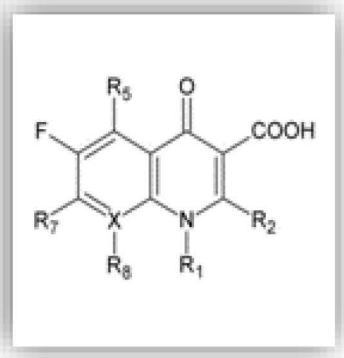

Joana Maria Ramalho de Almeida e Sousa

Evaluation of Intranasal Administration of Fluoroquinolones and their Potential in the Treatment of Chronic Rhinosinusitis: Ciprofloxacin and Levofloxacin

Tese de Doutoramento em Ciências Farmacêuticas, ramo de Farmacologia e Farmacoterapia,
orientada pelo Professor Doutor Amílcar Celta Falcão Ramos Ferreira e pelo Professor Doutor Gilberto Lourenço Alves,
apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Julho 2017

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Universidade de Coimbra

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FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

The experimental work presented in this thesis was developed under the scientific supervision of Professor Amílcar Celta Falcão Ramos Ferreira and Professor Gilberto Lourenço Alves at the Laboratory of Pharmacology, Faculty of Pharmacy, University of Coimbra and at the Health Sciences Research Centre, Faculty of Health Sciences, University of Beira Interior.

O trabalho experimental apresentado nesta tese foi desenvolvido sob a orientação científica do Professor Doutor Amílcar Celta Falcão Ramos Ferreira e do Professor Doutor Gilberto Lourenço Alves no Laboratório de Farmacologia da Faculdade de Farmácia da Universidade de Coimbra e no Centro de Investigação em Ciências da Saúde da Faculdade de Ciências da Saúde da Universidade da Beira Interior.

• U C •

CNC
CENTER FOR NEUROSCIENCE AND CELL BIOLOGY
UNIVERSITY OF COIMBRA, PORTUGAL

CICS-UBI
Centro de Investigação em Ciências da Saúde
Health Sciences Research Centre

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Pelo sonho é que vamos,
comovidos e mudos.

Chegamos? Não chegamos?
Haja ou não haja frutos,
pelo sonho é que vamos.

Basta a fé no que temos.
Basta a esperança naquilo
que talvez não teremos.
Basta que a alma demos,
com a mesma alegria,
ao que desconhecemos
e ao que é do dia a dia.

Chegamos? Não chegamos?
– Partimos. Vamos. Somos.

Sebastião da Gama

À minha Família

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Eterna Saudade…

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LIST OF ABBREVIATIONS

ANM	Anterior nasal mucosa
AUC	Area under the concentration-time curve
AUC_{all}	AUC from time zero to the last time point of the study
AUC_{extrap} (%)	Percentage of AUC extrapolated from time of last measurable concentration to infinity
$AUC_{t\text{-}inf}$	AUC extrapolated from time of last measurable concentration to infinity
$AUC_{0\text{-}inf}$	AUC from time zero to infinity
$AUC_{0\text{-}t}$	AUC from time zero to time of last measurable drug concentration
$AUC_{0\text{-}24h}$	AUC from time zero to 24 h
Bias	Deviation from nominal values
C_{last}	Last observed drug concentration
C_{max}	Maximum concentration
CAZS	Citric acid/zwitterionic surfactant
CIP	Ciprofloxacin
CNS	Central nervous system
CRS	Chronic rhinossinusitis
CV	Coefficient of variation
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
EPOS	European Position Paper on Rhinosinusitis and Nasal Polyps
ESS	Endoscopic sinus surgery
FD	Fluorescence detection
FDA	US Food and Drug Administration
FISH	Fluorescent in-situ hybridization
FQ	Fluoroquinolone
GA^2LEN	Global Allergy and Asthma European Network
GAT	Gatifloxacin
H. influenza	*Haemophilus influenza*
HPLC	High performance liquid chromatography
ID	Individual
IgE	Immunoglobulin E

IN	Intranasal
IS	Internal standard
IV	Intravenous
k_e	Apparent elimination rate constant
LC	Liquid chromatography
LEV	Levofloxacin
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOM	Lomefloxacin
M. catarrhalis	*Moraxella catarrhalis*
MCC	Mucociliary clearance
MIC	Minimum inhibitory concentration
MOX	Moxifloxacin
MRT	Mean residence time
NOR	Norfloxacin
$P_{octanol/water}$	Partition coefficient between 1-octanol ans water
PAE	Post-antibiotic effect
P. aeruginosa	*Pseudomonas aeruginosa*
PF-127	Pluronic F-127
PNM	Posterior nasal mucosa
QC	Quality control
r^2	Coefficient of determination
RP-HPLC	Reversed-phase high performance liquid chromatography
S. aureus	*Staphylococcus aureus*
S. pneumonia	*Streptococcus pneumonia*
t_0	Time zero
t_{max}	Time to reach the maximum concentration
$t_{1/2}$	Elimination half-life
TEA	Triethylamine
TRO	Trovafloxacin
US	United States
w/v	Weight/volume
w/w	Weight/weight

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PUBBLICATIONS

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ABSTRACT

Chronic rhinosinusitis is a persistent inflammation of the nasal cavity and paranasal sinuses mucosa with high prevalence and significant impact in patients´ quality of life. Although the exact etiology and pathophysiology of this multifactorial and difficult to treat disease are still unclear, there is growing evidence about the involvement of bacterial biofilms and their association with the refractory and chronic nature of the disease. Bacteria in biofilms show an increased resistance to antibiotics that can be as high as 1000-fold that of their corresponding planktonic form, which explains the lack of effectiveness of systemic administration of antibiotics in this clinical condition. High concentrations required to eradicate bacteria are difficult to attain without significant risks of systemic toxicity using the conventional routes of administration. Intranasal administration emerges as an alternative option to deliver drugs directly to the target site (sinonasal mucosa) achieving high local concentrations with minimal systemic exposure and consequent systemic adverse effects.

The aim of the present thesis was to evaluate the potential of intranasal administration of fluoroquinolones by comparing their pharmacokinetic behavior after intranasal and intravenous delivery in Wistar rat biological matrices adequate to probe the risks and benefits of this topical strategy. The progression of experimental work led to the selection of ciprofloxacin and levofloxacin which are two of the most commonly used and well-known fluoroquinolones. To support the *in vivo* pharmacokinetic studies and thus achieve the above objectives, a high performance liquid chromatography method coupled with fluorescence detection was developed and validated to quantify ciprofloxacin and levofloxacin in rat nasal mucosa, plasma and olfactory bulb, probing drug efficacy as well as systemic and central nervous system safety, respectively.

For intranasal administration a thermoreversible *in situ* gel was used to deliver ciprofloxacin and levofloxacin to Wistar rats at a dose of 0.24 mg/kg, a much lower dose than that administered by intravenous route, namely 10 mg/kg.

After intranasal administration, markedly higher concentrations were attained in the anterior nasal mucosa compared to those found in the posterior region. This

heterogeneous deposition pattern of formulation in nasal cavity contrasts with the intravenous homogeneous distribution and makes the application site more advantageous for this topical approach.

Dose-normalized concentrations and exposure pharmacokinetic parameters obtained for nasal mucosa were two or one order of magnitude higher – in anterior and posterior nasal regions, respectively – by intranasal administration than by intravenous route. A similar comparison for plasma and olfactory bulb lead to the conclusion of a lower systemic exposure after topical intranasal administration and of a possible contribution of drug direct nose-to-brain transport that should be carefully taken into account. The results confirm the significant advantage of topical intranasal administration to deliver ciprofloxacin and levofloxacin to the biophase in comparison with the intravenous administration. Given the typically lower doses used for intranasal route, both systemic and central nervous system safe profiles were also demonstrated by the minimal or negligible values attained with the intranasal dose of 0.24mg/kg.

Therefore, intranasal administration of topical-acting fluoroquinolones may represent a promising and safe alternative approach to be implemented in the management of chronic rhinosinusitis.

KEYWORDS: Chronic rhinosinusitis, ciprofloxacin, high-performance liquid chromatography, *in vivo* studies, intranasal administration, levofloxacin, pharmacokinetics.

