0.00	9.40	7.00	14.0	9.50	4.90	10.0	11.0	9.60	8.00	0.00	17.9	9.30	11.5	25.6
Final TEWL (g/m^2/h)	2.80	12.0	0.00	14.0	11.4	9.90	18.8	18.5	12.1	12.2	14.5	12.2	18.5	0.00	21.0	15.0	25.6	14.7
Donor compartment (µg)	42.2	40.7	41.3	40.7	4.68	42.3	5.76	5.06	3.39	44.5	11.9	44.3	1.63	50.6	5.36	4.20	1.21	7.94
Skin (µg)	18.7	14.2	18.7	48.4	7.26	11.8	52.4	11.8	17.0	21.7	72.0	18.7	10.3	15.7	1.73	6.93	3.21	2.65
Mass Balance (%)	91.4	108	90.0	103	112	103	117	59.6	102	90.9	93.8	109	108	94.5	107	97.3	78.9	110
Negative control formulation																		
Initial TEWL (g/m^2/h)	14.5	12.1	0.00	18.4	27.3	22.1	22.4	20.4	10.1	9.20	9.80	22.6	4.40	5.10	6.60	9.20	4.20	4.70
Final TEWL (g/m^2/h)	23.8	20.5	9.90	6.70	8.00	0.00	17.8	26.9	14.2	10.4	15.4	17.2	26.2	19	12.3	40.2	8.60	7.90

Donors	4	4	5	5	6	6	7	7	8	8	9	9	10	10	11	11	12	12
RP1																		
Donor compartment (µg)	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	11.9	N.D	10.1	9.42
Skin (µg)	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	14.8	N.D	18.7	19.3
Mass Balance (%)	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	112	N.D	94.4	99.7

Key: Green label – Compliant results; Red label – Non-compliant results. N.D. – Not determined.

As confirmed in the pilot studies, the developed method proved to be sensitive, being able to detect changes as a function of differences in drug delivery. According to a workshop hosted by the FDA, IVPT sensitivity studies should solely be performed during IVPT method development, in order to shed light into the method discriminatory capacity. On the other hand, EMA requires that IVPT method sensitivity be demonstrated during both pilot and pivotal studies (Raney, 2021b). In fact, marked differences were denoted between the nominal strength formulations and the negative control formulation, as the 5 mg/g cream bifonazole formulation solely registered permeation for a single donor. Based on these results, however, it was not possible to calculate a CI to numerically express inequivalence.

Mass balance results were generally compliant with the established criteria (please Table 6.24), however, for some skin pieces, the extraction procedures did not meet the prescribed values. The evaluation of drug concentration in heat-isolated epidermis samples is challenging, due to several reasons: (i) interference coming from the biological matrix; (ii) need for a very sensitive analytical method and; (iii) need to perform IVPT studies under finite dose conditions. These reasons, combined with the strict 90-110% recovery criteria by EMA, contributed to this occurrence (Demurtas *et al.*, 2020; European Medicines Agency, 2018).

Overall, TEWL results were compliant with the 20 $g/m^2/h$ threshold. Nevertheless, some borderline results were registered at the end of the IVPT experiment in some skin pieces. These however, were not correlated with an enhanced permeation rate. Moreover, no leaks were observed in the excised human membranes. Solely donor 9 tested with the RP1 formulation displayed in the beginning of the IVPT uncompliant TEWL results, which led to the exclusion of these results.

The comparative permeation and flux profiles are presented in Fig.6.15.

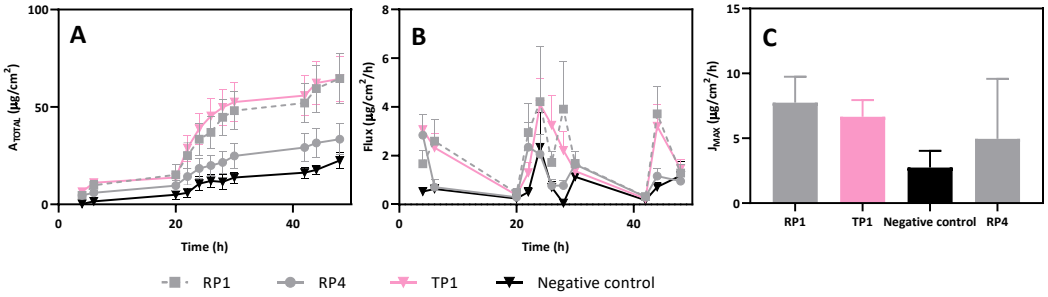


Fig.6.15 – Permeation profiles for all tested formulations in pivotal IVPT studies. B – Flux profiles attained during IVPT pivotal studies. C – Maximum flux attained during IVPT pivotal studies. Results report to the mean ± SEM calculated from duplicate sites from the same donor. RP1 = 8 donors; RP4 = 9 donors; TP = 9 donors; Negative control formulation = 4 donors. Two replicates per donor were always considered.

Fig.6.15A shows that the permeation profile of RP1 resembles the one attained with TP1, nevertheless differences between the RP batches are evident.

Despite the IVRT profiles of these two RP batches are equivalent according to FDA standards (Table 6.10), their rheological behaviour was statistically different, with RP1 exhibiting a more fluid behaviour when compared to RP4 (Fig.6.15). For this reason, the viscosity profile of RP1 was more similar to that of the TP. These rheological differences may have played a role in the permeation profile, as the RP batch with more structured consistency (RP4) displayed lower permeation when compared to the less viscous one (RP1).

The statistical analysis of the IVPT pivotal test is summarized in Table 6.25. As IVPT data does not follow a normal distribution, they should be log transformed prior to any calculation. Two statistical approaches were considered to analyze the permeation results: the EMA and the FDA. Both approaches advise a paired comparison, in which the differences between the TP and RP, in permeation endpoints, should be individually calculated for each donor.

In the European approach, the variability within subjects is calculated as the difference between each individual T-R and arithmetic mean of the two replicates per donor, per formulation. On the other hand, the FDA follows a scaled average bioequivalence approach (SABE). A SABE analysis attempts to standardize the difference due to the observed variability in the reference product. To be applicable, the within-subject standard deviation (S_{WR}), calculated for each IVPT endpoint attained with the RP formulation, should be higher than 0.294 (FDA, 2016c; Pensado *et al.*, 2019). According to this approach, bioequivalence can then be inferred if the geometric mean ratio (GMR) falls within the range [0.8, 1.25] for the selected bioequivalence margin and if the upper bound of the 90% confidence interval (SCI_{UB}) for the

quantity, $(\mu_T - \mu_R)^2 - \sigma^2_{WR}\,(\ln(1.25)/0.25)^2$, is less than or equal to zero. μ_T and μ_R regard the population means of the test and reference products, respectively, and σ^2_{WR} refers to the reference population variance (Pensado *et al.*, 2019). The calculations pertaining to the A_{TOTAL} parameter between RP1 and TP1 can be found in Appendix B.

Table 6.25 – 90% confidence interval calculated for J_{MAX} (μg/cm²/h) and A_{TOTAL} (μg/cm²) at the end of the permeation experiment (48 h) for bifonazole cream formulations following EMA and FDA approaches.

		J_{MAX} (μg/cm²/h)	Status	A_{TOTAL} (μg/cm²)	Status	Acceptance criteria
Approach used	Pairwise comparison					
EMA	RP1 vs TP1	69.15 – 98.38	NC	78.06 – 113.55	C	90% CI [69.84 – 143.19]
	RP4 vs TP1	128.15 – 221.97	NC	179.01 – 286.86	NC	
	RP1 vs RP4	38.43 – 73.57	NC	31.07 – 57.25	NC	
FDA	RP1 vs TP1	S_{WR} = 1.091 SCI_{UB} = - 0.2403 GMR = 0.8248	C	S_{WR} = 0.779 SCI_{UB} = - 0.1763 GMR = 0.9414	C	$SCI_{UB} < 0$ $GMR \in [0.8 - 1.25]$
	RP4 vs TP1	S_{WR} = 1.012 SCI_{UB} = - 0.3566 GMR = 1.6866	NC	S_{WR} = 0.899 SCI_{UB} = 0.4744 GMR = 2.2660	NC	
	RP1 vs RP4	S_{WR} = 1.078 SCI_{UB} = 1.9888 GMR = 0.4512	NC	S_{WR} = 0.925 SCI_{UB} = 2.5677 GMR = 0.3919	NC	

Key: J_{MAX} – Maximal flux; A_{TOTAL} – Cumulative drug amount permeated at the end of the IVPT study. RP4 vs TP1 = 9 donors; RP1 vs RP4 = 8 donors. In the EMA approach the 90% CI were calculated based on the geometric mean of the duplicate values obtained per donor. In the FDA approach: SCI_{UB} – upper bound of the 90% confidence interval; A_{TOTAL} and J_{MAX} are reported as the anti-logarithm of the arithmetic mean (lower-upper 90% confidence interval) of the natural log-transformed values; Green label – Compliant results; Red label – Non-compliant results.

According to EMA, a wider 90% confidence interval, up to a maximum of 69.84 – 143.19, may be accepted when high variability is observed with low strength and limited diffusion drug products. In this context, according to Table 6.25, compliance is registered with the A_{TOTAL} endpoint in the RP1 *vs.* TP1 pairwise combination. Nevertheless, a borderline, but still uncompliant result is attained for J_{MAX} in the same products. All the remaining product comparisons failed to document bioequivalence, even the one portraying the different RP batches. Therefore, according to the European criteria, none of the products permeation profile can be regarded as bioequivalent. This scenario is slightly different when addressing the FDA approach, where RP1 *vs.*TP1 can be considered as bioequivalent, but RP4 *vs*. TP1 and RP1 *vs*. RP4 continue to present inequivalent results.

According to these results, the selection of RP batches is not irrelevant, especially whenever involved in a topical generic product R&D program aiming at an abridged bioequivalence demonstration. Furthermore, the statistical approach to follow is also of outmost importance.

6.4B Concluding Remarks

In this work, we have investigated both the experimental procedures and regulatory mechanisms underlying the bioequivalence assessment of bifonazole 10 mg/g cream formulations.

According to European and American regulatory agencies, semisolid dosage forms that exhibit a complex microstructure, such as creams, should present Q1-Q4 equivalence, in addition to local availability assessment. In order to comprehensively address several scenarios that may occur in daily practice, an initial sample of RP batches was considered, together with a Q1/Q2 formulation (TP), a Q1 formulation (CPA), and finally, a bifonazole cream formulation with Q1/Q2 differences (CPB).

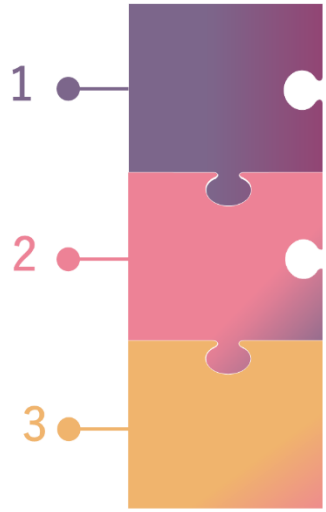
The product microstructure was evaluated in rheological studies. Given the high inter-batch RP variability, the initial pool of RP batches was expanded to obtain a detailed rheological characterization of the product at different lifecycle stages. In this analysis, the equivalence reporting to the RP itself failed to be supported. Not surprisingly, the rheology profiles between the RP and the other products were also not equivalent. The high variability registered in the rheological studies motivated the determination of the release profile for all formulations. Interestingly, the product performance showed equivalent results between the RP and the formulation with the same qualitative composition (TP and CPA). Nevertheless, it should be noted that equivalence was not generally supported when comparing RP batches, according to the EMA requirements.

Enlarging the RP batch pool was then a critical step in establishing equivalence of product performance. Given the results obtained, IVPT studies were carried out using two RP batches with opposite rheological profiles along with TP, since this formulation exhibited Q1 and Q2 sameness. The IVPT results were then analysed according to two statistical approaches – EMA and FDA. Equivalence was registered for RP1 *vs.* TP1 as per FDA requirements. Nevertheless, equivalence for RP4 *vs.* TP1 and RP1 *vs.* RP4 failed to be supported. This highlights that the selection of RP batches for this specific case study is a critical step in documenting bioequivalence of TGP. IVPT equivalence according to the EMA approach was not documented for any of the product comparisons considered. Because the FDA approach accounts for both RP and donor variability, it is more appropriate for IVPT data.

Considering all results, the strategy used here adequately supported bioequivalence. However, depending on the pharmacotherapeutic class of the drug and the complexity of the formulation, this stepwise protocol may not entirely fit all products. Envisioning an enhanced

equivalence assessment, efficacy model-based approaches that reflect the clinical outcomes of the products studied, in this particular case for antifungal activity, could be useful.

6.5 Highlights



Documenting the EPE of TGP is a cornerstone in EMA draft guideline. If equivalence fails to register, RP variability should be evaluated since semisolid dosage forms, especially those that regard multiphasic systems, exhibit intrinsic variability. To better characterize the RP, enlarging the pool of batches can be a reliable strategy.

For the selected case studies – bifonazole cream and diclofenac emulgel – documenting local availability equivalence using EMA equivalence criteria was not possible. Nevertheless, a different scenario was observed when applying the SABE approach which is recommended by the FDA. For both products the within-subject standard deviation was higher than 0.294, which classifies the products as highly variable. For bifonazole products equivalence could be successfully following the SABE approach. On the contrary, for diclofenac products equivalence failed to register even when addressing the criteria by both agencies.

The diclofenac TP used in this study displays an equivalent pharmacokinetic profile towards the RP. Therefore, the observed differences in Q3, Q4 and local availability parameters are not expected to translate into clinically significant differences.

7 CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

In this section, a general overview of the main results is given, together with the main conclusions. Finally, appropriate suggestions for future work are recommended.

Topically applied dosage forms, commonly developed to exert a local action, have been used throughout history for cosmetic and therapeutic purposes. Indeed, they are one of the oldest medicinal dosage forms known to human civilization. Their straightforward administration strengthens patient compliance, besides offering significant therapeutic benefits without systemic side effects. As described in Chapter 5, the dermatological drug delivery market is expected to register a compound annual growth rate of 7% between 2019 and 2024. Despite this performance, innovation in topical drug formulations for skin diseased lagged behind other pharmaceutical product classes. In fact, the regulatory mechanisms underlying the development and approval of topical generic products are complex. As outlined in the introductory chapter, there are several motives that are immediately apparent. However, in our perspective, there are two key aspects that are of critical importance:

- As the skin is the target site of most topical semisolid formulations, undetectable or extremely low amounts of drug can be measured systemically. In this context, it is highly understandable that the gold standard method for establishing bioequivalence of TGP significantly relied on comparative clinical studies, in which the efficacy of the test product is documented if i) it demonstrates superior performance to placebo and ii) if an equivalent efficacy profile to that of RP is achieved. Nonetheless, the clinical response to topical drugs is highly dependent on (patho) physiological factors and product application procedures. Furthermore, the excipients in topical formulations may exert a pharmacological action themselves. All these factors impair the comparative assessment of the clinical profile of a TP towards a RP.