RESUMO

A rinossinusite crónica é uma inflamação persistente da mucosa da cavidade nasal e dos seios perinasais com elevada prevalência e um impacto significativo na qualidade de vida dos doentes. Embora a etiopatologia desta doença mulifatorial e difícil de tratar permaneça ainda por elucidar, existe evidência crescente do envolvimento de biofilmes bacterianos, sendo este o aspeto mais apontado para explicar a natureza crónica e refratária da doença. Com efeito as bactérias em biofilmes revelam uma resistência aos antibióticos que pode ser até 1000 vezes superior à da correspondente forma planctónica, o que explica a ineficácia terapêutica da administração sistémica de antibióticos. Utilizando vias convencionais de administração, as elevadas concentrações necessárias para erradicação das bactérias dificilmente se atingem sem riscos significativos de toxicidade sistémica. A administração tópica intranasal surge assim como uma estratégia alternativa para a entrega direta de fármacos na biofase (mucosa naso-sinusal) por forma a obter elevadas concentrações locais e exposição sistémica mínima, sem os efeitos adversos/tóxicos associados.

O objetivo central desta dissertação foi a avaliação do potencial da administração intranasal de fluoroquinolonas, através da análise do risco/benefício envolvidos neste tipo de administração, utilizando matrizes biológicas estreitamente relacionadas com a segurança e a eficácia dos fármacos e comparando o respectivo comportamento farmacocinético após administração intranasal e intravenosa dos mesmos. A progressão do trabalho experimental conduziu à seleção da ciprofloxacina e levofloxacina, duas das fluoroquinolonas mais conhecidas e frequentemente utilizadas na clínica. Sendo a bioanálise um passo essencial de suporte aos estudos *in vivo* farmacocinéticos, procedeu-se ao desenvolvimento e validação de uma técnica de cromatografia líquida de alta eficiência com deteção por fluorescência, para quantificar a ciprofloxacina e levofloxacina na mucosa nasal, plasma e bolbo olfativo de ratos Wistar.

Um gel com propriedades termorreversíveis, capaz de gelificar na cavidade nasal, foi utilizado para a entrega tópica intranasal de uma dose de 0,24 mg/kg de ciprofloxacina e de levofloxacina, muito inferior à de 10 mg/kg administrada por via intravenosa.

Após administração intranasal as concentrações obtidas na mucosa nasal da região anterior foram acentuadamente mais elevadas do que as encontradas na região posterior, esta distribuição heterogénea da formulação na cavidade nasal contrasta com a distribuição homogénea observada por administração intravenosa e põe em evidência a situação mais vantajosa para o local mais próximo da aplicação tópica.

As concentrações e os parâmetros de exposição farmacocinéticos normalizados à dose obtidos na mucosa nasal por administração intranasal foram superiores aos da administração intravenosa - duas ordens de grandeza na região anterior e uma ordem de grandeza na região posterior. Uma comparação idêntica (portanto independente da dose) entre ambas as vias para o plasma e bolbo olfactivo permitiu inferir que a exposição sistémica foi inferior por administração intranasal e que a estreita relação do nariz com o sistema nervoso central não deve ser ignorada face os resultados obtidos no bolbo olfactivo. Confirma-se portanto a vantagem significativa da administração intranasal para entrega da ciprofloxacina e levofloxacina na biofase, face à administração intravenosa. Os valores negligenciáveis ou mínimos atingidos no plasma e bolbo olfactivo após administração de uma dose de 0,24 mg/kg demonstram um perfil seguro tanto a nível sistémico como a nível do sistema nervoso central.

Em conclusão, a administração intranasal de fluoroquinolonas demonstrou ser uma abordagem promissora e segura, podendo representar um contributo importante no tratamento tópico da rinossinusite crónica.

PALAVRAS-CHAVE: Administração intranasal, ciprofloxacina, cromatografia líquida de alta eficiência, estudos *in vivo*, farmacocinética, levofloxacina, rinossinusite crónica.

CHAPTER I

GENERAL INTRODUCTION

I.1. CHRONIC RHINOSINUSITIS

I.1.1. DEFINITION AND DIAGNOSIS

Rhinosinusitis is a group of disorders characterized by inflammation of the nasal cavity and paranasal sinuses mucosa. The term sinusitis was replaced by the currently accepted term rhinosinusitis, because sinusitis is usually preceded by and almost always accompanied by rhinitis. Therefore, as they coexist, the correct terminology used is now rhinosinusitis instead of sinusitis. Rhinosinusitis is traditionally classified by duration as acute (< 4 weeks), subacute (4-12 weeks) or chronic (> 12 weeks, with or without exacerbations) (Benninger et al., 2003; Cauwenberge et al., 2006; Fokkens et al., 2007; Rosenfeld et al., 2015).

Chronic rhinosinusitis (CRS) is increasingly recognized as a significant public health problem with a considerable negative impact on patients' quality of life and socio-economic costs. This is a complex disease and, due to the heterogeneity of its presentation, its definition is challenging (Alobid et al., 2008; Lee and Lane, 2011; Mattila, 2012). In the last decade, a number of international or national guidelines, position papers and consensus documents have been developed on epidemiology, diagnosis and treatment of CRS, summarizing the current knowledge. Two representative examples of such documents are the 2012 update of the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS), initiative of the European Academy of Allergology and Clinical Immunology, and the 2015 updated Clinical Practice Guideline from the American Academy of Otolaryngology-Head and Neck Surgery Foundation. Their common objective is to standardize definitions, improve diagnostic accuracy and guide the management of CRS (Bachert et al., 2014; Fokkens et al., 2012; Rosenfeld et al., 2015).

The diagnosis of CRS is based on the presence, for more than 12 consecutive weeks, of at least two of the following symptoms – nasal blockage/congestion/obstruction, nasal discharge (anterior/posterior nasal drip), facial pain/pressure or reduction or loss of smell (hyposmia) – in combination with either endoscopic signs or computed tomography evidences of nose and sinuses inflammation. According to the EPOS definition criteria, one of the two first symptoms is mandatory.

Symptoms alone can be non-specific and mimicked by order diseases, such as migraine or allergic rhinitis; therefore their confirmation by objective findings is necessary for accuracy of diagnosis. Nasal endoscopy signs include mucopurulent discharge, mucosal edema/obstruction and nasal polyps primarily in middle meatus, whereas computed tomography scanning further detects mucosal thickening/abnormalities/changes, anatomical variations within ostiomeatal complex and/or paranasal sinuses (Cain and Lal, 2013; Fokkens et al., 2012; Piromchai et al., 2013; Rosenfeld et al., 2015). Besides this accepted clinical definition of CRS, most epidemiologic studies are based on symptomatology alone, due to practical limitations in obtaining large population level studies that include endoscopic and radiographic examinations.

I.1.2. EPIDEMIOLOGY AND SOCIOECONOMIC IMPACT

Epidemiologic studies play an important role in assessing CRS distribution, investigating risk factors and supporting health policies. Estimating the true prevalence of CRS is demanding and still a matter of debate. This is due to the paucity of large scale studies and to the shortcomings in the current epidemiologic methods which vary widely, making a direct comparison amongst them difficult and less reliable (Bachert et al., 2014; Cauwenberge et al., 2006; DeConde and Smith, 2017; Hastan et al., 2011). In 2008, the first European international multicentre prevalence study of CRS was conducted as part of the Global Allergy and Asthma European Network (GA^2LEN) project by sending a symptom-based questionnaire consistent with the EPOS definition criteria of CRS to 12 countries. The overall prevalence of CRS was 10.9%, ranging from 6.9% (in Finland, Helsinki) to 27.1% (in Portugal, Coimbra) (Hastan et al., 2011). Two other surveys took place, in China and in São Paulo (Brazil); the overall prevalence of CRS by EPOS criteria – the one validated for epidemiological studies – was 8.0% and 5.5%, respectively (Pilan et al., 2012; Shi et al., 2015). In a national survey in Korea, the overall prevalence of CRS, defined as the presence of nasal obstruction and nasal discharge for more than 3 months together with endoscopic objective findings, was 6.95% (Kim et al., 2011). The largest study was carried out in 2012, in the United States (US), based on data from the US National Health Interview Survey. The reported prevalence of 12.0% corresponds to the percentage of participants who had been told by a doctor that they had sinusitis, thus it probably reflects the occurrence of both

acute and chronic rhinosinusitis (Blackwell et al., 2014; Rosenfeld et al., 2015). The relationship between both age or gender and CRS prevalence was inconsistent among the above mentioned studies. However, CRS was found to be more common in smokers than in nonsmokers, as referred by several epidemiological surveys, which points to a significant risk factor. Moreover, medical conditions such as allergic rhinitis, asthma and chronic obstructive pulmonary disease were also associated with higher prevalence of CRS, indicating possible risk factors for developing the disease.