- Multiphasic systems, such as creams, can be considered as highly complex dosage forms, since their quality attributes present a myriad of interdependencies. These are affected by the chemical characteristics of both API and excipients, by the physicochemical properties of the formulation, and also by the manufacturing process itself. All these parameters ultimately influence product performance. Under these circumstances, managing product variability is quite challenging.

In an attempt to stimulate the increase of topical generic options in the market, but at the same time to circumvent the previously appointed singularities, the regulatory recommendations concerning TGP development and approval undergone several noteworthy amendments. As reviewed in this thesis, these have been promoted by a broad range of multidisciplinary initiatives, such as the Strawman decision tree, the topical classification system, as well as by multiple FDA and EUFEPS workshops. The main documents reflecting the efforts of all these meetings are the FDA non-binding product specific draft guidelines, as well as the EMA draft guideline on quality and equivalence of topical products.

Although these guidelines are primarily aimed at promoting the acceptance of *in vitro*/*in vivo* surrogate methods for topical bioequivalence assessment, there are considerable differences between the recommendations from both agencies. The FDA considers product specificity, while EMA only provides general recommendations that should be adopted on a case-by-case basis. Despite the scope of the EMA, rather strict criteria are established that do not take into account the intrinsic variability characteristics of topical products. This aspect deeply conditions a successful translation of document *per se* into practice.

Against this background, the objective of this thesis was to provide a comprehensive insight into the implications of the intended regulatory directives. Nevertheless, since there are countless particularities and consequently numerous challenges at all stages of the current topical BE establishment, the work herein presented specifically aimed to provide a framework addressing the development and validation of rheological, IVRT and IVPT methods. Additionally, an effort was made to propose solutions able to circumvent some of the observed regulatory constraints.

Specifically and in summary, the main achievements of this thesis are:

Proposal of a practical approach to develop and validate the acquisition of the rheological profile of a semisolid dosage form (Chapter 3).

As part of this approach, rheometer qualification studies procedures were carried out, alongside with the definition of critical operational parameters, carefully selected through a

risk assessment analysis, as well as identification of suitable critical analytical attributes. Furthermore, the importance of an adequate documentation of the rheological method precision, selectivity and sensitivity is overall demonstrated. Our results indicate that geometry configuration, sample application mode and temperature are critical method variables that should be carefully optimized during rheology pilot studies. For the selected model formulation – a hydrocortisone cream – the rheological endpoints that proved to have an enhanced discriminatory capacity pertained to the thixotropic relative area, oscillatory yield point, flow point, as well as viscosity related endpoints. Nevertheless, it is important to fine tune the most suitable endpoints according to formulation technological features.

Within this framework, an actual case study is presented to document the rheological equivalence between a RP and a TP. This example clearly demonstrates the difficulties in directly applying the EMA acceptance criteria to rheological quantitative quality characteristics.

Application of aQbD principles in the development of an IVRT test (Chapter 4).

After the establishment of an analytical target profile through a risk assessment analysis, the critical analytical attributes (*in vitro* release rate, cumulative amount released at initial/final time point and dose depletion) and critical method variables (receptor medium, membrane and dose regimen) were identified. Based on the results of a 3x2x3 factorial design, Tuffryn membranes, PBS:Ethanol release medium (80:20, v/v), and a dose of 300 mg were found to be suitable parameters for establishing the release profile without compromising the discriminatory capacity of the method. These optimal conditions were then adopted during validation studies, which attempted to meet all regulatory requirements (membrane inertness, linearity, precision, robustness and discriminatory power evaluation).

By considering an aQbD approach, the time and cost associated with IVRT method development can be minimized; in addition, it offers a robust and regulatory-oriented platform for method development. The absence of IVRT method development protocols often impairs TGP submissions; consequently, this approach can be a reliable strategy to overcome such deficiencies.

In order to carry out the experimental work based on the EMA draft guideline criteria, a critical and reflected appraisal of this document had to be held. This analysis revealed that setting criteria for a 90-111% confidence interval, a 10% variation coefficient, and achieving a 70% release may not be practical for many topical drug products. Moreover, the inclusion of the IVRR as a product CQA requires the performance on a daily routine basis of IVRT tests, which

might prove to be too demanding for generic manufacturers. These observations formed the basis for the study design presented in Chapter 5.

Characterization of topical products batch-to-batch variability (Chapter 5).

Although both manufacturers and regulators actively strive for negligible batch-to-batch differences, there are still products where batch variability is strongly perceived. In this chapter, a panel of 8 reference blockbuster semisolid topical products, with three batches each, was characterized in terms of globule size, pH, rheological attributes and *in vitro* performance.

According to EMA criteria, all investigated RPs revealed marked batch-to-batch differences, which *a priori* compromises the documentation of extended pharmaceutical equivalence. Based on the observed results, there is an evident need to establish reasonable microstructure sameness criteria, which account the intrinsic variability of the RP being studied. Expanding the criteria for statistical acceptability of Q3/Q4 endpoints for highly variable products is a key point for successful implementation of the EMA draft guideline in practice.

Bioequivalence assessment flowchart proposal (chapter 6).

This chapter attempts to draw attention to an effective iterative search to determine the most appropriate strategy for evaluating topical bioequivalence on a case-by-case basis. For this purpose, three case studies were considered – dimethindene maleate 1 mg/g gel, bifonazole 10 mg/g cream and a diclofenac 20 mg/g emulgel, in an attempt to address a wide range of formulations with distinctive technological features, as well as targeting sites. The RPs for these formulations were compared with commercially available generic/comparator products or alternatively with test products. All methods used in this chapter were validated according to the rationales described in Chapters 3 and 4.

The dimethindene formulation embodied a simple formulation. Despite equivalence pertaining to Q4 was established, high variability was observed for some rheology endpoints, especially for the different RP batches. Therefore, equivalence could not be established for Q3 as per EMA requirements. In this context, it is important to determine if there are some rheology endpoints that can be waived, and if there is a possibility to establish reasonable criteria that are overall feasible for generic manufacturers and at the same time safe for the patient.

The bifonazole cream is a biphasic semisolid system with higher technological complexity, when compared to the dimetindene formulation. As greater rheological variability was

observed in the RP, the initial pool of RP batches was strategically enlarged to enable a more detailed characterization. Nevertheless, the actual impact of these rheological differences on product performance appeared to be negligible, as the IVRT comparative results (RP *vs.* TP) successfully determined Q4. Product efficacy studies were then conducted and the resulting data evaluated according to the EMA and FDA approaches. Even though the products were considered equivalent when applying the FDA scaled average bioequivalence assessment (SABE) criteria, the same scenario was not found when the EMA guideline was applied.

Finally, for the diclofenac formulation, a highly complex product, equivalence pertaining to rheology was not established. In terms of product performance, equivalence was only found for some batch combinations and when a broader acceptance criterion (75-133%) was applied. The IVPT studies also failed to demonstrate equivalence. Nevertheless, since the generic product used in the present study displayed an equivalent pharmacokinetic profile to the RP, the observed differences in Q3, Q4 and local availability parameters are not expected to translate into clinically significant differences.

The strategy herein considered can be summarized in the next decisional flowchart, please see Fig.7.1.

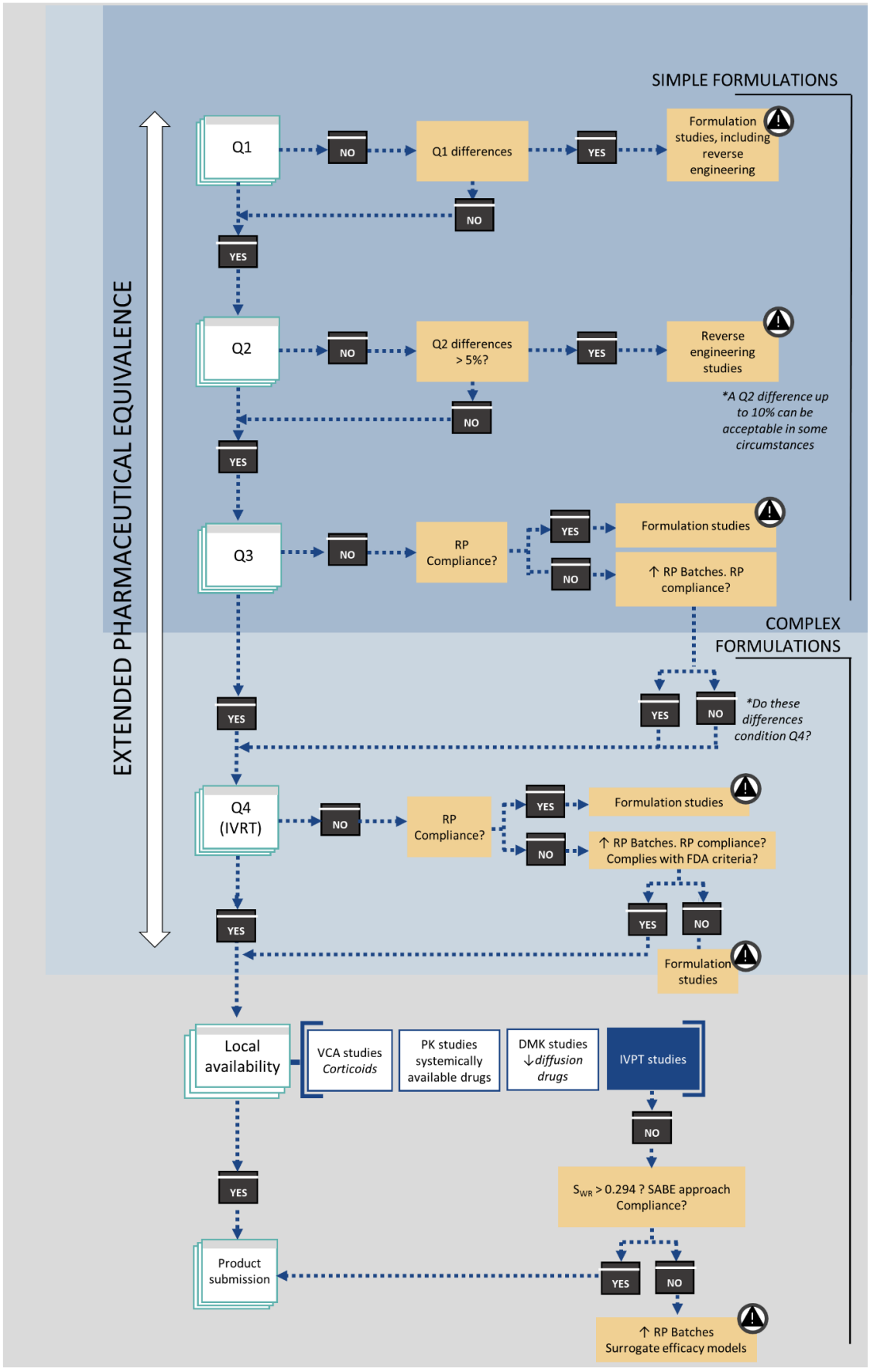


Fig.7.1 – Proposed bioequivalence assessment flowchart.

For simple formulations that predominantly target the skin surface, such as the dimetindene maleate 1 mg/g gel formulation, BE can be sustained by establishing Q1, Q2, Q3 and Q4 equivalence. If equivalence cannot be registered in terms of microstructure or performance parameters, RP variability should be adequately characterized to establish an adequate number of batches, as well as reasonable acceptance criteria.

Regarding more complex formulations, such as creams or emulgels, the same procedure must be applied. Nevertheless, for these products, equivalence regarding product efficacy must also be sustained. For that, IVPT studies should be performed. If equivalence is not registered, the variability of the RP should be determined by evaluating if S_{WR}>0.294. Under these circumstances, the pool of batches may be increased. The strategy herein employed properly supported bioequivalence for the bifonazole cream formulation. However, depending on the pharmacotherapeutic class of the drug, as well as formulation complexity, this stepwise protocol may not entirely fit all products, as portrayed in the diclofenac emulgel case study. Envisioning an enhanced equivalence assessment, efficacy model based approaches reflecting the clinical outcomes of the studied products, may be of value.

Overall, in our opinion, to be able to consider a biowaiver from clinical endpoint studies for topical generic products, further work and discussion with EMA are required. The document in its current form does not take into account the intrinsic variability of these products, since the statistical criteria regarding microstructure, performance and efficacy parameters fail to register even when only addressing the RP. In this work, we intended to develop a general framework that aims to surpass some of the limitations of the draft guideline. This approach should be extended to other topical products and lay the foundation for validation procedures needed to ensure the appropriate selection of product-specific bioequivalence assessment protocols.

7.2 Future work

The studies performed in the present thesis provided new insights into the field of topical bioequivalence, and as might be expected, each conclusion laid the groundwork for addressing new challenges. Furthermore, the state-of-the-art workshops hosted by the FDA in the past year, have been systematically emphasizing the regulatory need to propose development and validation strategies addressing all methods involved in Q3- local availability equivalence demonstrations. Altogether, the knowledge gathered throughout this thesis raised even more questions. The next paragraphs attempt to summarize the main areas where further research would be valuable.

Development of methods to determine particle size and particle size distribution and respective validation procedures.

Monitoring the particle size in semisolid formulations may be extremely challenging. As previously explained throughout Chapter 4, image analysis by manual microscopy is the most direct method for assessing particle size and morphology due to the very limited sample preparation procedures. Nevertheless, in this thesis, no formal development and validation strategies concerning microscope-based technique specially tailored for topical products were explored. Due to increasing regulatory pressure to include this parameter as part of the quality specification for stability, as well as a CQA for formulation, the development of suitable validation procedures is a point of concern that frequently impairs topical generic drugs submission and approval procedures. Even though the FDA guidance "Technical Performance Assessment of Digital Pathology Whole Slide Imaging Devices" provides some insight, more direct procedures specific to topical products are needed. Moreover, due to the extremely laborious nature of this technique, it would be useful to explore surrogate methods such as the automated microscopy and imaging of topical products, as this technique would largely reduce operator variability and considerably improve statistical robustness.

Development of methods concerning evaluation of the drug physical state within the formulation and respective validation procedures.

As discussed in Chapter 4, the API may be in solid or dissolved state, or both, depending on drug solubility. For complex semisolid products containing suspended actives, such as the acyclovir formulation studied in the above mentioned chapter, the ratio of dissolved to suspended API is expected to influence skin permeation, especially for products applied to diseased skin. Furthermore, suspended actives and emulsion globules are both prone to change over the shelf life of the product, thus conditioning the stability profile. Even though no permeation experiments with this product were performed, we were able to conclude that the API appeared to be essentially dissolved into the lipid matrix as demonstrated by DSC experiments, although rectangular drug crystals were observed microscopically. For this product, statistical differences regarding particle/droplet size had an impact on the release profile, while these did not seem to affect the rheology profile. It is important to further investigate the relationships between drug crystallization – viscosity profile – release and permeation mechanisms. Furthermore, it would be beneficial to evaluate these parameters together at the different lifecycle stages of the product. As documented in the bifonazole cream rheological studies, the microstructure of a topical product is prone to change during

its shelf-life. Therefore, further studies are needed to document the actual impact of these differences on the efficacy profile of the product.