CRS is a common chronic disease affecting millions of patients worldwide and the consequent health costs may reach a few billions of dollars per year (Smith et al., 2015). It represents a burden to individuals since it affects their health-related quality of life with increased physical suffering and diminished social activities. These patients have worse health utility scores than those with chronic diseases such as congestive heart failure, chronic obstructive pulmonary disease, angina, or chronic back pain (Alobid et al., 2008; DeConde and Smith, 2017; Rosenfeld et al., 2015). CRS is also recognized as a socioeconomic burden represented by both direct and indirect costs. Direct costs to individuals/society reflect the money spent on medication, doctor´s visits, surgeries, diagnostic tests or general costs for medical care systems. Indirect costs affecting economy result from increased absenteeism and decreased work productivity of patients with CRS (Bachert et al., 2014; Rosenfeld et al., 2015).

CRS is a long-term debilitating disease and the socioeconomic burden is continuously rising; these facts, together with its high prevalence, justify the need for control and efficient treatment of CRS.

I.1.3. ETIOLOGY AND PATHOGENESIS

Multiple theories have been proposed regarding the underlying pathogenesis of CRS. It is widely believed that CRS is not a single disease entity but occurs as a variety of histological and clinical presentations. In fact, the causes of inflammation in CRS are diverse and multifactorial, with a significant overlap of host and environmental factors (Lee and Lane, 2011; Wood and Douglas, 2010). Notwithstanding the multiple etiologies contributing to its development, a common *sequela* of CRS is the disruption of normal sinonasal epithelial function. Several studies revealed an indubitable decrease of sinonasal

mucociliary clearance (MCC) in CRS patients (Chen et al., 2006). Changes in mucus composition, impairment of ciliary function or narrowed sinus ostia affect the effectiveness of the sinonasal MCC phenomenon (*section I.2.1.3.*), which constitutes the first line of defense from foreign material and is responsible to maintain the healthy state of paranasal sinuses (Kern and Decker, 2013). Ineffective sinonasal MCC clearance leads to impaired mucus drainage from paranasal sinuses. Retained sinonasal secretions cause poor sinus ventilation and accumulation of debris and by-products of inflammation, which in turn stimulates microbial colonization and produces further inflammation, leading to a vicious cycle that is difficult to break and aggravates even more this chronic disease (Aukema and Fokkens, 2004; Bhattacharyya, 2002; Chen et al., 2006). As a consequence, when CRS is clinically diagnosed the identification of the initial causes is difficult to establish.

Currently, it is believed that CRS inflammation is likely to stem from the defective capability of individuals to maintain the homeostasis, continuously challenged by environmental triggers (e.g. allergens, pollutants, microbes and viruses), across sinonasal mucosa via their mechanical barrier or immune responses. Although the exact etiology and pathophysiology of CRS remain unclear, it is assumed that this chronic disease results most probably from a complex interaction between host and environmental factors, a number of them being presented herein (Foreman et al., 2012; Suh and Kennedy, 2011).

I.1.3.1. Host Factors

Anatomical variations/anomalies

The ostiomeatal complex is an important anatomical area of the middle meatus of the nasal cavity, which collects the mucus coming from several important paranasal sinuses and is involved in the pathophysiology of those sinuses (*section I.2.1.2.*). An open sinus ostium is important to allow particles and microorganisms, eventually coming into contact with sinonasal mucosa, to be removed by MCC. Anatomic variations of the nose and paranasal sinuses may obstruct mucus outflow and impair drainage and ventilation. Amongst these variations detected by computed tomography imaging, nasal septal deviation and pneumatization of certain structures such as *concha bullosa* (air filled cavity

within middle turbinate bone) and Haller cells (atypical ethmoid air cells) are commonly observed (Carniol et al., 2015; Cauwenberge et al., 2006).

Genetic disorders

CRS is a common problem in patients with Kartagener's syndrome and cystic fibrosis. These genetic diseases predispose patients to CRS due to alterations in ciliary function or in mucus viscoelastic properties. Kartagener's syndrome is a primary ciliary dyskinesia, a hereditary disorder that affects the structure of cilia, making them immotile. This results in mucus *stasis*, a precursor of the development of CRS. Cystic fibrosis is another genetic disorder caused by a mutation of the cystic fibrosis transmembrane regulator gene that codifies a membrane protein (Cl^- channel), affecting the electrolyte transport, which results in production of highly viscous mucus. Patients affected by cystic fibrosis exhibit deficient MCC due to difficulty of the cilia to adequately transport the thick mucus (Aukema and Fokkens, 2004; Cain and Lal, 2013; Kern and Decker, 2013).

Immune deficiencies or dysregulations

Patients with an immunocompromised system (e.g. hypogammaglobulinemia, immunosuppressive cancer treatments) are at higher risk of developing difficult-to-treat CRS. On the other hand, CRS is also often associated with hypersensitive reactions of immune system, such as allergy and aspirin sensitivity. Allergic immune response has been shown to increase the depth of the liquid phase of nasal mucus (plasma exudation); as a result, cilia tips cannot reach the overlying gel phase, causing mucus *stasis*. Moreover swelling of sinonasal mucosa is thought to obstruct sinus ostia leading to poor ventilation and mucus *stasis*. Although the precise role of allergy and its causal relation to CRS are not unambiguously established, addressing this condition in patients with CRS will enhance, to some extent, the success of treatment. Several studies have reported an association between atopy and CRS and some authors suggest that allergic rhinitis predisposes to CRS, although this remains difficult to prove (Aukema and Fokkens, 2004; Cauwenberge et al., 2006; Kern and Decker, 2013). Some patients may also have aspirin sensitivity associated with asthma and CRS with nasal polyps. Patients with this so called Samter´s triad show an exacerbated response to aspirin or other non-steroidal anti-inflammatory drugs, consisting

of an exaggerated increase of leukotrienes which induces nasal mucosa edema and mucus secretion (Carniol et al., 2015; Wood and Douglas, 2010).

I.1.3.2. Environmental Factors

Nasal irritants and tobacco smoke

Airborne pollutents, such as ozone, sulfur dioxide and formaldehyde are nasal irritants, known to cause inflammation of sinonasal mucosa and negatively affect MCC (Hamilos, 2011). Similarly, exposure to tobacco smoke decreases ciliary beat frequency and increases mucus production, significantly reducing MCC clearance. Moreover, long-term exposure to tobacco smoke has been reported to cause mucosa hyperplasia and increased epithelial permeability; this, together with the immunosuppressive effect of tobacco smoke, enhances the occurrence of sinonasal infection. Active and second-hand smokers are therefore prone to CRS and patients with CRS should definitely avoid smoking (Kern and Decker, 2013; Reh et al., 2012).

Pathogens

Viral or bacterial infection is widely recognized as a direct cause of acute rhinosinusitis. On the contrary, although the infectious component of CRS has long been accepted, the level of pathogen involvement is still unclear (Bachert et al., 2014; Cain and Lal, 2013; Comstock et al., 2010). Traditionally, it has been proposed that CRS is an extension of unresolved acute infection. Acute rhinosinusitis is frequently viral in its early stage and, in a number of cases, progresses to a secondary acute bacterial infection. If the infection is not properly treated the inflammatory process can persist with time and foster the development of CRS (Brook, 2011).

The bacteriology of CRS differs from that of acute rhinosinusitis. In acute rhinosinusitis, the standard common bacteria found in sinonasal mucosa are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis.* In the case of CRS, the predominant bacteria include *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, various gram-negative rods, such as *Pseudomonas aerugionosa* as well as anaerobic bacteria, such as *Peptostreptococcus* and *Propionibacterium*. Polymicrobial infection is

common in CRS and mixed populations of the above bacteria have been encountered, making CRS more difficult to eradicate than acute rhinosinusitis infection (Bhattacharyya, 2002; Manes and Batra, 2012; Thanasumpun and Batra, 2015). The exact role of bacteria in CRS remains unknown; however, bacterial infection is an important factor as it may be implicated either as a causative event or as a disease modifier, worsening and prolonging the inflammation to a chronic state. Several levels of bacteria involvement in development of CRS have been proposed: they can cause direct bacterial infection resulting in inflammation and symptoms, produce bacterial by-products leading to injury of mucosa, expose the host to superantigens or be implicated in the formation of biofilms (Bachert et al., 2014; Bhattacharyya, 2002; Dlugaszewska et al., 2016; Manes and Batra, 2012). These biofilms protect bacteria from host defense and antibiotics, and are responsible for the persistence of infection with an additional capacity to release free-floating planktonic bacteria, a source of re-infection and recurrent acute exacerbations in CRS (Bachert et al., 2014; Bachert and Holtappels, 2015). Recent studies show that *S. aureus* can also be present intracellularly within sinonasal mucosa, releasing exotoxins that may function as superantigens: they induce a massive immune response by activating an abnormally large number of T lymphocytes, resulting in a polarized cytokine response towards the production of eosinophilia and immunoglobulin E (IgE) (T-helper 2 inflammation). These superantigens amplify the inflammatory condition of CRS. The role of fungi in CRS is controversial because their presence is ubiquitous in both healthy and CRS patients and they fail to respond to antifungal therapy. Further research information addressing this condition is required and not all guidelines explicitly discuss the management of fungal CRS (Bhattacharyya, 2002; Carniol et al., 2015).

I.1.4. CLASSIFICATION

CRS is usually broadly classified by the presence or absence of endoscopically visible nasal polyps in nasal cavity or paranasal sinuses. The two groups of patients, CRS with nasal polyps and CRS without nasal polyps, are not only clinically (phenotypically) but also histologically different. In Caucasian population, CRS without nasal polyps is the most common form of CRS encountered in the majority of cases and is characterized by fibrosis of mucosa and a T-helper 1 cytokine profile (e.g. increased interferon-γ levels). On the

other hand, in the same ethnic race, CRS with nasal polyps typically shows tissue edema and a predominant T-helper 2 inflammatory response with high tissue eosinophilia and increased levels of interleukine-5, interleukine-13 and IgE, being strongly associated with asthma and aspirin sensitivity (Bachert et al., 2014; Cain and Lal, 2013; Watelet et al., 2007). Nasal polyps are grape-like structures which consist of outgrowths of loose connective tissue, edema, inflammatory cells and some glands and capillaries, covered with several types of epithelium (Aukema and Fokkens, 2004). Differences in both categories and the presence of associated diseases are key elements for the management of this chronic inflammation.

I.1.5. BACTERIAL BIOFILMS

Bacterial biofilms have been found in nasal cavity and paranasal sinuses mucosa of CRS patients and their role in the persistence and recalcitrance of CRS is increasingly recognized (Dlugaszewska et al., 2016; Suh et al., 2010). Mucosa of these patients is markedly more damaged compared with mucosa of patients without biofilms; the damage ranges from morphologic changes in cilia array to reduced ciliary beat frequency or complete absence of cilia (Dlugaszewska et al., 2016; Fastenberg et al., 2016).

A bacterial biofilm is an organized community of bacteria adherent to an inert or biological surface and encased in an extracellular polymeric matrix. The development of bacterial biofilms starts with the attachment of sessile planktonic bacteria to a surface due to weak interactions. They subsequently undergo a phenotypic change allowing for a stronger binding, using adhesins. Once attached, bacteria begin to proliferate forming microcolonies and secrete an extracellular matrix composed of polysaccharides, nucleic acids (namely DNA) and proteins, enveloping bacteria. When bacterial density reaches a critical value, communication between them occurs, triggering a phenomenon known as *quorum sensing*, which leads to a mature biofilm phenotype. This phenotype is morphologically characterized by the formation of bacterial towers, disposed as layers of live bacteria interpenetrated with a network of water channels which nourish the individual bacteria. Bacteria can detach from mature biofilms; this release of planktonic bacteria results in their spread to other locations, enabling the whole cycle to start again,

thereby causing re-infection in the host (*Figure I.1.1.*) (Cohen et al., 2009; Fastenberg et al., 2016; Suh et al., 2010).

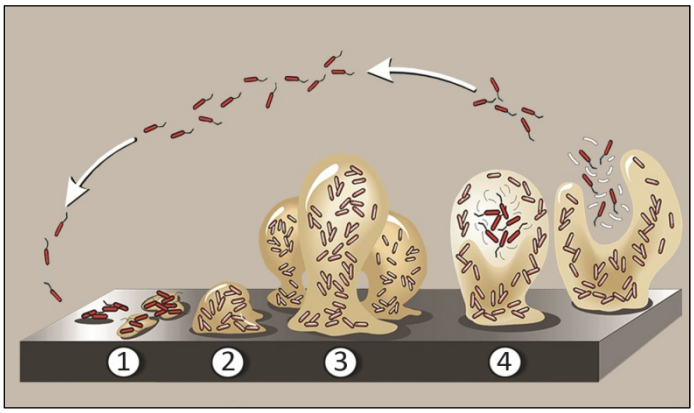

Figure I.1.1. Diagram of bacterial biofilm development: (1) Attachment; (2) Proliferation/Growing; (3) Maturation of biofilm; (4) Dispersion of planktonic bacteria (Adapted from Stoodley et al., 2002).

Increased resistance of bacterial biofilms to antibiotics is one of the most significant causes for the interest in biofilm research. Several mechanisms have been proposed to explain this resistance. One possibility is that the extracellular polymeric matrix constitutes a physical barrier impairing the antibiotic penetration; however some studies indicate that antibiotics diffuse efficiently down the water channels of the biofilm. Moreover, within the biofilm, the positively charged antibiotics are attracted by the negative charge of the matrix polymers, thereby deactivating the antibiotic. Another proposed mechanism is the low metabolic state of bacteria in the basal layers (core) of the biofilm, as a stress response to nutrient and oxygen depletion or waste-product accumulation. Since most antibiotics target metabolically active or dividing bacteria, the "dormant state" of these bacteria causes antibiotic resistance. The above mentioned *quorum sensing* is a further method of resistance; bacteria in biofilms share information within the community via intercellular signaling, contributing to adaptative responses. After the first contact with antibiotics, peripheral bacteria send signaling molecules to deeper located bacteria, which alter genetic expression to a resistant phenotype (e.g. up-regulation of efflux pumps). Biofilm structure and physiology also allow for gene transfer between bacteria and for mutational mechanisms, important in the development of antibiotic resistance. All of these mechanisms encourage persistence of bacteria for long periods despite conventional

antibiotic treatment. Giving all these properties, bacteria biofilms may be responsible for the chronic infection in CRS and also explain its intermittent acute exacerbations and its refractory nature (Cohen et al., 2009; Fastenberg et al., 2016; Kilty and Desrosiers, 2008).

To support the theory of bacterial biofilms implication in CRS, several studies have been conducted to demonstrate the presence of bacterial biofilms either in animal models of CRS, on paranasal sinuses stents removed from CRS patients, or from biopsied sinus mucosal specimens of patients undergoing endoscopic sinus surgery (ESS). Many of the latter studies have shown the presence of mucosal biofilms in the majority of samples of CRS patients and failed to identify them in healthy controls. However not all patients with CRS have biofilms and conversely biofilms have been detected in healthy control patients (Fastenberg et al., 2016; Foreman et al., 2012). These discrepancies may reflect different biofilm imaging methods (e.g. scanning or transmission electron microscopy and confocal laser scanning microscopy), different tissue preparation methodologies or unrecognized sinonasal inflammation in selected controls, but they also reinforce the multifactorial nature of CRS (Ramakrishnan et al., 2015). Having established the presence of biofilms in nasal cavity and paranasal sinuses of CRS patients, the need for detailed characterization, including identification of individual bacterial species present in biofilms, became clear. While imaging techniques are important to study the morphology of biofilms, species-specific techniques, such as fluorescent *in-situ* hybridization (FISH), are required for this identification purpose. To date, the bacteria most commonly identified in the form of biofilm are *S. aureus*, *P. aeruginosa*, *H. influenza* and *S. pneumonia* (Foreman et al., 2009; Healy et al., 2008; Sanderson et al., 2006). Due to technical constraints, FISH-based studies can only use a limited number of probes to identify species within biofilms; thus the selection of probes must be based on previous microbiological data and, on the other hand, it may overlook other important pathogens involved in CRS. New molecular-based techniques have been developed to characterize the bacterial diversity present in sinonasal tissue and mucus samples (Fastenberg et al., 2016; Foreman et al., 2012). In one of these studies, the authors have identified up to 20 different microbials per sample and their results suggest that anaerobe involvement may be more frequent than presumed (Stephenson et al., 2010). Nonetheless, the dominant presence of *S. aureus* in biofilms is corroborated by other studies; in the largest studies, *S. aureus* was identified in at least

50% of biofilms in CRS patients (Foreman et al., 2009; Stephenson et al., 2010; Tan et al., 2013). The intracellular uptake of this pathogen into sinonasal epithelium has also been demonstrated to occur and it is believed to play a role of a second reservoir for re-infection; their association with biofilms in sinonasal mucosa of CRS patients indicates a link between intracellular bacteria and biofilm colonization (Tan et al., 2013). The effect of bacterial biofilms on post-operative outcomes of CRS patients has been evaluated (Bendouah et al., 2006; Prince et al., 2008; Psaltis et al., 2008). For instance, Psaltis et al. (2008) concluded that patients with biofilms had worse pre-operatively conditions as well as worse post-operative symptoms and endoscopic evidence of inflammation. Moreover, Prince et al. (2008) found a statistically significant positive correlation between biofilm formation and the number of prior ESSs; Bendouah et al. (2006) demonstrated that biofilm-forming capacity by *S. aureus* and P. *aeruginosa* was associated with unfavorable evolution after ESS.