Similar to particle size analysis, the development of a validation strategy concerning the DSC analysis were not implemented in this thesis. In the future, it would be of value to simultaneously present the thermograms of the placebo (negative control), as well as those of a sample with the drug crystalized in a formulation vehicle (positive control), in addition to the pure drug. Moreover, the presentation of alternative methods, such as thermogravimetric analysis and X-ray powder diffraction and X-ray diffraction microscopy, to complement the DSC analysis would be useful to provide a more detailed characterization of the API physical state.

As addressed in chapter 2, the API physical state within the formulation is closely linked with product metamorphosis, an event highlighted in the EMA draft guideline. The lack of clarification of the methods required to characterize this phenomenon is a major obstacle for manufacturers of topical generic products. It is important to investigate the CQA of the formulation affecting the product transformation and to find the most useful tools to characterize these CQA throughout the event, in order to study the actual impact on product performance and product efficacy according to the drug saturation degree. For all the above mentioned reasons, more research in this field is highly needed.

Development and validation of pharmacodynamic assays to evaluate product efficacy

As documented in Chapter 6, the 80-125% CI criterion is very difficult to achieve in IVPT studies in many circumstances, even when only different batches of RP are studied. The reasons that explain the observed variability have been extensively discussed in the above mentioned chapter. Nevertheless, under these factual circumstances and given the unwillingness of the regulatory authorities, at least in the near future, to allow a wider acceptance criteria, it would be of great interest to propose and develop pharmacodynamic tests capable of characterizing efficacy profile of products. These would provide an alternative, complementary and product-specific approach to assessing therapeutic efficacy of bioequivalence. Several interesting examples, specially tailored to topical antifungals, such as the TurChub, ChubTur, TurSh and RoMar *in vitro* models, have proven useful for this application. As detailed in the introductory chapter, spectrophotometric methods such as the near-infrared offers appealing research opportunities in this field.

Development and validation of computational tests to evaluate topical bioequivalence

Recently, the FDA accepted a virtual bioequivalence assessment of a diclofenac sodium topical gel (1%). Although this study was accepted alongside with Q1, Q2, and Q3 similarity documentation to the reference product, as well as an *in vivo* bioequivalence study with pharmacokinetic endpoints, the importance of computational studies is expected to increase in the upcoming years. This case study, by proposing good practices for model verification and validation, when intending a physiologically-based pharmacokinetic modelling simulation, is highly relevant under a regulatory point of view.

To conclude, as science and regulatory policy do not exist in vacuum, every stakeholder – academia, industry and regulatory agencies – have a role to play. In general, this thesis contributes to a broader comprehension of the regulatory limitations that still have to be addressed when establishing bioequivalence of topical generic products.

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APPENDIX A

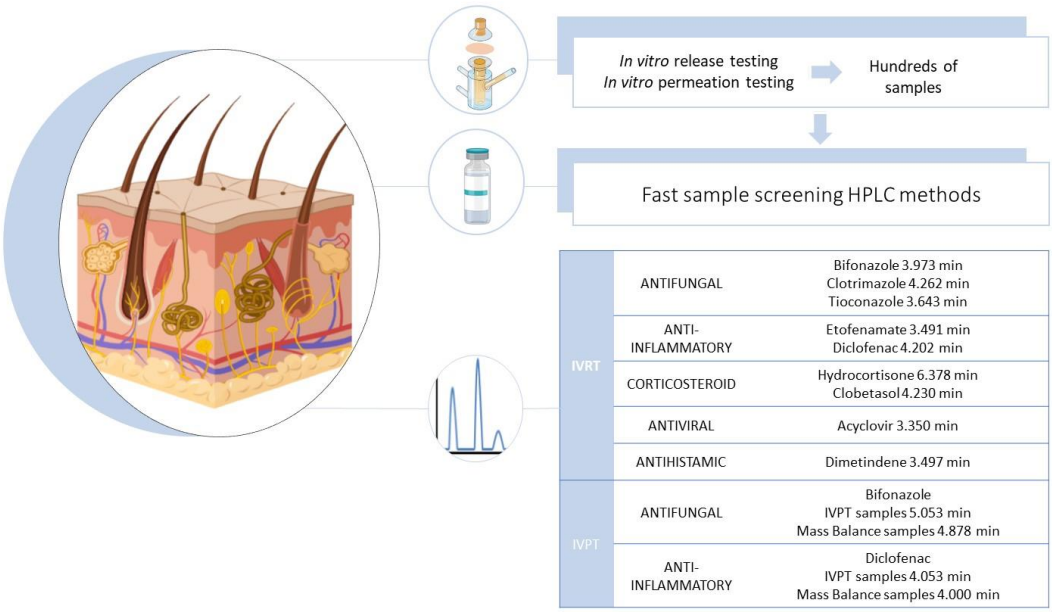

Fig. A1.1 – Graphical abstract: Appendix A1.

This appendix has been adapted from the following publications:

Miranda, M., Cardoso, C., Vitorino, C. 2020. "Fast Screening Methods for the Analysis of Topical Drug Products." Processes 2020, Vol. 8, Page 397 8 (4): 397. https://doi.org/10.3390/PR8040397.

Miranda, M., Veloso, C., Brown, M., Pais, A.A.C.C., Cardoso, C., Vitorino, C. "Topical bioequivalence: Experimental and regulatory considerations following formulation complexity" (submitted manuscript).

Tailoring bioequivalence – A topical antifungal case-study (manuscript in draft).

M.M and C.V conceived the idea and established the research design and implementation. M.M performed the experimental parts of the work and wrote the first draft of the manuscript. Supervision, resources, data curation, review and editing was provided by C.V. Funding acquisition was provided by C.C and C.V.

Introduction

Considering the recent regulatory requirements, the overall importance of IVRT/IVPT methods regarding topical product development is undeniable, especially when addressing particulate systems. For each IVRT/IVPT study, several hundreds of samples are generated. Therefore, developing rapid reversed-phase high-performance liquid chromatography (RP-HPLC) methods, able to provide a real-time drug analysis of IVRT/IVPT samples, is a priority. This will avoid stability issues and provide timely evaluation as well as real-time assessment of specification conformity. From a quality control perspective, developing rapid HPLC methods for IVRT samples can facilitate routine decision making, especially when out-of-trend (OOT) or out-of-specification (OOS) results occur.

Taking this information into account, the first part of the present appendix presents a framework for the selection of an appropriate IVRT medium, based on active pharmaceutical ingredient (API) physicochemical characteristics. Furthermore, a partial validation of the analytical methods developed for Chapters 4, 5, and 6 is herein presented. The second part of the appendix addresses a partial validation of the analytical methods developed for the IVPT/mass balance samples processed in Chapter 6.

Appendix A1

A1.1 Materials and Methods

A1.1.1 Materials

Bifonazole, clotrimazole, tioconazole, etofenamate, sodium diclofenac, clobetasol propionate, micronized hydrocortisone, acyclovir and dimetindene maleate, were kindly supplied by Laboratórios Basi. Water was purified with a Millipore MILLI-Q reagent water system and filtered through a 0.22 µm nylon filter before use. All other chemicals were of analytical grade or equivalent.

A1.1.2 Methods

A1.1.2.1 Instrumentation and Chromatographic Conditions

A Shimadzu LC-2010HT apparatus equipped with a quaternary pump (LC-20AD), an autosampler unit (SIL-20AHT), an oven (CTO-10AS), and a detector (SPD-M2OA) was employed to quantify all the drugs. Three columns were used for the analysis: (i) a XBridge™ C18 with 5 µm particle size, 2.1 mm internal diameter, a 150 mm length; (ii) a LiChrospher® 100 RP-18 with 5 µm particle size, 4.6 mm internal diameter and 125 mm length; and finally; (iii) a LichoCART® 250 RP-18 with 5µm particle size, 4.6 mm internal diameter and 250 mm length. All columns were supported with a SecurityGuard cartridge. Table A1.1 summarizes the specific analytical conditions used for each drug product.

Table A1.1 – Analytical conditions. All developed methods are isocratic and relate to a 10 µL injection volume.

Drug	Analytical conditions
Bifonazole	Column: XBridge™ C18 5µm (2.1 x 150 mm) Mobile phase: Buffer solution (2 mL of phosphoric acid with 980 mL of ultrapurified water, adjusted to pH 3.2 ± 0.05 with trimethylamine) and acetonitrile (68:32, v/v) Flow: 0.5 mL/min Run time: 5.5 Wavelength: 210 nm Oven temperature: 30ºC Injection volume: 10 µL
Clotrimazole	Column: XBridge™ C18 5µm (2.1 x 150 mm) Mobile phase: Methanol: 25 mM dipotassium hydrogen phosphate 75:25 m v/v, pH = 7.5 Flow: 0.4 mL/min Run time: 5.5 min Wavelength: 210 nm Oven temperature: 30ºC Injection volume: 10 µL

Drug	Analytical conditions
Tioconazole	Column: XBridge™ C18 5µm (2.1 x 150 mm) Mobile phase: water: methanol (20:80, v/v) Flow: 0.4 mL/min Run time: 5.5 min Wavelength: 218 nm Oven temperature: 30°C Injection volume: 10 µL
Etofenamate	Column: LiChrospher® 100 RP-18. 5µm (4.6 mm × 125 mm) Mobile phase: methanol: acetic acid 2% (80:20, v/v) Flow: 1 mL/min Run time: 5.5 min Wavelength: 287 nm Oven temperature: 30°C Injection volume: 10 µL
Sodium diclofenac	Column: LiChrospher® 100 RP-18. 5µm (4.6 mm × 125 mm) Mobile phase: methanol: 2% acetic acid (75:25, v/v) Flow: 1 mL/min Run time: 7 min Wavelength: 280 nm Oven temperature: 30°C Injection volume: 10 µL
Clobetasol propionate	Column: LiChrospher® 100 RP-18. 5µm (4.6 mm × 125 mm) Mobile phase: methanol: 2% acetic acid (70:30, v/v) Flow: 0.35 mL/min Run time: 5.5 min Wavelength: 240 nm Oven temperature: 30°C Injection volume: 10 µL
Hydrocortisone	Column: XBridge™ C18 5µm (2.1 x 150 mm) Mobile phase: water and acetonitrile (75:25, v/v) Flow: 0.35 mL/min Run time: 8 min Wavelength: 247 nm Oven temperature: 30°C Injection volume: 10 µL
Acyclovir	Column: XBridge™ C18 5µm (2.1 x 150 mm) Mobile phase: water and methanol (95:5, v/v) Flow: 0.4 mL/min Run time: 6 min Wavelength: 247 nm Oven temperature: 30°C Injection volume: 10 µL
Dimetindene maleate	Column: LichoCART® C18 5µm (4.6 x 250 mm) Mobile phase: Buffer solution (Potassium dihydrogen phosphate (10 mM), dipotassium hydrogen phosphate (40 mM), adjusted to pH 7.7 using 200µL of trimethylamine) with acetonitrile and methanol (78.7 : 19.3 : 2, v/v) Flow: 0.7 mL/min Run time: 7 min Wavelength: 250 nm Oven temperature: 40°C Injection volume: 30 µL

A1.1.2.2 Preparation of Stock Solutions, Calibration Standards, and Quality Controls

All stock solutions were prepared by weighing approximately 10 mg of drug in 10 mL of an appropriate solvent, yielding ca. 1 mg/mL concentration.

Methanolic stock solutions were prepared for diclofenac, clobetasol, tioconazole, etofenamate, and clotrimazole. Alternatively, for bifonazole, hydrocortisone and dimetindene maleate, acetonitrile stock solutions were prepared instead. For acyclovir, due to its high hydrophilicity, the stock solution was directly prepared in water. Two working standards were considered for each drug. These were prepared by further dilution of each stock solution with the corresponding release medium used in IVRT studies. Table A1.2 summarizes the release medium used for each drug.

Table A1.2 – Release medium composition used for IVRT studies.

Drug	Release medium
Bifonazole	PBS/ethanol (50:50, v/v, pH = 7.4)
Clotrimazole	PBS/ethanol (50:50, v/v, pH = 7.4)
Tioconazole	PBS/ethanol (50:50, v/v, pH = 4.5)
Etofenamate	PBS/ethanol (70:30, v/v)
Sodium Diclofenac	PBS/ethanol (80:20, v/v, pH = 7.4)
Clobetasol propionate	PBS/ethanol (50:50, v/v, pH = 7.4)
Hydrocortisone	Water/ethanol (70:30, v/v)
Acyclovir	PBS
Dimetindene maleate	PBS/ ethanol (80:20 v/v, pH = 7.4).

Six to ten standard solutions were considered for each calibration curve. Moreover, as quality controls, five replicates of three different concentration standards were used.

Since the focus of the present work was to quantify IVRT samples, preliminary *in vitro* tests were performed to determine the appropriate range for the calibration standards. Table A1.3 summarizes the concentrations used for each molecule. In all cases, IVRT and standard samples were directly injected. All stock and working solutions were freshly prepared each day.

A1.1.2.3 Method Validation

Validation studies were performed according to the International Council for Harmonization (ICH) guidelines for each drug substance (CDER, 1994; ICH, 2005). These included system suitability, limits of detection, and quantification, linearity, accuracy, precision, robustness, and stability assessments.

Specificity and Selectivity

Specificity, *viz.*, the ability to accurately measure the analyte in the presence of all potential sample components, was evaluated by comparing the chromatograms of three different control matrices: (i), blank receptor medium solution (negative control, C_n), (ii) quality control solutions prepared in receptor medium (positive controls, C_p), and (iii) release medium solution of an IVRT run conducted with the tested formulations (matrix positive control, C_{pm}). The main purpose of this assessment was to ensure that the integrity of each active substance retrieved from IVRT samples was not compromised by any formulation excipient.

The negative controls (C_n) were analysed to confirm the absence of any detectable drug concentration. The mean retention time and concentration values (regarding the nominal concentrations) for the C_p and C_{pm} were used to set the acceptance criteria for the drug retention time, and the difference in both parameters should not exceed 15% (Tiffner *et al.*, 2018).

System Suitability

System suitability was evaluated by injecting the same standard solutions six times. The following requirements were considered: relative standard deviation (RSD) of the detector response and retention time for all standard injections was not more than 2%, capacity factor (k´) was higher than 2, tailing factor (T) of drug peak was not more than 2.0, and theoretical plate number (N) was higher than 2000.

Limits of Detection and Quantification

A specific calibration curve obtained from six standard solutions was traced in order to determine the limits of detection (LOD) and quantification (LOQ). The following expressions were used:

$$LOD = 3.3 \times \frac{\sigma}{S} \quad \text{(A1.1)}$$

$$LOQ = 10 \times \frac{\sigma}{S} \quad \text{(A1.2)}$$

where σ is the standard deviation of the response and S the slope of the calibration curve (ICH, 2005).

Linearity

To test the linearity of the detector response, a set of six to ten calibration standards were prepared for each drug, in the corresponding release medium. A specific range for each active substance was adopted, taking into account IVRT results. Please see Table A1.3.

Table A1.3 – Concentrations used for calibration curve, quality control, and limits of detection (LOD) and limits of quantification (LOQ) standards.