This highlights the importance of bacterial biofilm eradication strategies in the management of CRS, particularly in patients refractory to pharmacological and surgical treatment.

I.1.6. MANAGEMENT AND TREATMENT OPTIONS

There is no standard management of CRS and treatment strategies include a variety of systemic and topical therapeutic approaches with different degrees of recommendation according to the type and severity of this multifactorial disease. International guidelines propose different managements of CRS for patients with and without nasal polyps while considering also other associated diseases (e.g. allergy or asthma) (Cain and Lal, 2013; Fokkens et al., 2012; Rosenfeld et al., 2015). The main goals of CRS treatment are reduction of sinonasal mucosal inflammation, control of infection and reestablishment of MCC within the paranasal sinuses. The initial use of pharmacological therapies (often referred as medical therapies) to its maximum possible extension followed, in case of failure, by surgical intervention, is generally accepted. The mainstay of pharmacological treatment is the use of topical corticosteroids and oral antibiotics (antibacterials) that may be combined with other adjunct therapies. Common pharmacological regimes include an 8 week course of topical intranasal (IN) corticosteroids and a 3-4 week course of broad-

spectrum or culture-directed oral antibiotics (antibacterials) (Schwartz et al., 2016; Serralheiro et al., 2013; Suh and Kennedy, 2011). ESS is reserved for CRS patients who were refractory to pharmacological therapy. The aims of ESS are to remove nasal polyps and debris from sinuses and to establish patency of sinuses ostia, opening and clearing the sinus drainage pathways and facilitating access for topical delivery into paranasal sinuses. However, there is a subpopulation of patients that are refractory to pharmacological and ESS therapies. This is a challenge calling for further understanding of the therapeutic options and development of new emerging medical strategies (Cain and Lal, 2013; Wood and Douglas, 2010).

I.1.6.1. Systemic Therapies

Oral corticosteroids

Corticosteroids are well known for their anti-inflammatory properties; they have the ability to inhibit cytokine and chemokine synthesis and release, to block epithelial cell activation and to reduce inflammatory cell infiltration through a variety of mechanisms. Therefore, their use in CRS therapy contributes to reduce mucosa edema, which may open obstructed paranasal sinuses thus increasing their ventilation and improving/reestablishing drainage of mucus by MCC (Mattila, 2012; Serralheiro et al., 2013; Suh and Kennedy, 2011).

Evidence supporting the efficacy of oral corticosteroids in patients with CRS without nasal polyps is limited and thus their use in these patients is optional. On the contrary, evidence for their use in patients with CRS with nasal polyps is far better established (Cain and Lal, 2013; Schwartz et al., 2016). However, although very effective in this group of patients, systemic corticosteroids have several side effects which increase with dose and duration of treatment, limiting their use to courses of short duration. A systematic review by Poetker et al. (2013) strongly recommends oral corticosteroids for short-term management of CRS with nasal polyps; they also found significant beneficial effects in the preoperative period by improving intraoperative visualization thus technically facilitating the ESS intervention and favoring its success. Likewise, oral corticosteroids administered in the immediate postoperative period were found to improve sinonasal endoscopic

appearance. The optimum dosage and duration of oral corticosteroids is unclear, but an average duration of 2 weeks of oral prednisolone or prednisone is usually prescribed in CRS with nasal polyps, most commonly as a tapered regimen (Poetker et al., 2013). Hissaria et al. (2006) studied the clinical efficacy of a 14-day course of 50 mg of prednisolone in subjects diagnosed with CRS with nasal polyps and found a significant improvement of nasal symptoms, nasal inflammation and a reduction in nasal polyps' size. The authors concluded that the short course implemented was effective and also safe (Hissaria et al., 2006). However, the safety profile of oral corticosteroids is still limited due to the lack of trials enrolling a large number of patients and longer periods of follow up. Therefore, patients should be informed and well aware of the possible systemic adverse effects of oral corticosteroids, namely decreased bone density, cataract formation, glaucoma, hyperglycemia, adrenal axis suppression, sleep disturbance and weight gain. Given the fact that CRS requires ongoing treatment over long periods, the risk-to-benefit ratio should always be assessed (Cain and Lal, 2013; Suh and Kennedy, 2011).

Oral and intravenous antibiotics

Bacterial infection is likely to play a role in the inflammatory process of CRS. Although the exact multifactorial etiology is unknown and may differ from patient to patient, the use of antimicrobials continues to be a fundamental strategic treatment in CRS and antibacterials are one of the most frequently prescribed drugs (Cain and Lal, 2013; Schwartz et al., 2016; Soler et al., 2012). Following a common usage in literature, the term antibiotic will be used here with the restricted sense of antibacterial.

The value of antibiotics is acknowledged to treat acute exacerbations of CRS; these sudden worsening of symptoms are associated with endoscopic evidence of purulent mucus drainage and a positive bacterial culture. There is no antibiotic specifically approved by the US Food and Drug Administration (FDA) for the treatment of CRS. Examples of the commonly used oral antibiotics include amoxicillin/clavulanic acid, cephalosporins, clindamycin, sulfamethoxazole/trimethoprim and fluoroquinolones (FQs) (e.g. ciprofloxacin, levofloxacin and moxifloxacin). A broad-spectrum antibiotic is routinely and empirically prescribed to cover the appropriate range of bacteria and thus decrease the bacterial load and treat the infection responsible for the acute exacerbation (Bachert et al.,

2014; Casey et al., 2015; Piromchai et al., 2013; Suh and Kennedy, 2011). Despite this common practice, high-level experimental evidence is still missing to support the use of oral antibiotics. The main drawback is the lack of placebo-controlled studies, since both physicians and patients are generally reluctant to participate in such studies due to a deep belief in the efficacy of oral antibiotics (Adelson and Adappa, 2013; Schwartz et al., 2016; Soler et al., 2012). A study by Legend et al. (1994) comparing ciprofloxacin (CIP) and amoxicillin/clavulanic acid showed no significant difference in the symptomatic clinical cure and bacteriological eradication rates. However, in patients with an initial positive culture, a significantly higher cure rate was observed for CIP compared to amoxicillin/clavulanic acid, 40 days after treatment (83.3% vs 67.6%) (Legent et al., 1994).

The EPOS 2012 guideline and the recent systematic review by Soler et al. (2012) do not agree on the relative benefit of oral antibiotics in the CRS with nasal polyps and CRS without nasal polyps groups. Notwithstanding, a short-term treatment with an average duration of 3-4 weeks is appropriate and consensually recommended for patients with persistent purulent mucus drainage and identified positive bacterial cultures to enable a culture-directed therapy as opposed to traditional symptom-based empiric therapy, thus increasing efficacy and reducing the risk for development of antibiotic resistance (Adelson and Adappa, 2013; Bachert et al., 2014; Fokkens et al., 2012; Mattila, 2012; Soler et al., 2012). However, efficacy is always limited, as clinical relapse of acute exacerbations occurs with variable frequency; moreover negative bacterial cultures do not exclude the eventual presence of dormant bacteria within biofilms (as secretions rather than biopsied mucosa are collected). The use of oral antibiotics in postoperative period is justified by the theoretical benefit of reducing the risk of infection during the healing phase and therefore an optional recommendation following ESS (Casey et al., 2015; Hull and Han, 2015; Lim et al., 2008).

Long-term, low dose, oral macrolide antibiotics receive special attention due to their anti-inflammatory properties, which are thought to be more effective for neutrophilic inflammation than for eosinophilic inflammation, therefore with greatest benefit in a subgroup with normal or low IgE. Their exact mechanism of action is unknown, but it probably involves down-regulation of cytokine production, neutrophil apoptosis and impairment of biofilm adherence. The main concern associated with this strategy is the

emergence of antibacterial resistance (Bhattacharyya and Kepnes, 2008; Crosby and Kennedy, 2015; Schwartz et al., 2016).

There is little evidence in literature to support the beneficial use of intravenous (IV) antibiotics in CRS. Experts recommend against their routine use in uncomplicated CRS, although they may be an alternative reserved for cases where surgery is not an option (Anand et al., 2003; Schwartz et al., 2016).

I.1.6.2. Topical Intranasal Therapies

CRS is a chronic disease that requires ongoing therapies and this may lead to systemic adverse effects and antibiotic resistance. The development of topical therapy enables direct drug delivery to the target site with the advantage of achieving higher local drug concentrations with minimal systemic exposure and thus systemic side effects. Therefore, topical IN therapies can potentially and gradually supplant some systemic options in treatment of CRS (Comstock et al., 2010; Lim et al., 2008; Varshney and Lee, 2017).

Topical intranasal saline

Topical saline solutions intranasally administered exert their beneficial effect by removing mucus, debris, bacterial biofilms, antigens and inflammatory mediators, improving symptoms by restoring normal MCC and reducing inflammation. Thus, saline solutions are employed as part of the topical treatment regimen for CRS. There are variations in the delivery method (e.g. sprays and irrigations), volume administered (high or low), sodium chloride concentration (hyper-, hypo- or isotonic) and frequency (Rudmik and Soler, 2015; Schwartz et al., 2016; van den Berg et al., 2014). The effect of hypertonic and isotonic nasal saline sprays (1 mL) on MCC and nasal airway patency was evaluated by Hauptman and Ryan (2007) in a randomized double-blind trial. Both solutions significantly improved MCC, as measured by the saccharine clearance test: 178 seconds decrease from baseline for hypertonic and 121 seconds decrease from baseline for isotonic solution. With respect to nasal airway patency (measured by acoustic rhinometry) isotonic saline solution had a significant positive effect, whereas hypertonic solution caused a decrease in nasal airway patency. This contradicts the previous hypothesis of an added benefit of nasal

decongesting effect through an osmotic mechanism; according to the author of this study, this may suggest that hypertonic saline induced neural responses leading to mucosa swelling. Both hyper- and isotonic solutions improved nasal symptoms; however, burning and irritation were reported after hypertonic saline solution administration (Adappa et al., 2012; Hauptman and Ryan, 2007).

A randomized controlled trial by Pynnonen et al. (2007) compared the symptoms and quality-of-life scores, two, four and eight weeks after administration of isotonic saline nasal irrigations (240 mL) and isotonic saline nasal sprays, as adjunct therapies to other usual medications. They found improvement in both groups although significantly better in patients using saline irrigations. This result may probably be attributed to the greater volume and forceful stream of saline nasal irrigations which facilitate mechanical debridement (Adappa et al., 2012; Pynnonen et al., 2007). A few studies evaluating the effect of nasal saline irrigations demonstrated improved early postoperative symptoms scores (Rudmik et al., 2011). Systematic reviews support the conclusion that saline nasal irrigations are beneficial either as a sole treatment or associated with topical corticosteroid therapy (Harvey et al., 2007). Topical nasal saline irrigations are often recommended in daily practice as an adjunctive therapy to topical IN corticosteroids for patients with and without nasal polyps (Rudmik et al., 2013; Rudmik and Soler, 2015).

Topical intranasal corticosteroids

Topical IN corticosteroid therapy is the mainstay of pharmacological treatment of CRS for patients with and without nasal polyps. The main advantage of this direct delivery is the possibility of obtaining higher local concentrations with limited systemic exposure and enabling its use for longer periods, thereby minimizing the potential adverse effects associated with the use of systemic corticosteroids.

Many types of topical IN corticosteroids have been used in the management of CRS and are often categorized into “standard” and “non-standard” therapies. The former comprise corticosteroid formulations that have been approved for nasal use – typically metered-dose nasal sprays – whereas the latter group lacks approval for the specific use in the nose. Commonly used approved corticosteroids nasal sprays include beclomethasone dipropionate, budesonide, flunisolide, triamcinolone acetonide, ciclesonide, fluticasone propionate, fluticasone furoate and mometasone furoate. They are often used outside

their approved indication as shown in *Table I.2.1.* (Rudmik et al., 2013, 2012; Schwartz et al., 2016; Varshney and Lee, 2017).

Several studies report the role of "standard" topical IN corticosteroid therapy on objective and subjective clinical outcomes of patients with CRS with or without nasal polyps. With regard to CRS with nasal polyps, Joe et al. (2008), Kalish et al. (2012) and Rudmik et al. (2012) performed systematic reviews and the results demonstrated reduction in polyp size and in polyp recurrence frequency after surgery and improvement in nasal peak inspiratory flow and symptom scores (Joe et al., 2008; Kalish et al., 2012; Rudmik et al., 2012). Concerning CRS without nasal polyps, similar systematic reviews have shown that standard topical IN corticosteroid therapy also results in improved nasal symptoms and that a subgroup analysis revealed more benefit with direct paranasal sinuses cannulation than with simple nasal delivery (Burton et al., 2012; Snidvongs et al., 2011). In view, of the high level of evidence for the clinical benefits of standard topical IN corticosteroids and safety profile, they are strongly recommended as the cornerstone of maintenance treatment of CRS by US and Europe guidelines (Fokkens et al., 2012; Rosenfeld et al., 2015; Rudmik and Soler, 2015).

Nonstandard topical IN corticosteroid therapies have the advantage of delivering higher volume and/or higher concentration solutions of topical intranasal corticosteroids to the mucosa compared to standard therapy. They include high-volume budesonide saline irrigations (0.5 mg/2mL in 240 mL of normal saline) and low-volume, high-concentration solutions, namely dexamethasone (0.1%, w/v) and prednisolone acetate (1%, w/v) ophthalmic drops and dexamethasone/CIP otic drops (0.1%/0.3%, w/v) (Rudmik et al., 2013; Schwartz et al., 2016). There are few studies demonstrating the clinical benefits of nonstandard corticosteroids. A large study evaluated the efficacy of postoperative IN corticosteroid irrigations in CRS. After ESS, patients received once daily irrigation therapy of either budesonide (1 mg) or betamethasone (1 mg) delivered in 240 mL of normal saline through a squeeze bottle (for at least 3 months) and have shown improved symptoms and endoscopy scores, especially in patients with high eosinophilia (Snidvongs et al., 2012). There is some concern of unwanted systemic corticosteroid adverse effects when using nonstandard IN corticosteroid therapies, due to the administration of higher doses in this treatment modality. However, studies do not support this fear. A retrospective study

conducted by DelGaudio and Wise (2006) included patients treated in an off-label fashion with IN dexamethasone or prednisolone ophthalmic drops or CIP/dexamethasone otic drops after ESS and concluded that they had a reduced need for revision surgery and that only one case of adrenal suppression occurred despite the high-dose applied (DelGaudio and Wise, 2006). Another study demonstrated that budesonide nasal irrigations following ESS did not confirm the suppression of hypothalamic-pituitary adrenal axis (by measuring cortisol levels). It has been claimed that high volume delivery techniques such as squeeze bottle or neti pot result in less than 5% of the solutions remaining in the sinuses and therefore the higher dose administered does not reflect the actual drug exposure (Rudmik et al., 2013; Welch et al., 2010). Notwithstanding the potential advantages of nonstandard IN corticosteroid therapies, more studies are needed to assess their safety profile before they are recommended not only as optional but as a routine strategy (Rudmik et al., 2013).

Topical intranasal antibiotics

The rationale of topical IN antibiotic therapy is to provide high concentrations of antibiotics directly to the sinonasal mucosa, potentially minimizing adverse effects associated with traditional oral administration. There is growing evidence of the role played by bacterial biofilms in the pathogenesis of CRS, particularly in case of refractory patients. In fact, the recalcitrant character of this disease may be taken as indicative of the biofilm involvement. Due to their nature, bacteria in biofilms show an increased resistance to antibiotics, that can be as high as 1000-fold that of their planktonic counterpart (Comstock et al., 2010; Ramakrishnan et al., 2015). Increasing the concentration of antibiotics may be a strategy to counter this resistance. Ha et al. (2008) investigated the activity of three antibiotics – mupirocin, vancomycin and CIP – against *in vitro* biofilms of several strains of *S. aureus* collected from CRS patients and found them to be susceptible to those agents. Mupirocin reduced biofilm mass by more than 90% at a concentration of 125 µg/mL or less, whereas CIP achieved 50% biofilm reduction for all biofilm strains with a concentration of 15.62 µg/mL or less after 24h of treatment (Ha et al., 2008). *In vitro* activity of moxifloxacin (MOX) against *S. aureus* biofilms was also assessed in a study conducted by Desrosiers et al. (2007). After visual confirmation of *in vitro* biofilm formation, plates were incubated with either a phosphate buffered saline solution or with

MOX solution at concentrations of 0.1x, 1x, 100x and 1000x the minimum inhibitory concentration (MIC) for 24h. The number of viable bacteria was similar for non-treated and MOX-treated biofilms at MIC and sub-MIC levels. On the contrary, supra-MIC levels apparently led to a dose-related reduction in the number of viable bacteria and at a concentration of 1000x MIC (100-200 μg/mL) a significant reduction of 99% in the number of viable bacteria was observed (Desrosiers et al., 2007). Chiu et al. (2007) used an animal model to evaluate the efficacy of tobramycin in eradicating biofilms of *P. aeruginosa*. Irrigations with tobramycin solutions with increasing concentrations ranging from 80- to 400-fold (80-400 μg/mL) the known MIC were performed at a rate of 20mL/min in rabbits with *P. aeruginosa* biofilms established in maxillary sinus mucosa. The results showed that, although clearance of viable bacteria was observed in the sinus lumen, viable bacteria attached to the sinus mucosa persisted, as shown by scanning electron microscopy analysis and by bacterial counts in disrupted sinus mucosa (Chiu et al., 2007). The combined results of the above described studies indicate that MIC is not effective against bacteria in the biofilm form and that increasing concentrations of antibiotics may be needed to eradicate biofilms. Although systemic treatment with such high concentrations would result in serious risks of toxicity, antibacterial agents can be delivered safely in high concentrations when applied topically on the biofilm surface. This makes IN delivery of topically acting antibacterial agents an interesting approach to treat CRS patients. However, further efforts are needed to investigate the methodology of delivery and treatment protocols to reach maximum positive results (Comstock et al., 2010; Desrosiers et al., 2007; Suh and Kennedy, 2011).