Drug	Calibration Curve Standards (µg/mL)	Quality Control Standards (µg/mL)	LOQ and LOD Standards (µg/mL)
Bifonazole	0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, and 150	0.25, 20, and 80	The calibration curve standards were used
Clotrimazole	0.05, 0.25, 0.5, 1, 5, 10, 25, 50, 75, and 100	0.1, 20, and 40	0.05, 0.25, 0.5,1, and 5
Tioconazole	0.1, 0.5, 1, 2.5, 10, 25, and 50	4, 8, and 16	The calibration curve standards were used
Etofenamate	10, 25, 50, 75, 100, and 200	20, 80, and 180	0.5, 1, 2, 2.5, 4, and 5
Sodium Diclofenac	10, 25, 75, 100, 150, and 200	20, 80, and 180	0.5, 1, 2, 2.5, 4, and 5
Clobetasol propionate	0.1, 0.25, 0.5, 1, 2, 5, and 10	0.1, 1.5, and 4	The calibration curve standards were used
Hydrocortisone	2.5, 5, 10, 20, 25, and 50	3, 15, and 40	0.25, 0.5, 1, 2, and 2.5
Acyclovir	5, 10, 25, 50, 75, 100, 150, and 300	20, 80, and 200	0.25, 0.5, 1, 2, and 5
Dimetindene maleate	10, 15, 20, 25, 30, 35, 40 and 50	12.5, 22.5 and 37.5	The calibration curve standards were used

At least four calibration curves were considered for each active substance. Linearity was determined through the calculation of a regression line, attained from the peak area as a function of the standard concentration, by the method of the least squares. To comply with regulatory requirements, curves that did not present a R^2 of at least 0.99 were not considered (USP, 2009).

Accuracy and Precision

Precision measures the closeness of agreement, i.e., the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample. This validation parameter was assessed by repeatability (intraday) and intermediate precision (interday) for three days, of the established quality control (QC) standards. Five samples of each concentration level were prepared. The RSD determined at each concentration level should not exceed 15%, except for the lower concentrations, where a maximum of 20% was allowed (Basso *et al.*, 2018; CDER, 1994).

To demonstrate accuracy, the closeness of agreement between the true value and the found value, Equation A1.3, was used. Afterwards, the mean bias percentage for the five replicates of the three QCs was calculated.

$$Accuracy = \frac{nominal\ concentration - observed\ concentration}{observed\ concentration} \times 100 \quad \text{(A1.3)}$$

The same acceptance criteria regarded for precision were also considered to establish the methods accuracy (CDER, 1994; ICH, 2005).

Ruggedness

To prove the methods ruggedness, five duplicates of the quality control samples were alternatively analysed using different HPLC equipment, a Shimadzu LC-10AD apparatus, equipped with a quaternary pump (LC-10AD), an autosampler unit (SIL-10ADVP), a CTO-10AVP oven, and a CBM-20 A detector.

Stability

Five replicates of quality control solutions were prepared to evaluate the stability of IVRT samples at room temperature storage (25°C) for 24 h in the autosampler and in short-term storage at 4°C for 72 h (Basso *et al.*, 2018; Carla Vitorino *et al.*, 2013). The same acceptance criteria established during precision and accuracy assessments were used.

A1.1.2.4 Method Applicability to *in vitro* Release Testing – Franz Cell Receptor Fluid Screening

The maintenance of sink conditions is crucial throughout the release experiments, therefore, screening different receptor medium (co-solvents and pH effects), according to the active substance physicochemical profile, should be a priority concern (Baert *et al.*, 2010).

In order to rationally establish solubility conditions to be tested, *in silico* studies were performed first. For that, the chemical structure of each API was introduced in Chemaxon® software (ChemAxon, Budapest, Hungary) to calculate the respective chemical descriptors, such as size, geometry, lipophilicity, solubility, and surface topology (Faria *et al.*, 2019).

For solubility studies, the adopted protocol was as described in Chapter 4.

A1.2 Results and Discussion

A1.2.1 Method Validation

A1.2.1.1 Specificity and Selectivity

Chromatographic separation of all drugs in their respective release medium was successfully achieved using the previously described analytical conditions, see Fig. A1.2.

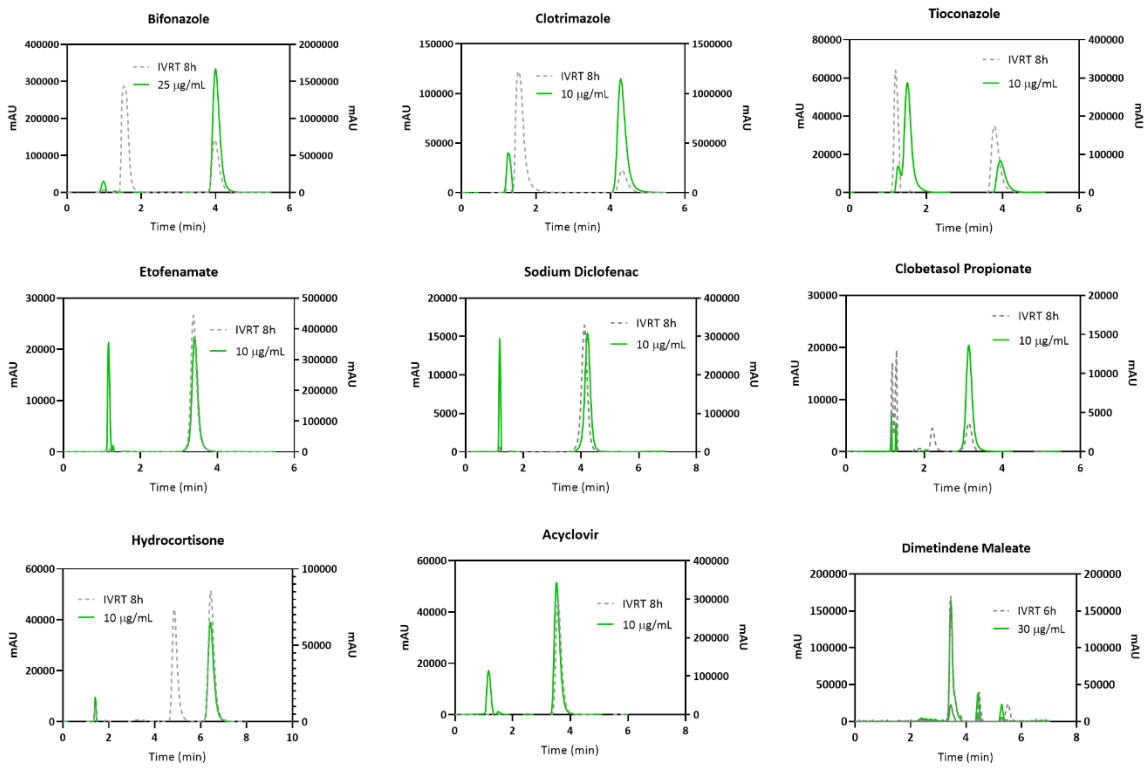

Fig. A1.2 – Representative chromatograms of a standard solution (solid green line) and *in vitro* release testing (IVRT) samples (dashed grey line).

Key: Right YY axis – standards; Left YY axis – IVRT samples.

Please note that the products used in this study are complex formulations with multiple ingredients. Preservatives such as benzyl alcohol or methyl parahydroxybenzoate, present in some of the studied products, absorb in the UV region. For this reason, additional peaks are present in IVRT samples of bifonazole, clobetasol, and tioconazole (pertaining to benzyl alcohol) and in hydrocortisone (corresponding to methyl parahydroxybenzoate). In all cases, drug peaks were resolved from the additional components, thus highlighting the specificity of the proposed methods. No interference with the selected release medium was observed.

To further illustrate the chromatographic similarity between the standards and IVRT samples, the retention times are presented in Table A1.4.

Table A1.4 – Retention times for standards (n=45) and IVRT samples (n=180).

Drug	Standards	IVRT Samples
	Retention time mean (min)	
Bifonazole	3.947 ± 0.09	4.012 ± 0.06
Clotrimazole	4.315 ± 0.04	4.296 ± 0.02
Tioconazole	3.697 ± 0.11	4.064 ± 0.21
Etofenamate	3.395 ± 0.02	3.390 ± 0.01
Sodium Diclofenac	4.166 ± 0.02	4.241 ± 0.01
Clobetasol propionate	4.241 ± 0.03	4.229 ± 0.04
Hydrocortisone	6.387 ± 0.02	6.438 ± 0.01
Acyclovir	3.432 ± 0.07	3.506 ± 0.03
Dimetindene maleate	3.947 ± 0.09	3.447 ± 0.04

Key: Please note that tioconazole retention time fluctuations may be ascribed to the hydrophobic interactions established with the matrix' (formulation) lipophilic components.

A1.2.1.2 System Suitability

System suitability tests aim to assess if the chromatograph and respective modules are able to generate acceptable accuracy and precision results. To provide quantitative data, several parameters should be taken into consideration. These include: number of theoretical plates, separation factor, resolution, tailing factor, and precision (Papadoyannis and Samanidou, 2004). System suitability results and acceptance criteria are summarized in Table A1.5.

Table A1.5 – System suitability test parameters. System suitability was evaluated by injecting the same standard solution six times.

Drug	Conc. (µg/mL)	Retention Time (min)		Peak Area		T. Plates	K´	T. Factor	Resolution
		Mean	RSD	Mean	RSD				
BFZ	50	3.973	0.09	9135580	0.08	1 898 400	3.057	1.684	2.09
CLT	50	4.262	0.07	8701986	0.13	1 721 502	2.393	1.634	4.10
TCZ	10	3.643	0.08	522268	0.44	1 289 836	2.060	1.889	4.86
ETF	100	3.491	0.24	2306243	0.14	1883362.8	1.957	1.140	5.70
DF	100	4.202	0.05	2188617	0.42	1 598 293	2.628	1.177	5.67
CLB	2	4.230	0.19	96246	1.12	1 368 004	1.812	1.703	7.50
HC	25	6.378	0.06	1779896	0.05	2 689 997	4.446	1.456	15.77
ACV	100	3.350	0.23	8187120	0.57	1 362 134	1.795	1.583	3.60
DM	25	3.451	0.09	157445	0.22	3 486 849	-	1.478	-
Acceptance criteria		-	≤ 2.0%	-	≤ 2.0%	> 1000	> 1000	≤ 2.0	> 2.0

Key: BFZ – Bifonazole; CLT – Clotrimazole; TCN – Tioconazole; ETF – Etofenamate; DF – Diclofenac; CLB – Clobetasol; HC – Hydrocortisone; ACV – Acyclovir; DM – Dimetindene Maleate; Conc – concentration; T. Plates – theoretical plates; K´ – capacity factor; T. Factor – tailing factor.

The precision of both peak area and retention time was compliant for all drugs, thus documenting the system's ability to detect and analyse the compounds in their respective release medium.

Column efficiency was also evidenced for all drugs, since the number of theoretical plates (T. Plates), tailing factor (T. Factor), and resolution were compliant with ICH criteria. Even though the capacity factor (k´) for acyclovir and clobetasol was slightly beneath 2.0, according to the British Pharmacopoeia, this value can still be accepted (k´ < 1.5) (C. Vitorino *et al.*, 2013).

A1.2.1.3 Limits of Detection and Quantification

Since different dosage forms were considered for the present study, different release mechanisms and release ranges are expected. Therefore, preliminary IVRT results were used to determine specific and plausible LOD concentrations. LOD and LOQ values are summarized in Table A1.6.

Table A1.6 – LOD and LOQ values.

Drug	LOD (ng/mL)	LOQ (ng/mL)
Bifonazole	1.46	4.43
Clotrimazole	0.35	1.06
Tioconazole	14.05	42.59
Etofenamate	0.44	1.33
Sodium Diclofenac	0.88	0.29
Clobetasol proprionate	2.52	7.63
Hydrocortisone	0.05	0.15
Acyclovir	0.04	0.12
Dimetindene maleate	1.39	4.20

A1.2.1.4 Linearity

IVRT sample concentration is interpolated by using the regression results obtained from calibration curves. These are often calculated through the least squares method that estimates the correlation coefficient (R^2) and the regression equation (Basso *et al.*, 2018; Gonçalves *et al.*, 2018). Table A1.7 depicts the results from linearity studies.

Table A1.7 – Results obtained from the regression analysis using the least squares method for all studied drugs. Results are expressed as mean ± standard deviation (SD), n=4. Please note that a weighted linear regression was performed on calibration data using $1/x^2$ as the weighting factor.

Drug	Mean R^2	Mean Slope	Mean Intercept
Bifonazole	0.9984 ± 0.0007	71972704 ± 846636	12227244 ± 6063944
Clotrimazole	0.9994 ± 0.0002	175242 ± 5754	1659 ± 749
Tioconazole	0.9987 ± 0.0003	61944 ± 2704	3271 ± 231
Etofenamate	0.9988 ± 0.0008	23376 ± 1055	56591 ± 31185
Sodium Diclofenac	0.9994 ± 0.0003	21958 ± 106	10595 ± 14365
Clobetasol proprionate	0.9970 ± 0.0008	52833 ± 1014	1160 ± 429
Hydrocortisone	0.9999 ± 0.0001	72492 ± 772	-2643 ± 834
Acyclovir	0.9996 ± 0.0001	73690 ± 2119	10926 ± 8039
Dimetindene maleate	0.9990 ± 0.0017	6316 ± 342	6558 ± 4917

All R^2 were superior to 0.99 and, therefore, evidence is provided regarding data quality and linearity in the proposed range.

A1.2.1.5 Accuracy and Precision

Calibration curve quality should also be documented through the evaluation of precision and accuracy. Both parameters should be assessed at the intraday and interday levels.

According to our results, displayed in Table A1.8, RSD values did not exceed 15%, even for the lowest concentration QC. The same trend was also verified for the accuracy results.

Table A1.8 – Intraday and interday accuracy and precision results for all drugs. Results are expressed as mean ± SD (n=5).

Drug		Intraday (n=5)			Interday (n=15)		
	Concentration CQ (µg/mL)	Measured Concentration (µg/mL)	Accuracy (%)	RSD (%)	Measured Concentration (µg/mL)	Accuracy (%)	RSD (%)
BFZ	0.25	0.26 ± 0.02	–5.41	7.97	0.26 ± 0.03	–4.92	11.52
	20	20.2 ± 0.4	–1.22	1.93	20.2 ± 0.5	–1.23	2.72
	80	82.5 ± 1.5	–3.13	1.77	82.6 ± 2.9	–3.33	3.52
CLT	0.1	0.11 ± 0.01	–3.25	12.28	0.11 ± 0.01	–3.25	12.91
	20	19.4 ± 0.4	9.07	1.86	19.5 ± 0.8	9.07	4.08
	40	38.9 ± 1.2	3.03	2.97	38.6 ± 2.8	3.56	7.37
TCZ	4	3.82 ± 0.13	4.51	3.42	3.8 ± 0.29	5.06	7.76
	8	7.68 ± 0.14	3.96	1.94	7.67 ± 0.30	4.19	3.94
	16	16.4 ± 0.4	–2.30	2.42	16.4 ± 0.6	–2.30	3.76
ETF	20	20.9 ± 0.9	–0.05	4.62	20.6 ± 2.1	–0.03	10.27
	80	79.2 ± 1.9	0.01	2.47	79.2 ± 2.6	0.01	3.49
	180	175 ± 3	0.03	2.11	175 ± 7	–1.19	4.08
DF	20	20.1 ± 0.3	–0.01	1.52	20.1 ± 0.5	–0.01	2.52
	80	83.31 ± 0.99	–0.04	1.17	83.3 ± 0.5	–0.04	3.95
	180	181 ± 3	–0.01	1.92	181 ± 4	–1.26	2.41
CLB	0.4	0.38 ± 0.02	3.75	7.22	0.39 ± 0.05	3.36	13.04
	1.5	1.38 ± 0.09	7.69	7.01	1.38 ± 0.15	8.30	10.71
	4	3.75 ± 0.16	6.21	4.39	3.75 ± 0.27	6.31	7.11
HC	3	3.18 ± 0.04	–6.11	1.23	3.16 ± 0.17	–5.23	5.53
	15	15.11 ± 0.2	–0.72	1.48	15.1 ± 0.3	–0.67	2.04
	40	40.2 ± 0.2	–0.61	0.54	40.2 ± 0.5	–0.55	1.18
ACV	20	20.7 ± 0.9	–3.48	4.47	20.6 ± 1.3	–2.84	6.27
	80	80.2 ± 0.9	–0.28	1.19	80.2 ± 1.4	–0.22	1.80
	200	203 ± 1	–1.72	0.56	204 ± 7	–1.87	3.61
DM	12.5	12.44 ± 0.17	0.49	1.41	12.44 ± 0.32	0.49	2.58
	22.5	22.38 ± 0.24	0.55	1.05	22.38 ± 0.42	0.55	1.89
	37.5	37.15 ± 0.25	0.93	0.68	37.15 ± 0.70	0.93	1.89

Key: BFZ – Bifonazole; CLT – Clotrimazole; TCN – Tioconazole; ETF – Etofenamate; DF – Diclofenac; CLB – Clobetasol; HC – Hydrocortisone; ACV – Acyclovir; DM – Dimetindene Maleate.

These results substantiate that the developed methods are accurate, reliable, and reproducible, since they all met the acceptance recommendations.

A1.2.1.6 Ruggedness

The ability of the method to provide reliable results despite minor variations in the analytical conditions can be defined as ruggedness or robustness. Nevertheless, both terms present slight differences (González *et al.*, 2014). ICH defines analytical method robustness as, "the

measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage" (CPMP/ICH/381/95, 2005). Furthermore, ICH provides examples of such variations. These include mobile phase composition/pH variations, different columns, and different temperature analysis or flow rates (CPMP/ICH/381/95, 2005; González *et al.*, 2014). USP defines ruggedness as a measure of reproducibility of the test results under the variation conditions normally expected from laboratory to laboratory and from analyst to analyst (United States Pharmacopoeia, 2005). According to these compendial definitions, the term ruggedness is more accurate when considering two different equipment types. Table A1.9 summarizes robustness results.

Table A1.9 – HPLC method robustness. Results are expressed as mean ± SD (n=4).

Drug	Concentration CQ (µg/mL)	Mean Concentration (µg/mL)	Accuracy (%)	RSD (%)
Bifonazole	0.25	0.26 ± 0.02	6.29	9.15
	20	18.4 ± 0.8	–8.05	4.44
	80	81.2 ± 0.5	1.49	0.59
Clotrimazole	0.1	0.11 ± 0.01	9.03	9.85
	20	17.5 ± 0.7	–12.46	3.92
	40	35.6 ± 0.3	–10.87	0.72
Tioconazole	4	3.99 ± 0.07	0.13	1.71
	8	7.56 ± 0.28	5.47	3.76
	16	16.0 ± 0.7	-0.08	4.12
Etofenamate	20	22.9 ± 0.5	13.77	2.37
	80	79 ± 1.4	–0.93	1.75
	180	171 ± 0	–4.95	0.24
Sodium Diclofenac	20	21.0 ± 0.3	4.84	1.36
	80	85.3 ± 1.0	6.62	1.21
	180	197 ± 10	9.18	5.05
Clobetasol Proprionate	0.4	0.41 ± 0.05	3.59	11.75
	1.5	1.37 ± 0.04	–8.75	3.09
	4	3.61 ± 0.04	–9.83	1.17
Hydrocortisone	3	3.44 ± 0.06	14.67	1.86
	15	15.2 ± 0.3	2.18	2.16
	40	40.1 ± 0.2	0.70	0.17
Acyclovir	5	4.59 ± 0.02	–8.17	0.63
	100	95.9 ± 0.1	–4.02	0.08
	200	206 ± 0	2.94	0.12
Dimetindene Maleate	12.5	12.19 ± 0.07	-2.70	0.55
	22.5	22.23 ± 0.03	-0.77	0.15
	37.5	37.63 ± 0.08	-0.10	0.22

Even though all results are in good acceptance with regulatory standards, some borderline results were verified for hydrocortisone, etofenamate and clotrimazole.

A1.2.1.7 Stability

Stability testing should mimic common sampling procedures, so as to provide reliable data analysis. The stability conditions assessed included autosampler and short-term stability. Results are presented in Table A1.10.

Table A1.10 – Autosampler and short-term stability of quality control (QC). Results are expressed as mean ± SD (n=5).

Stability condition	Autosampler				Short term stability		
Drug	**Concentration CQ (µg/mL)**	**Mean Concentration (µg/mL)**	**Accuracy (%)**	**RSD (%)**	**Mean Concentration (µg/mL)**	**Accuracy (%)**	**RSD (%)**
BFZ	0.25	0.23 ± 0.01	–8.43	6.29	0.29 ± 0.01	15.96	3.94
	20	19.9 ± 0.3	–.29	1.64	20.7 ± 0.4	3.17	2.07
	80	84.2 ± 0.8	5.22	0.94	85.5 ± 0.8	6.83	0.96
CLT	0.1	0.11 ± 0.01	7.67	5.37	0.10 ± 0.01	–0.41	13.45
	20	19.5 ± 0.2	–2.68	0.94	20.2 ± 0.8	1.14	4.08
	40	38.7 ± 0.6	–3.30	1.44	40.5 ± 3.3	1.26	8.10
TCZ	4	3.41 ± 0.15	–14.81	4.50	3.49 ± 0.18	–12.73	5.19
	8	7.76 ± 0.63	–2.94	8.12	7.41 ± 0.35	–7.33	4.75
	16	16.3 ± 0.3	1.72	1.57	17.4 ± 1.2	8.98	6.72
ETF	20	21.9 ± 0.7	–0.09	3.06	20.2 ± 0.1	1.03	0.75
	80	92.8 ± 3.0	–0.16	3.28	78.8 ± 1.7	–1.54	2.20
	180	198 ± 5	–0.10	2.66	178 ± 1	–0.87	0.35
DF	20	20.4 ± 0.6	2.01	3.00	20.0 ± 0.1	0.04	0.67
	80	84.4 ± 2.7	5.50	3.20	82.3 ± 0.6	2.82	0.69
	180	180 ± 5	–0.15	2.60	184 ± 0.0	2.05	0.20
CLB	0.4	0.34 ± 0.01	–14.13	2.70	0.33 ± 0.02	–16.24	6.19
	1.5	1.35 ± 0.03	–10.01	2.02	1.41 ± 0.06	–5.94	3.99
	4	3.72 ± 0.08	–7.06	2.18	3.91 ± 0.21	–2.24	5.43
HC	3	3.06 ± 0.05	1.96	1.77	3.40 ± 0.05	13.36	1.66
	15	15.1 ± 0.5	0.82	3.25	15.2 ± 0.3	1.24	2.04
	40	39.9 ± 0.3	–0.31	0.79	40.1 ± 0.7	0.21	0.27
ACV	20	21.8 ± 1.7	8.80	7.83	22.7 ± 0.3	13.50	1.42
	80	87.6 ± 0.5	9.55	0.57	85.3 ± 0.8	6.59	0.93
	200	215 ± 0.0	7.78	0.14	209 ± 2	4.27	0.77
DM	12.5	12.17 ± 0.05	-2.64	0.38	12.53 ± 0.25	-0.17	2.02
	22.5	22.51 ± 0.17	0.04	0.75	22.40 ± 0.31	-0.97	1.40
	37.5	37.42 ± 0.04	-0.20	0.10	37.66 ± 0.13	-0.17	0.36

Key: BFZ – Bifonazole; CLT – Clotrimazole; TCN – Tioconazole; ETF – Etofenamate; DF – Diclofenac; CLB – Clobetasol; HC – Hydrocortisone; ACV – Acyclovir; DM – Dimetindene Maleate.

According to the results, it can be inferred that the analytes are stable under the studied conditions, since precision and accuracy requirements were met.

A1.2.2 Method Applicability to *in vitro* Release Testing – Franz Cell Receptor Fluid Screening

In Silico Studies: Using Chemical Predictors to Support IVRT Development

In order to determine the most appropriate conditions for solubility studies, the physicochemical properties of each molecule were firstly predicted *in silico* and used to infer their ionization, lipophilicity, permeability and solubility status, please see Table A1.11.

Table A1.11 – *In silico* prediction of several physicochemical descriptors obtained from the molecule structure using Chemaxon software.

API	M	Log P	S (mg/mL)	pKa	H donors	H Acceptors	Lipinski Rule	Log D			Solubility and pH (mg/ml)		
								4.6	6.5	7.4	4.6	6.5	7.4
CLT	344.84	5.84	0.000703	6.26	0	1	X	5.3	5.7	5.8	0.03	0	0
BFZ	310.40	5.23	0.000791	6.36	0	1	X	4.7	5.07	5.2	0.05	0	0
TCZ	387.70	5.30	0.000552	6.48	0	2	X	4.72	5.1	5.3	4.6	0	0
ETF	369.34	4.86	0.0187	15.12	2	4	C	4.9	4.9	4.9	0.02	0.02	0.02
DF	296.15	4.26	0.0149	4.00	2	3	C	3.6	1.8	1.1	0.07	4.8	37.9
CLB	466.97	4.18	0.0017	13.59	1	4	C	4.2	4.2	4.2	0	0	0
HC	362.47	1.28	0.408	12.59	3	5	C	1.3	1.3	1.3	0.4	0.4	0.4
ACV	225.21	-1.03	9.1	11.98	3	7	C	-1.04	-1.03	-1.03	9.3	9.1	9.1
DM	408.5	4.03	0.356	9.7	0	2	C	0.06	0.71	1.46	408.5	408.5	72.23

Key: BFZ – Bifonazole; CLT – Clotrimazole; TCN – Tioconazole; ETF – Etofenamate; DF – Diclofenac; CLB – Clobetasol; HC – Hydrocortisone; ACV – Acyclovir; DM – Dimetindene Maleate; S – intrinsic solubility (water at 25 ^{o}C); C – Compliant; NC – Non-compliant; M – Molar Mass.

The distribution coefficients (Log D) were determined within a physiological range of pH values compatible with skin application. The Log D is the appropriate descriptor for ionizable compounds, since it relates to the solubility of all chemical forms depending on the selected pH. Hydrocortisone, etofenamate, acyclovir, and clobetasol, as neutral actives, do not present changes in LogD at different pH values. By contrast, all antifungal drugs plus diclofenac and dimetindene show opposite behaviours. For diclofenac, the Log D at 4.6 was higher than the Log D at 6.5, since the non-ionized form is predominant at an acidic pH (Carrer *et al.*, 2018). The reverse situation is observed for all antifungal drugs and dimetindene maleate, where an increase of pH is followed by an increase of the Log D. The non-ionized form of bifonazole, tioconazole, clotrimazole and dimetindene maleate is predominant at more basic pH.

According to the Lipinski rule of five, it is more likely for a compound to exhibit an enhanced permeability if the number of hydrogen bond donors/acceptors is no more than 5 or 10, respectively, the molecular weight does not exceed 500 Da, and the LogP is not more than 5. Hydrocortisone, etofenamate, acyclovir, clobetasol, diclofenac and dimetindene comply with

these requirements, while the other active substances (antifungals) are expected to have poorer permeability (Faria *et al.*, 2019). Regarding lipophilicity, different profiles can be identified. Hydrocortisone and acyclovir exhibit low/moderate lipophilicity, while the other compounds are predominantly more lipophilic.

The solubility of the compounds was carefully inspected in order to select the most appropriate IVRT conditions. Dimetindene maleate, acyclovir and hydrocortisone, display the highest intrinsic solubility amongst the studied drugs (acyclovir and dimetindene are very soluble and hydrocortisone is freely soluble) (The European Pharmacopoeia Commission, 2019). Even though PBS (pH = 7.4) could be a suitable choice for the release medium of these actives, two additional circumstances had to be considered:

- The Topical and Transdermal Drug Products Product Performance Tests section in the USP suggests a water/ethanol (70:30, v/v) release medium for a hydrocortisone cream (USP, 2009). Therefore, this particular release medium was instead considered.
- The dimetindene maleate 1 mg/g gel is an antihistaminic drug which reduces swelling and sooths skin irritation. Since it should be administered 2-4 times a day, the IVRT study timeframe was shorten to 6h (PAR, 2015b). In this context, to prompt drug release, the addition of a cosolvent was equated. Please note that, dimetindene maleate solubility is impacted by the pH, nevertheless at pH= 7.4 its solubility is still very high, therefore screening studies solely contemplated the impact of cosolvents.

For acyclovir, a release medium comprising PBS was selected.

Diclofenac and etofenamate can be classified as sparingly soluble, hence the effects of two co-solvents, ethanol and propylene glycol, were tested. Etofenamate Log D displays no alteration within the considered pH range, therefore, a pH =7.4 was solely considered. The solubility of these two actives was then assessed in the following conditions: PBS/co-solvent 80:20 and 70:30, v/v.

On the other hand, diclofenac solubility is impacted by the pH, consequently, screening studies were performed at a pH values of 3.6, 5.5, and 7.4 in the condition PBS/co-solvent 80:20, v/v.

Clobetasol is considered slightly soluble and its Log D is not affected by pH. Taking this into account, the following conditions were tested: PBS/ethanol 70:30 and 50:50, v/v.

Antifungal molecules present the lowest solubility, as they are considered as very slightly soluble molecules. As previously mentioned, these molecules do not exhibit change in the Log D at a physiological pH compatible with skin application. However, for tioconazole, at a

pH = 4.6, the molecule evidences some degree of solubility. Therefore, the effect of the same co-solvents was screened in the following conditions: PBS/co-solvent 70:30, 60:40, and 50:50, v/v, for both bifonazole and clotrimazole. Alternatively, for tioconazole, PBS/co-solvent ratios of 80:20, 70:30, and 50:50, v/v, were tested at a pH = 7.4. Since ethanol provided the highest solubility results, the PBS/ethanol 80:20 and 50:50, v/v, conditions were repeated, but at a pH of 4.5. Solubility results are presented in Fig. A1.3.