There is a lack of high quality studies regarding the efficacy of topical IN antibiotics in clinical practice. Very few randomized controlled trials have been published to date and studies are heterogeneous with regard to topical antibacterial agent, dose, regimen, delivery method, patient selection and outcome measures. Moreover, there is also sparse or no information about either pharmacokinetics and systemic bioavailability or bacterial eradication rates of intranasally administered antibiotics. The benefit of topical antibiotic therapy is therefore difficult to establish and EPOS 2012 does not recommend it for routine use. However, systematic reviews suggest a higher level of evidence existing in postsurgical patients and in culture-directed therapy. This opens the way to the use of this therapy as a potential option in refractory CRS patients, those who were unresponsive to

traditional pharmacological and surgical therapies (Fokkens et al., 2012; Lim et al., 2008; Rudmik et al., 2013; Soler et al., 2012). The most commonly implicated pathogens in refractory CRS are *S. aureus* and *P. aeruginosa*; patients harboring those bacterial biofilms have worse outcomes and present difficult to treat infections. Commonly used topical antibiotics are mupirocin and aminoglycosides (e.g. tobramycin and gentamycin), as well as, less frequently, cephalosporins and FQs. They are usually delivered as nasal sprays, irrigations and nebulized aerosols and examples of clinical studies involving these drugs are described in *Table I.1.1*. Larger and better designed clinical studies are required to evaluate this emerging treatment strategy (Bachert et al., 2014; Bendouah et al., 2006; Craig and Goyal, 2015; Schwartz et al., 2016; Varshney and Lee, 2017).

Alternative and innovative topical intranasal therapies

The growing evidence of bacterial biofilm involvement in CRS and the paucity of industry development of novel antibiotics have boosted the research on new eradication strategies and topical delivery methods.

Biofilms can be mechanically disrupted by using *surfactants* such as baby shampoo and citric acid/zwitterionic surfactant (CAZS). Surfactants are amphoteric molecules that decrease the surface tension, thereby reducing the viscosity of mucus and solubilizing the biofilm. Chiu et al. (2008) investigated the effect of 1% baby shampoo saline irrigations for 4 weeks postoperatively and found improvement in mucus thickness and postnasal drainage; 10% of patients reported minor nasal and skin irritations and discontinued its use (Chiu et al., 2008). A subsequent study by Farag et al. (2013) compared a surfactant solution of 1% baby shampoo and a hypertonic saline solution, both irrigated following ESS. Although both groups demonstrated similar improvements in symptoms, the surfactant group had poor tolerability and lower compliance rate due to the side effects (headaches and nasal burning) (Farag et al., 2013). In the case of CAZS, an *in vivo* study conducted by Le et al. (2008) found an initial reduction in biofilm surface area coverage when CAZS was delivered via a hydrodebrider, a pressurized irrigation system, followed by rapid re-growth after treatment discontinuation (Le et al., 2008). A main concern about this surfactant is its toxic effect on cilia, causing deciliation and temporarily neutralizing MCC, as observed in a rabbit model (Tamashiro et al., 2009).

Table I.1.1. Examples of topical intranasal antibiotics for chronic rhinosinusitis (CRS).

Author(s)	Study Design / Patients	Study Groups	Treatment Protocol	Delivery Method	Results/Conclusions
Sykes et al., 1986	Randomized controlled trial Stable CRS	(1) Dexamethasone+tramazoline+neomycin (2) Dexamethasone+tramazoline vs. Propellant alone	(1) 20 µg+120 µg+100 µg (2) 20 µg+120 µg Per nostril QID For 2 weeks	Nasal spray	More effective than placebo but no difference between treated groups in respect to symptoms and objective findings
Moss and King, 1995	Cystic fibrosis patients with CRS	(1) Tobramycin vs. Surgery alone	(1) 40 mg/mL TID For 7–10 days	Intra-operative antral irrigation	Reduced need for revision ESS
Desrosiers and Salas-Prato, 2001	Randomized controlled trial Refractory CRS	(1) Tobramycin (2) Saline alone	(1) 4 mL (20 mg/mL) TID For 4 weeks	Large-particle nebulizer	Equivalent improvements in both groups; Nasal congestion in tobramycin group
Scheinberg and Otsuji, 2002	Retrospective study Refractory patients, stable CRS	(1) Cefuroxime (2) Ciprofloxacin (3) Levofloxacin (4) Tobramycin	(1) 285 mg TID (2) 90 mg BID (3) 70 mg BID (4) 95 mg BID For 3-6 weeks	Nebulization	Symptom scores improve Side effects infrequent, mild and transient
Vaughan and Carvalho, 2002	Retrospective study Refractory patients, acute exacerbation of CRS	(1) Ceftazidime (2) Ciprofloxacin (3) Levofloxacin (4) Ofloxacin (5) Tobramycin (6) Gentamycin vs. Standard therapy (oral and IV)	(1) 550 mg BID (2) 90 mg BID (3) 70 mg BID (4) 90 mg BID (5) 95 mg BID (6) 95 mg BID For at least 3 weeks	Nebulization	Improvement in symptoms with nebulized antibiotics Infrequent and mild side effects Average 17 weeks infection free period compared with average 6 weeks for standard therapy
Uren et al., 2008	Prospective study Refractory CRS patients with *S. aureus* positive culture	(1) Mupirocin	(1) 200 mL (500 µg/mL) BID For 3 weeks	Nasal irrigation squeeze bottle	Improved endoscopic findings Negative swab cultures for *S. aureus* Minimal adverse effects
Ezzat et al., 2015	Prospective controlled study Refractory CRS patients with positive cultures and/or bacterial biofilm	(1) Ofloxacin vs. Without treatment	(1) 3 drops (3mg/mL) per nostril TID For 12 weeks	Nasal drops (commercially available as eye drops)	80% of treated group showed marked improvement in (middle meatus) nasal mucosa structure with disappearance of biofilm

BID, twice daily; ESS, endoscopic sinus surgery; IV, intravenous; TID, three times daily; QID, four times daily.

Enzymes targeting important components of biofilm matrix are also under investigation. For example, *dispersin B* degrades poly-N-acetylglucosamine, a polysaccharide produced by *S. aureus* that is essential during the formation of biofilm matrix, forming a charge barrier which repeals mucus antimicrobial peptides of the host immune system (Fastenberg et al., 2016).

Manuka honey is a natural product with inherent antibacterial properties which has demonstrated in vitro efficacy against *S. aureus* and *P. aeruginosa* biofilms. This is partially due to the high concentration of methylglyoxal in this type of honey, although other components still unidentified may also contribute. Clinical studies to assess the efficacy of manuka honey in CRS patients are still awaited (Fastenberg et al., 2016; Schwartz et al., 2016).

Xylitol is a natural sugar alcohol without inherent antimicrobial properties; however, when delivered to the airway epithelia it reduces the salt concentration of mucus, enhancing the activity of innate immune factors. The EPOS 2012 guideline supports the use of xylitol nasal irrigations, although several systematic reviews do not provide formal recommendation due to limited evidence of clinical efficacy (Schwartz et al., 2016).

Drug eluting stents have emerged as an innovative IN therapy, offering the advantages of continuous slow drug release to the sinonasal mucosa and acting as mechanical spacers to improve ostial patency of the implanted area. Propel Sinus Implant® is a self-expanding implant consisting of a bioabsorbable polymer (of polylactide) for controlled release of embedded mometasone furoate (0.37 mg) that has been recently approved by FDA for CRS patients undergoing ESS. Drug eluting stents are promising as a component in postoperative CRS management, given the current interest raised by the emergency of new biomaterials (Varshney and Lee, 2017).