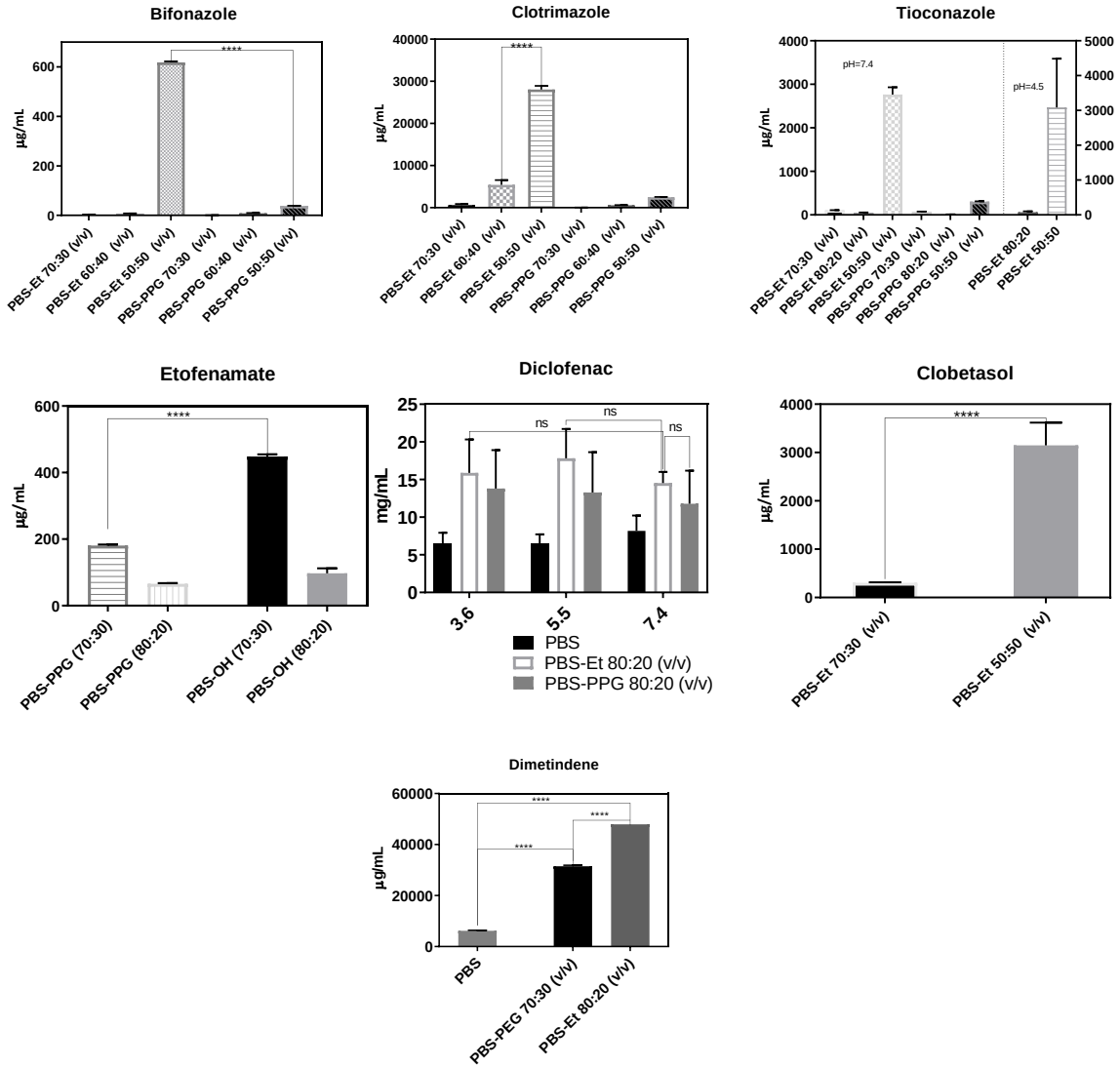

Fig. A1.3 – Solubility of the active substances in each medium. The results are expressed as mean ± standard error of the mean (SEM) (4≤n≤7). Since the data are normally distributed (Shapiro-Wilk normality test), a two-way ANOVA with a Tukey multiple comparison test was employed to statistically compare the means. The differences among the means were considered significant for values of p<0.1.

Ethanol was herein selected due to its prevalence in the literature, while the selection of propylene glycol was mainly related to the presence of this component in the qualitative formulation of some products. In fact, among the polyvalent alcohols, propylene glycol is the most frequently used co-solvent in topical products (de Melo *et al.*, 2017). Nevertheless, for all drugs, ethanol demonstrated a higher solubility.

For etofenamate, PBS/ethanol pH = 7.4 at a 70:30, v/v, ratio was selected as the release medium. For bifonazole, clotrimazole, and clobetasol, a 50:50, v/v, ratio of PBS/ethanol pH = 7.4 enabled the optimal solubility conditions. For tioconazole, the same medium displayed good results, however, a pH = 4.5 was selected. Finally, for diclofenac and dimetindene maleate, a PBS/ethanol 80:20, v/v, at a pH = 7.4 was selected.

IVRT

The analytical methods presented in this appendix were used to monitor drug concentrations in Chapters 4, 5 and 6. To meet formal requirements, topical product release mechanisms should follow the Higuchi model, in other words, drug release should exhibit linearity with respect to the square root of time (EMA, 2018b; Higuchi, 1961). As portrayed in the above mentioned chapters, for all products, this criterion was fulfilled (Naik *et al.*, 2016).

A1.3 Conclusions

Considering the current regulatory background, product performance evaluation techniques, such as IVRT, are becoming increasingly common in all stages of topical products' lifecycles. In this context, one of the principal contributions of the developed analytical methods relies on the establishment of a portfolio of HPLC methods specifically tailored for commercially available topical products. The analytical methods presented here are specific, linear, accurate, reliable, and reproducible. Due to their simplicity and a high sample throughput potential, this database can constitute a useful tool for (i) fast screening of formulation performance, and (ii) timely analysis of batch compliance, supporting a routine implementation. Lastly, an *in silico* approach was successfully developed to assist the IVRT release medium selection.

Appendix A2

A2.1 Materials and Methods

A2.1.1 Materials

Bifonazole and diclofenac diethylammonium, were kindly supplied by Laboratórios Basi. Skin samples used for IVPT experiments were obtained from two different sources: (i) Centro Hospitalar de Lisboa Central, where the experimental protocol was approved by the Bioethics Committee. Written informed consent forms have been obtained from the participants involved in this study (Process number 447/2017); (ii) Genoskin®. The tissue was also obtained from plastic reduction surgeries. In both skin sources, after tissue excision, all specimens were transported in saline solution (normal saline) under refrigeration (for less than 24 hours). Water was purified with a Millipore MILLI-Q reagent water system and filtered through a 0.22 µm nylon filter before use. All other chemicals were of analytical grade or equivalent.

A2.1.2 Methods

A2.1.2.1 Instrumentation and Chromatographic Conditions

A Shimadzu LC-10AD apparatus, equipped with a quaternary pump (LC-10AD), an autosampler unit (SIL-10ADVP), a CTO-10AVP oven and a CBM-20 A detector was used to monitor drug concentrations in IVPT samples. Chromatographic analysis was conducted in isocratic mode. The next table describes the analytical conditions used. All columns were supported with a SecurityGuard cartridge.

Table A2.1 summarizes the specific analytical conditions used for each drug product.

Table A2.1 – Analytical conditions. All developed methods are isocratic and relate to a 10 µL injection volume.

Drug	Analytical conditions
Bifonazole	Column: XBridge™ C18 5 µm (2.1 mm x 150 mm) Mobile phase: Buffer solution (900 mL of a sodium dihydrogen phosphate solution (29 mM) with 100 mL of a orthophosphoric acid solution (25 mM), adjusted to pH 3.2 using trimethylamine) and acetonitrile (60:40, v/v). Flow: 0.35 mL/min Run time: 8 min Wavelength: 210 nm Temperature: 40ºC
Diclofenac diethylammonium	Column: LiChrospher® 100 RP-18. 5 µm (4.6 mm × 125 mm) Mobile phase: methanol: 2% acetic acid (75:25, v/v) Flow: 1 mL/min Run time: 7 min Wavelength: 280 nm Temperature: 30ºC

A2.1.2.2 Preparation of Stock Solutions, Calibration Standards, and Quality Controls

Bifonazole and diclofenac stock solutions for were prepared as previously described and two working standards were considered for each drug. These were prepared by further dilution of the stock solution with the corresponding permeation medium/ extraction solvent used in IVPT studies, see Table A2.2.

Table A2.2 – Release medium/ extraction solvent used for IVPT studies.

Drug	Medium
Bifonazole	IVPT Samples: PBS-PEG-400 (60:40, v/v pH=7.4) Mass Balance samples: Acetonitrile
Diclofenac diethylammonium	IVPT Samples: PBS (pH=7.4) Mass Balance samples: Methanol

Six to ten standard solutions were considered for each calibration curve. Moreover, as quality controls, five replicates of three different concentration standards were used.

Since the focus of the present work was to quantify IVPT/ mass balance samples, preliminary *in vitro* tests were performed to determine the appropriate range for the calibration standards. In all cases, IVPT and standard samples were directly injected. All stock and working solutions were freshly prepared each day.

A2.1.2.3 Method Validation

Validation studies were performed according to the International Council for Harmonization (ICH) guidelines for each drug substance (CDER, 1994; ICH, 2005). These experimental

procedures reporting to system suitability, limits of detection, and quantification, linearity, accuracy, precision, robustness, and stability assessments were previously reported in section A1.

A2.1.2.4 Method Applicability to *in vitro* permeation Testing

The maintenance of sink conditions is crucial throughout the release experiments, therefore, solubility studies were performed. The adopted protocol was as described in section 4.2.2.3.

A2.2 Results and Discussion

A.2.2.1 Specificity/selectivity

Chromatographic separation of all drugs was successfully achieved using the described analytical conditions, see Fig. A2.1.

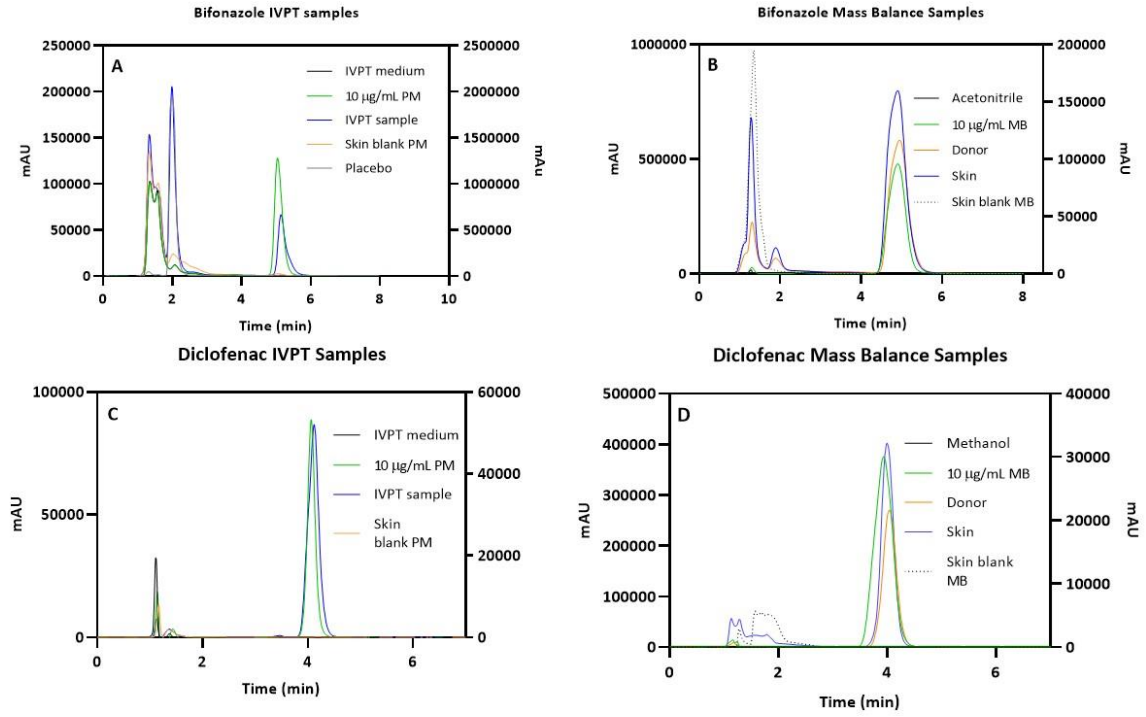

Fig. A2.1 – Representative chromatograms obtained in IVPT studies.

Key: A – Bifonazole IVPT studies chromatograms. Right Y axis – IVPT medium, 10 µg/mL standard and IVPT sample; Left Y axis – skin blank PM and placebo. B – Bifonazole IVPT mass balance studies chromatograms. Right Y axis – 10 µg/mL standard, donor chamber and skin. Left Y axis – Acetonitrile and skin blank. C – Diclofenac IVPT studies chromatograms. Right Y axis – permeation medium, IVPT sample and blank skin; Left Y axis – 10 µg/mL standard. D – Diclofenac IVPT mass balance studies chromatograms. Right Y axis – methanol, blank skin; donor chamber and skin; Left Y axis – 10 µg/mL standard.

Please note that for each IVPT study two calibration curves were made, since this study equates two different types of samples: (i) IVPT samples, and (ii) mass balance samples.

Moreover, it should be pointed out that these products regard complex formulations with multiple ingredients. Preservatives such as benzyl alcohol present in bifonazole, absorb in the UV region. For this reason, additional peaks are present in IVPT samples of bifonazole. Moreover, IVPT samples also display additional peaks related to the skin matrix. Nevertheless, in all cases, drug peaks were resolved from the additional components, thus highlighting the specificity of the proposed methods.

To further illustrate the chromatographic similarity between the standards and IVRT samples, the retention times are presented in Table A2.3.

Table A2.3 – Retention times (min) for standards and IVRT/IVPT samples.

Drug	IVRT Samples	IVPT Samples	IVPT mass balance samples
Bifonazole	Standards: 3.947 ± 0.09 (n=45) Samples: 4.012 ± 0.06 (n=180)	Standards: 5.141±0.104 (n=30) Samples: 5.053±0.044 (n=389)	Standards: 4.878±0.0029 (n=23) Samples: 4.916±0.04 (n=62)
Diclofenac	Standards: 4.166 ± 0.02 (n=45) Samples: 4.241 ± 0.01 (n=180)	Standards: 4.053 ± 0.89 (n=45) Samples: 4.12 ± 0.03 (n=421)	Standards: 3.978 ± 0.06 (n=45) Samples: 4.00±0.03 (n=70)

A.2.2.2 System Suitability

System suitability results and acceptance criteria are summarized in Table A2.4.

Table A2.4 – System suitability test parameters. System suitability was evaluated by injecting the same standard solution six times.

Drug	Conc. (µg/mL)	Retention Time (min)		Peak Area		T. Plates	K´	T. Factor	Resolution
		Mean	RSD	Mean	RSD				
BFZ_IVPT samples	10	5.166	0.12	1 502 906	0.75	1 807 869	6659.5	1.620	5.459
BFZ_MB samples	10	4.879	0.13	2 786 524	0.42	625 715	2.727	1.09	2.635
DF_IVPT samples	5	4.040	0.63	312 078	1.19	4 163 049	2.578	1.213	10.439
DF_MB samples	50	3.818	1.61	2 822 057	1.27	1 334 386	2.497	1.070	6.924
Acceptance criteria		-	≤ 2.0%	-	≤ 2.0%	> 1000	> 1000	≤ 2.0	> 2.0

Key: BFZ – Bifonazole; BFZ_MB samples – Bifonazole mass balance samples; DF – Diclofenac; DF_MB samples – Diclofenac mass balance samples; Conc – concentration; T. Plates – theoretical plates; K´ – capacity factor; T. Factor – tailing factor.

The precision of both peak area and retention time was compliant for all samples, thus documenting the system's ability to detect and analyse the compounds in their respective matrices. Column efficiency was also evidenced for all drugs, since the number of theoretical plates (T. Plates), tailing factor (T. Factor), and resolution were compliant with ICH criteria (CDER, 1994).

A.2.2.3 Limits of Detection and Quantification

LOD and LOQ values are summarized in Table A2.5.

Table A2.5 – LOD and LOQ values.

Drug	LOD (ng/mL)	LOQ (ng/mL)
Bifonazole IVPT samples	1.01	3.05
Bifonazole Mass balance samples	0.38	1.16
Diclofenac IVPT samples	4.93	14.93
Diclofenac mass balance samples	2.28	5.36

A.2.2.4 Linearity

Sample concentration was interpolated by using the regression results obtained from calibration curves. These are often calculated through the least squares method that estimates the correlation coefficient (R^2) and the regression equation (CDER, 1994; CPMP/ICH/381/95, 2005). Table A2.6 depicts linearity studies results.

Table A2.6 – Results obtained from the regression analysis using the least squares method for all studied drugs. Results are expressed as mean ± standard deviation (SD), n > 4. Please note that a weighted linear regression was performed on calibration data using $1/x^2$ as the weighting factor.

Condition	Range	Mean R^2	Mean slope	Mean intercept
Bifonazole IVPT samples	0.05-5 µg/mL	0.9999 ± 0.0001	127,368 ± 32,561	18,554 ± 2871
	5 - 50 µg/mL	0.9997 ± 0.0003	219,863 ± 61,655	-516,812 ± 278,696
Bifonazole Mass Balance Samples	0.25-100 µg/mL	0.9999 ± 0.0001	273,785 ± 2825	29,925 ± 24,776
Diclofenac IVPT samples	0.01-100 µg/mL	0.9999 ± 0.0001	62,365 ± 845	291 ± 215
Diclofenac Mass Balance Samples	1-200 µg/mL	0.999 ± 0.0012	59,351 ± 818	10,387 ± 4427

All R^2 were superior to 0.99 and, therefore, evidence is provided regarding data quality and linearity in the proposed range.

A.2.2.5 Accuracy and Precision

The evaluation of precision and accuracy was assessed at both intraday and interday levels.

According to our results, displayed in Table A2.7, RSD values were compliant even for the lowest concentration QC. The same trend was also verified for the accuracy results. These results substantiate that the developed methods are accurate, reliable and reproducible (CDER, 1994; CPMP/ICH/381/95, 2005).

Table A2.7 – Intraday and interday accuracy and precision results for all drugs. Results are expressed as mean ± SD (n=5).

Drug		Intraday (n=5)			Interday (n=15)		
	QC	Measured concentration	Accuracy (%)	Precision (%)	Measured concentration	Accuracy (%)	Precision (%)
Bifonazole IVPT samples	0.5	0.49 ± 0.02	2.85	3.33	0.49 ± 0.06	1.68	12.02
	2	1.89 ± 0.09	5.65	4.84	1.88 ± 0.10	5.80	5.24
	20	21.1 ± 0.7	5.38	3.53	21.0 ± 0.9	-5.22	4.18
Bifonazole Mass Balance Samples	2.5	2.44 ± 0.02	2.49	0.96	2.44 ± 0.16	2.49	6.67
	20	20.2 ± 0.2	-0.93	0.89	20.2 ± 0.3	-0.93	1.49
	60	59.5 ± 0.5	5.38	0.79	59.5 ± 1.0	0.76	1.76
Diclofenac IVPT samples	0.1	0.1 ± 0.01	-1.75	6.65	0.1 ± 0.01	-1.02	10.46
	2.5	2.48 ± 0.05	0.70	2.10	2.48 ± 0.06	0.70	2.44
	20	20.3 ± 0.3	5.38	1.53	20.3 ± 0.4	-1.41	2.24
Diclofenac Mass Balance Samples	2.5	2.5 ± 0.06	-0.25	2.55	2.47 ± 0.18	1.31	7.10
	30	30.0 ± 0.6	-0.13	2.16	30.0 ± 0.9	-0.13	3.09
	80	79.3 ± 0.9	5.38	1.15	79.3 ± 1.1	0.88	1.44

A.2.2.6 Stability

Stability testing should mimic common sampling procedures, so as to provide reliable data analysis. The stability conditions assessed included autosampler and short-term stability. Results are presented in Table A2.8. According to the results, it can be inferred that the analytes are stable under the studied conditions, since precision and accuracy requirements were met.

Table A2.8 – Autosampler and short-term stability of quality control (QC) samples. Results are expressed as mean ± SD (n=5).

Stability condition		Autosampler			Short term stability		
Drug	**QC**	**Measured concentration**	**Accuracy (%)**	**Precision (%)**	**Measured concentration**	**Accuracy (%)**	**Precision (%)**
Bifonazole IVPT samples	0.5	0.47 ± 0.03	-3.07	7.15	0.47 ± 0.02	-6.09	4.25
	2	2.19 ± 0.13	9.39	6.04	2.13 ± 0.03	6.40	1.55
	20	20.7 ± 0.5	3.66	2.58	20.5 ± 1.15	2.73	5.59
Bifonazole Mass Balance Samples	2.5	2.59 ± 0.05	3.47	1.74	2.60 ± 0.03	3.92	1.22
	20	20.1 ± 0.14	0.46	0.70	20.2 ± 0.07	0.99	0.35
	60	59 ± 0.47	-1.66	0.79	58.81 ± 0.1	-1.99	0.17
Diclofenac IVPT samples	0.1	0.09 ± 0.01	-11.97	5.93	0.10 ± 0.004	-3.68	3.99
	2.5	2.51 ± 0.05	0.46	2.08	2.49 ± 0.015	-0.40	0.59
	20	20.3 ± 0.5	1.47	2.32	20.2 ± 0.3	1.02	1.47
Diclofenac Mass Balance Samples	2.5	2.4 ± 0.04	-4.08	1.68	2.44 ± 0.02	-2.47	0.99
	30	30.7 ± 0.5	2.41	1.66	29.8 ± 1.5	-0.77	5.11
	80	81.1 ± 0.8	1.36	0.96	77.6 ± 0.6	-3.00	0.81

APPENDIX B

The present Appendix describes the statistical approaches used for confidence interval calculations.

90% Confidence Interval Calculation according to SUPAC-SS criteria (FDA)

In Chapter 4, to evaluate the sensitivity of the diclofenac emulgel IVRT method, the confidence intervals (CI) pertaining to the lower/higher strengths formulations *vs.* the nominal formulation were calculated based on the Wilcoxon Rank Sum/Mann-Whitney rank test. This statistical test, described in the SUPAC-SS guidance, regards a nonparametric test. According to the FDA, this is suitable for IVRT data since it minimizes the presence of outliers, which, as extensively reviewed throughout this thesis, are expected to occur in IVRT experiments (Conover, 1999; FDA, 1997). The next example regards the Wilcoxon Rank Sum/Mann-Whitney rank test for the 90% CI computed between the 1% *vs.* 0.5% diclofenac formulations, described in Chapter 4.

The first step evolved in the computation of this CI is to form a 144 element matrix, which corresponds to the 12 x 12 individual TP/RP ratios. This is illustrated in Table B.1, where the IVRR of the 1% diclofenac formulation (RP) are listed down the left margin of the table, and IVRR values of the lower strength formulation (TP) are displayed across the top of the table. All the individual T/R ratios regard the entries in the body of the table.

Table B.1 – 90% Confidence interval calculation according to the Wilcoxon Rank Sum/Mann-Whitney rank test (FDA).

	402.1	453.5	529.8	421.9	425.3	405.0	490.3	475.5	511.7	516.9	444.4	404.4
655.9	0.6131	0.6915	0.8078	0.6432	0.6484	0.6175	0.7475	0.7249	0.7802	0.7881	0.6775	0.6165
767.9	0.5236	0.5906	0.6899	0.5494	0.5538	0.5274	0.6385	0.6191	0.6664	0.6731	0.5787	0.5266
704.8	0.5705	0.6435	0.7518	0.5986	0.6035	0.5747	0.6957	0.6746	0.7261	0.7334	0.6305	0.5738
636.3	0.6319	0.7128	0.8326	0.6630	0.6684	0.6365	0.7705	0.7472	0.8042	0.8124	0.6984	0.6355
652.8	0.6160	0.6948	0.8116	0.6463	0.6515	0.6204	0.7511	0.7284	0.7839	0.7919	0.6808	0.6195
604.9	0.6648	0.7498	0.8759	0.6975	0.7031	0.6696	0.8106	0.7861	0.8460	0.8546	0.7347	0.6686
644.8	0.6236	0.7034	0.8216	0.6543	0.6596	0.6281	0.7603	0.7373	0.7936	0.8016	0.6892	0.6271
803.0	0.5008	0.5648	0.6598	0.5254	0.5297	0.5044	0.6106	0.5921	0.6373	0.6438	0.5534	0.5036
724.6	0.5549	0.6259	0.7312	0.5822	0.5869	0.5589	0.6766	0.6562	0.7062	0.7134	0.6133	0.5581
649.8	0.6188	0.6979	0.8153	0.6492	0.6545	0.6232	0.7545	0.7316	0.7875	0.7954	0.6838	0.6223
654.4	0.6145	0.6930	0.8096	0.6447	0.6499	0.6189	0.7492	0.7265	0.7820	0.7899	0.6791	0.6179
617.0	0.6518	0.7351	0.8588	0.6838	0.6894	0.6565	0.7947	0.7707	0.8295	0.8379	0.7203	0.6555

The second step to determine the confidence interval is to rank these 144 individual TP/RP ratios from lowest to highest. In the third step, the 43rd and the 121st ordered individual ratios regard the lower and upper limits, respectively, of the 90% CI for the ratio of the median TP IVRR over the median IVRR for RP.

For this specific case study, the CI corresponds to 0.6271 to 0.7861, or in percentage terms to 62.71% to 78.61%.

90% Confidence Interval Calculation according to EMA criteria

To calculate the CI according to EMA directives, the procedures described in the bioequivalence guideline were followed (EMA, 2010). For this, one should take into account that the terms sequence, subject within sequence, period and formulation do not apply, since we are considering *in vitro* studies. In the guideline it is stated that a non-parametric analysis is not acceptable (EMA, 2010).

The next example regards the calculation of the 90% CI, following EMA directives, of the IVRR of batch 1 *vs.* IVRR of batch 2 of the hydrocortisone cream studied in Chapter 5 (HC_1 *vs*. HC_2).

Firstly, the data was natural log transformed. Then, the means and the standard deviations were calculated. This was followed by obtaining the ratio of the two back-transformed averages for IVRR, please see Table B.2.

Table B.2 – Step 1 for 90% CI calculation according to EMA bioequivalence guideline. The data herein presented corresponds to the IVRR (μg/cm^2/√h) attained for hydrocortisone cream batch 1 and batch 2.

	IVRR HC_1	**IVRR HC_2**	**Ln (IVRR HC_1)**	**Ln (IVRR HC_2)**
	69.227	76.133	4.237	4.332
	74.593	87.596	4.312	4.473
	84.603	80.719	4.438	4.391
	76.921	77.496	4.343	4.350
	69.583	81.129	4.243	4.396
	88.825	77.973	4.487	4.356
	63.901	90.563	4.157	4.506
	70.721	74.324	4.259	4.308
	68.501	77.622	4.227	4.352
	69.916	81.112	4.247	4.396
	59.210	78.366	4.081	4.361
	75.428	84.530	4.323	4.437
Mean			4.279	4.388
SD			0.111	0.058
Exp Mean			72.20	80.50
Ln Ratio average			-0.109	

The next step included the calculation of the 90% confidence interval, according to Equations (B.1) and (B.2).

$$S_p = \sqrt{\frac{(n_1-1)\times s_1^2+(n_2-1)\times s_2^2}{n_1+n_2-2}} \quad \text{(B.1)}$$

$$\frac{\overline{X_1}}{\overline{X_2}} \pm t_{1-\alpha/2,n_1+n_2-2,} S_p \sqrt{\frac{1}{n_1}+\frac{1}{n_2}} \quad \text{(B.2)}$$

where $\bar{X}$ is the mean value to evaluate the test ($\overline{X_1}$) or reference product ($\overline{X_2}$), t1-α/2 is the Student's t value for α = 0.90, s is the standard deviation, and n the number of observations.

Please take into account that in this example the terms TP and RP do not apply, since all data was attained with different batches of the RP. Nevertheless, for CI calculations aiming bioequivalence documentation the ratios should be calculated as TP/RP.

Table B.3 – Step 4 for 90% CI calculation according to EMA bioequivalence guideline. The data herein presented corresponds to the IVRR (μg/cm^2/√h) attained for hydrocortisone cream batch 1 *vs.* batch 2.

S_p	0.089
$t_{1-\alpha/2,n_1+n_2-2,}S_p\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}$	0.06
Lower CI	-0.171
Upper CI	-0.046
Antilog	
Lower CI (%)	84.27
Upper CI (%)	95.46

90% Confidence Interval based on the pairwise difference between population means.

In Table 5.9, the 90% confidence intervals regarding the microstructure parameters were calculated based on the difference between population means.

The following formula was used:

$$90\%\ CI = (M_1 - M_2) \pm t \times \sqrt{\left(\frac{s_p^2}{n_1}\right) + \left(\frac{s_p^2}{n_2}\right)} \quad \text{(B.3)}$$

where, M_1 and M_2 regard the sample means of the parameter being considered, t corresponds to the t statistic determined by confidence level, s the standard deviation and n the sample size.

This calculation assumes that the two population variances being compared are equal (i.e., it uses a pooled standard deviation in order to calculate the standard error portion of the confidence interval calculation). If the CI encloses the value 0, we can ensure equivalence similitude.

For the clotrimazole case study in Chapter 3 and in Chapter 6, for simplicity, the CI values were expressed in percentage and calculated according to EMA approach.

90% Confidence Interval calculated through the SABE approach (FDA)

As mentioned in Chapter 6, the CI calculations pertaining to the IVPT data were determined following two approaches – the EMA and the FDA approach. The next section summarizes the main procedures used to estimate the 90% CIs of IVPT endpoints according to the FDA approach. All calculations followed the FDA acyclovir guidances, as well as the work by A. Pensado and collaborators (FDA, 2016c; Pensado *et al.*, 2019).

The example herein selected refers to the A_{TOTAL} endpoint retrieved from pivotal IVPT data for the pairwise bifonazole 10 mg/g cream comparison RP1 *vs.* TP1. This rationale is also applicable to J_{MAX}. In this study, the above mentioned products were tested in the skin from 8 donors and 2 replicates were always considered, nr=2 and n=8. Please note that the results attained from the pilot study were not regarded for the statistical analysis, as per FDA requirements (FDA, 2016c).

Bioequivalence calculations were performed based on this specific study design – a balanced study with *nr* replicated measurements of each formulation in a total of *n* subjects for a metric determined in the k^{th} replicated site treated with formulation *i* on subject *j* (M_{ijk}).

M_{ijk} regards a metric which always represents a positive number, such as the mass per unit area of drug permeated at the end of the IVPT study (A_{TOTAL}). To evaluate bioequivalence in such conditions, M_{ijk} was naturally log-transformed, which will be further defined as Z_{ijk}, then it was determined in each subject and then averaged across all subjects as follows:

$Z_{\mathrm{ijk}} = \ln(M_{\mathrm{ijk}})$ = naturaly log transformed value of M_{ijk}

$\bar{Z}_{ij} = \frac{1}{nr}\sum_{k=1}^{nr} Z_{ijk}$ = mean of the log-transformed metric for formulation *i* in each subject *j*

$\bar{M}_{ij} = \exp(\bar{Z}_{ij})$ = Geometric mean of the metric for formulation *i* in each subject *j*

$\bar{I}_j = \bar{Z}_{1j} - \bar{Z}_{2j}$ = Difference between formulations 1 and 2 of the mean of the log transformed metric in each subject *j*

$\bar{I} = \frac{1}{n}\sum_{j=1}^{n} \bar{I}_j$ = Mean of the differences between formulations 1 and 2 of the log transformed metric averaged over n subjects

$s_i^2 = \frac{1}{n-1}\sum_{j=1}^{n}(\bar{I}_j - \bar{I}_j)^2$ = inter-subject variance of the differences between formulations 1 and 2 of the log transformed metric

The within variance for a formulation I measured in subject j is calculated as follows:

$s_{wi}^2 = \frac{1}{n(nr-1)} \sum_{j=1}^{n} \sum_{k=1}^{nr} \left[Z_{ijk} - \bar{Z}_{ij} \right]^2$= within subject variance of the log transformed metric for formulation i

Note that for a balaced design, estimates for $\bar{I}$ and s_i are the same for $\bar{I}_j$ calculated from the difference of the mean of the log trnasformed metric (i.e. $\bar{I}_j = \bar{Z}_{1j} - \bar{Z}_{2j}$) or calculated as the mean of randomely matched replicates, such as $\bar{I}_j = \frac{1}{nr} \sum_{k=1}^{nr} (Z_{1jk} - Z_{2jk})$ as specified in (Pensado *et al.*, 2019).

The following tables specify the calculations performed when evaluating bioequivalence of the bifonazol 10 mg/g cream RP1 and the test product TP1. Furthermore, the determination of the within subject variance for RP1 is listed on Table B.6.

Traditional average bioequivalence assessment

Traditionally, formulation 1 and 2 are considered to be equivalent for a margin m if:

$$\left| \bar{I} \pm \delta_{i,90\%} \right| \leq \ln(m)$$

$\delta_{i,90\%} = \frac{s_i \times t_{0.95,n-1}}{\sqrt{n}}$ = projected half-width of the 90% confidence interval (CI) for the population mean difference. In this, $t_{0.95,n-1}$ regards the 95% percentile of the student´s t distribution with n-1 degrees of freedom. The value m, traditionaly corresponds to m=1.25 which corresponds to the [0.8 - 1.25] confidence interval.

Results are presented as the geometric mean ratio (GMR) of the selected metric for formulation 1 compared with formulation 2 and the projected lower and upper 90% CIs for the population mean ratio ($GMR_{90\%,upper}$ and $GMR_{90\%,lower}$ respectively) calculated as:

$$GMR = \exp(\bar{I})$$

$$GMR_{90\%,lower} = \exp(\bar{I} - \delta_{90\%}) \text{ and } GMR_{90\%,upper} = \exp(\bar{I} + \delta_{90\%})$$

Example calculations of this traditional BE assessment methodology for RP1 and TP1 are provided in Table B.5. Even though the GMR, as well as the lower and upper CI can be naturaly or base 10 logarithmic transformed, given that the anti-log step is consistent with the type of log transformation used in this work the natural log transformation was in all cases selected. The reasons that support this selection are related with the scaled average bioequivalence procedure which is specific to the type of logarithmic transformation (Pensado *et al.*, 2019).

Scaled average bioequivalence assessment (SABE)

As previously mentioned in Chapter 6, this methodology applies when the within subject standard deviation for the RP is S_{w2}>0.294. In this condition, the RP and the TP can be considered bioequivalent if the geometric mean ratio (GMR), calculated as previously described, falls within the range [1/m, m] for the selected bioequivalence margin (which is traditionally set to 1.25) and if the upper 95% confidence interval (SCI_{UB}) for the quantity $(\mu_1 - \mu_2)^2 - \sigma_{w2}^2 \left(\frac{\ln(m)^2}{0.25}\right)$ is less than, or equal to zero.

The parameters μ_1 and μ_2 regard the population means of the test and reference product, respectively, and σ_{w2}^2 corresponds to the variance of the reference population. Please note that all parameters should be calculated for the log transformed metric. The calculation of SCI_{UB} as described by Pensado *et al.* is following presented (Pensado *et al.*, 2019):

$$\theta = \left[\frac{\ln(m)}{\sigma_{w0}}\right]^2$$

$$X = \bar{I}^2 - \frac{s_I^2}{n}$$

$$Y = -\theta s_{w2}^2$$

$$X'_\beta = \left(|\bar{I}| + t_{0.95,n-1}\sqrt{\frac{s_I^2}{n}}\right)^2$$

$$Y'_\beta = -\theta \frac{n(nr-1)s_{w2}^2}{\chi^2_{0.95,n(nr-1)}}$$

$$V = \left(X'_\beta - X\right)\left|X'_\beta - X\right| + \left(Y'_\beta - Y\right)\left|Y'_\beta - Y\right|$$

$$SCI_{UB} = X + Y + \left(\frac{|V|}{V}\right) \times \sqrt{|V|}$$

In this, $\sigma_{w0} = 0.25$ (regulatory constant), $t_{0.95,n-1}$ is defined as the traditional ABE assessment and $\chi^2_{0.95,n(nr-1)}$ corresponds to the 95th percentile of the Chi-Square distribution with *n*(*nr*-1) degrees of freedom.

Example SABE calculations for RP1 and TP1 are provided in Table B.7. As previously mentioned, all calculations herein presented assume that the data was naturally log transformed.

Table B.4 – Bifonazole amounts (µg/cm^2) and the log transformed values retrieved in the end of the IVPT study (48 h) in the skin replicate samples (Q_{ijk}, replicate k of formulation i in donor j) from each of the skin of the 8 donors used for the RP1 *vs.* TP1 batch comparison.

	RP1 - A_{TOTAL} (µg/cm^2)						TP1 - A_{TOTAL} (µg/cm^2)					
Donor number	Q_{ij1}	Q_{ij2}	$\ln(Q_{ij1})$	$\ln(Q_{ij2})$	$\ln \overline{Q_{ij}}$	$\overline{Q_{ij}}$	Q_{ij1}	Q_{ij2}	$\ln(Q_{ij1})$	$\ln(Q_{ij2})$	$\ln \overline{Q_{ij}}$	$\overline{Q_{ij}}$
4	30.32	13.41	3.41	2.60	3.00	20.17	3.59	64.27	1.28	4.16	2.72	15.20
5	23.30	64.48	3.15	4.17	3.66	38.76	23.77	4.72	3.17	1.55	2.36	10.59
6	162.16	32.83	5.09	3.49	4.29	72.96	121.37	43.43	4.80	3.77	4.28	72.60
7	51.55	55.08	3.94	4.01	3.98	53.29	74.19	60.56	4.31	4.10	4.21	67.03
8	21.74	144.18	3.08	4.97	4.03	55.99	85.47	11.08	4.45	2.41	3.43	30.78
10	65.17	166.82	4.18	5.12	4.65	104.27	150.41	25.84	5.01	3.25	4.13	62.34
11	81.32	19.34	4.40	2.96	3.68	39.66	134.93	92.76	4.90	4.53	4.72	111.88
12	83.85	22.92	4.43	3.13	3.78	43.84	104.88	122.14	4.65	4.81	4.73	113.18
Mean					3.88						3.82	
SD					0.49						0.89	
90% CI					0.00						0.60	
Lower CI					2.96						3.22	
Upper CI					4.80						4.42	
Anti log												
Mean, $\overline{Q_i}$					48.55						45.70	
Lower CI					19.33						25.12	
Upper CI					121.90						83.14	

Table B.5 – Traditional bioequivalence evaluation of the TP1 *vs*. RP1 (designated as formulations 1 and 2, respectively). The calculations were performed based on the difference between both formulations of the log transformed bifonazole amounts retrieved at the end of the IVPT study $\overline{I_J}$.

Donor number	$\overline{I_J} = \overline{Z_{1J}} - \overline{Z_{2J}}$ (µg/cm²)
4	-0.2830
5	-1.2970
6	-0.0049
7	0.2295
8	-0.5983
10	-0.5144
11	1.0371
12	0.9484
$\bar{I}$	-0.0603
S_i	0.7913
$\delta_{I,90\%}$	0.5301
$\bar{I} - \delta_{I,90\%}$	-0.5904
$\bar{I} + \delta_{I,90\%}$	0.4697
GMR	0.9414
$GMR_{90\%\ lower}$	0.5541
$GMR_{90\%upper}$	1.5996

Table B.6 – Calculation of the within subject variance (S^2_{W2}) for the A_{TOTAL} parameter.

$[Z_{2jk} - Z_{2j}]^2$		
Donor number	**K=1**	**K=2**
4	0.1662	0.1662
5	0.2591	0.2591
6	0.6379	0.6379
7	0.0011	0.0011
8	0.8948	0.8948
10	0.2208	0.2208
11	0.5157	0.5157
12	0.4205	0.4205
Sum	6.232	
S^2_{W2}	0.779	
S_{W2}	0.883	

Table B.7 – Scaled average bioequivalence (SABE) calculations comparing the bifonazole RP1 *vs.* TP1 batch (designated as formulations 1 and 2, respectively) for the log transformed bifonazole amount (A_{TOTAL}) for m = 1.25.

σ_{w0}	m	θ	n	nr	$t_{0.95,(n-1)}$	$\chi^2_{0.95,nr(n-1)}$					
0.25	1.25	0.797	8	2	1.8946	15.51					
Calculated values	$\bar{I}$	S_i^2	S_{w2}	X	Y	X'_β	Y'_β	$X'_\beta - X$	$Y'_\beta - Y$	V	SCI_{UB}	GMR
	-0.0603	0.6262	0.8826	-0.0746	-0.6206	0.3486	-0.3202	0.4232	0.3005	0.2694	-0.1763	0.9414

90% Confidence Interval for IVPT data (EMA)

As previously mentioned, the CI calculations pertaining to the IVPT data were determined following two approaches – the EMA and the FDA approach. The next section summarizes the main procedures used to estimate the 90% CIs of IVPT endpoints according to the EMA approach.

Firstly, the data was natural log transformed. Then, the arithmetic mean of all individual T-R differences was calculated. Subsequently, the variability within subjects was calculated as the difference between each individual subject difference T-R and the previously determined mean. These squared differences were summed to obtain the sum of squares. The sum of squares was divided by n-1 degrees of freedom, to obtain the variance of the differences.

The standard error of the differences was obtained by dividing the variance by n and then calculating the square root. The confidence interval was attained by the usual expression

$$X \pm t \times \frac{s}{\sqrt{n}} \tag{B.4}$$

wherein X is the previously calculated mean of all individual T-R differences , *t* is the *t*-value reporting to a 90% CI with n-1 degrees of freedom, s regards the standard error calculated as previously described, and finally, n is the sample size.

Table B.8 – Bifonazole amounts (µg/cm^2) and the log transformed values retrieved in the end of the IVPT study (48 h) in the skin replicate samples (Q_{ijk}, replicate k of formulation i in donor j) from each of the skin of the 8 donors used for the RP1 *vs.* TP1 batch comparison.

	RP1 - A_{TOTAL} (µg/cm^2)						TP1 - A_{TOTAL} (µg/cm^2)					
Donor number	Q_{ij1}	Q_{ij2}	$\ln(Q_{ij1})$	$\ln(Q_{ij2})$	$\ln \overline{Q_{ij}}$	$\overline{Q_{ij}}$	Q_{ij1}	Q_{ij2}	$\ln(Q_{ij1})$	$\ln(Q_{ij2})$	$\ln \overline{Q_{ij}}$	$\overline{Q_{ij}}$
4	30.32	13.41	3.41	2.60	3.00	20.17	3.59	64.27	1.28	4.16	2.72	15.20
5	23.30	64.48	3.15	4.17	3.66	38.76	23.77	4.72	3.17	1.55	2.36	10.59
6	162.16	32.83	5.09	3.49	4.29	72.96	121.37	43.43	4.80	3.77	4.28	72.60
7	51.55	55.08	3.94	4.01	3.98	53.29	74.19	60.56	4.31	4.10	4.21	67.03
8	21.74	144.18	3.08	4.97	4.03	55.99	85.47	11.08	4.45	2.41	3.43	30.78
10	65.17	166.82	4.18	5.12	4.65	104.27	150.41	25.84	5.01	3.25	4.13	62.34
11	81.32	19.34	4.40	2.96	3.68	39.66	134.93	92.76	4.90	4.53	4.72	111.88
12	83.85	22.92	4.43	3.13	3.78	43.84	104.88	122.14	4.65	4.81	4.73	113.18

Table B.9 – EMA IVPT data bioequivalence evaluation of the TP1 *vs*. RP1 (designated as formulations 1 and 2, respectively). The calculations were performed based on the difference between both formulations of the log transformed bifonazole amounts retrieved at the end of the IVPT study $\overline{I_J}$.

Donor number	$\overline{I_J} = \overline{Z_{1J}} - \overline{Z_{2J}}$ (µg/cm²)
4	-0.2830
5	-1.2970
6	-0.0049
7	0.2295
8	-0.5983
10	-0.5144
11	1.0371
12	0.9484
$\bar{I}$	-0.0603

Table B.10 – EMA IVPT data bioequivalence evaluation of the TP1 *vs*. RP1 (designated as formulations 1 and 2, respectively). The calculations were performed based on the difference between both formulations of the log transformed bifonazole amounts retrieved at the end of the IVPT study $\overline{I_J}$.

Donor number	$[\overline{I_J} - \bar{I}]^2$ (µg/cm²)
4	0.0496
5	1.5293
6	0.0031
7	0.0840
8	0.2894
10	0.2062
11	1.2043
12	1.0176
Sum	4.3835
Lower CI (%)	78.06
Upper CI (%)	113.55