I.1.6.3. Other Therapies

Systemic and topical intranasal *antifungals* are not recommended for routine use in CRS according to published systematic reviews. In fact, a meta-analysis performed by Sacks et al. (2012) demonstrated that oral terbinafine *versus* placebo showed no efficacy in terms of symptomatic and radiographic improvement. Moreover, topical IN administration of amphotericin B led to symptom outcomes that were more favorable in the placebo

group than in the group subject to the treatment (Sacks et al., 2012). Another meta-analysis corroborated these findings with no difference found in symptom scores, nasal endoscopy and computed tomography results with topical IN amphotericin B (Isaacs et al., 2011). Reported adverse effects for systemic and topical intranasal antifungals outweight any potential benefit, therefore authors do not support antifungal therapies in the management of CRS (Isaacs et al., 2011; Lim et al., 2008; Sacks et al., 2012).

Other therapies are employed in the management of this chronic disease, such as mucolytics, decongestants, antihistamines and antileukotrienes. For example, antihistamines are effective and commonly used for the treatment of allergic rhinitis, being an important component of CRS therapy in patients with both diseases. The complex and diverse nature of CRS makes it a challenging disease to manage, calling for an individual assessment of the disease and treatment approach. Topical IN therapy represents, however, a wealth of opportunities with recognized advantages (Piromchai et al., 2013; Suh and Kennedy, 2011).

I.2. NOSE AS A ROUTE FOR DRUG DELIVERY

I.2.1. STRUCTURE AND FUNCTION OF HUMAN NOSE AND PARANASAL SINUSES

Due to the anatomical, histological and physiological characteristics of the nose, the IN administration is an attractive alternative option for drug delivery with a widespread interest among the scientific community. The basic concepts related to these unique features of the nose and adjacent cavities (paranasal sinuses) developed in the next section will serve as background for the entire *section I.2.*.

I.2.1.1. Anatomy and Histology of Human Nose

The nose is a complex organ of the upper respiratory tract. The external part of the human nose is supported and shaped by a framework of bone and cartilage, namely the nasal bones and the upper and lower lateral cartilages. This protruding structure bears the nostrils and builds up its pyramidal conformation. The external nose is important as it can create sufficient resistance against the transmural pressure of inspiration and control the entrance of air into the respiratory tract (Jones, 2001; Watelet and Cauwenberge, 1999).

Inhaled air flows through the nostrils and enters the nasal cavity which is sagittally divided into two symmetrical and non-connected halves (called also nasal passages) by the nasal septum. The nasal cavity extends posteriorly to the nasopharynx, where the two nasal passages join together. Anatomically, the ethmoid and sphenoid bones form the roof of the human nasal cavity and the palatine its floor, while the septum wall and lateral wall limit each of the two halves of the nasal cavity. The total surface area of the human nasal cavity is about 150 cm^2 and the total volume is about 15 mL (Mygind and Dahl, 1998; Pires et al., 2009; Watelet and Cauwenberge, 1999).

There are three distinct areas in the nasal cavity, the nasal vestibule, the olfactory region and the respiratory region (*Figure I.2.1.*). The nasal vestibule is the most anterior part of the nasal cavity and corresponds to the region just inside the nostrils. It is covered by a stratified squamous and keratinized epithelium and has nasal hairs that block the inhalation of large airborne particles. This area of the nasal mucosa may function like the

epidermis in the skin by protecting the underlying tissues from atmospheric agents; the absorption of drugs in this poorly vascularized region is limited (Bitter et al., 2011; Pires et al., 2009; Serralheiro et al., 2012). Between the nasal vestibule and the respiratory region, the nasal cavity becomes lined by a transitional epithelium which is composed of non-ciliated pseudostratified columnar cells with microvilli. In this intermediate space (called atrium), there is the narrowest portion of the entire respiratory tract called the nasal valve (or internal ostium), with a cross-sectional area of about 30 mm^2 on each halve. The nasal valve accounts for approximately 50% of the total resistance to respiratory airflow from the nostril to the alveoli and any changes will compromise the nasal airflow (Mygind and Dahl, 1998; Pires et al., 2009).

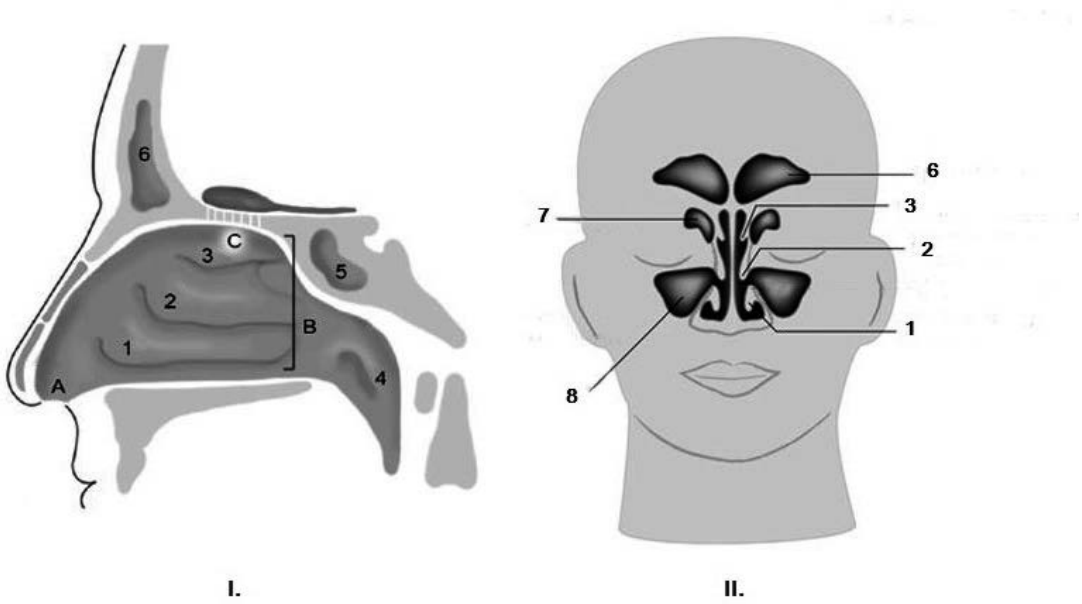

Figure I.2.1. Sagittal section (I) and frontal section (II) showing the human nasal cavity and paranasal sinuses: (A) Nasal vestibule; (B) Respiratory region; (C) Olfactory region; (1) Inferior turbinate; (2) Middle turbinate; (3) Superior turbinate; (4) Nasopharynx; (5) Sphenoid sinus; (6) Frontal sinus; (7) Ethmoid sinus; (8) Maxillary sinus (Adapted from Serralheiro et al., 2012).

The respiratory region is the largest area of the nasal cavity, representing 80-90% of its total surface area. This region presents three nasal conchae or turbinates (inferior, middle and superior) that divide the nasal cavity in three meatus (inferior, middle and superior). The nasal turbinates are bony structures projected from the lateral nasal walls into the airway lumen, forming a folded structure that increases the inner surface area of the nasal cavity and plays an important role in filtering, humidification and warming the

inspired air. Moreover, this anatomy creates turbulence, providing a close contact between the inhaled air and the respiratory surface. The respiratory region is highly vascularized and lined by a pseudostratified columnar epithelium. Given these anatomical characteristics – large surface area and rich vascular nature – the respiratory region is the most permeable area of nasal cavity and therefore a privileged place for drug systemic absorption, with the additional advantage of circumventing gastrointestinal and first-pass hepatic metabolism (Bitter et al., 2011; Pires et al., 2009; Serralheiro et al., 2012).

The olfactory region is restricted to the upper part of the nasal cavity, below the cribriform plate of the ethmoid bone, extending over part of the septum and superior turbinate, with a surface area of less than 10% of the total nasal cavity. Its nasal mucosa is also pseudostratified with rich vascularization but contains specialized olfactory receptor cells that are responsible for smell perception. As will be detailed later along with the histology of the olfactory mucosa, this region offers a direct and unique pathway to the brain and is therefore a valuable strategy for targeting neurological diseases (Bitter et al., 2011; Pires et al., 2009; Watelet and Cauwenberge, 1999).

Nasal respiratory mucosa

The nasal respiratory mucosa is constituted by the epithelium, basement membrane and lamina propria. The surface epithelium has four types of cells: ciliated and non-ciliated pseudostratified columnar cells, goblet cells and basal cells (*Figure I.2.2.*). The latter, which are the progenitors of the other cell types, lie on the basement membrane and do not reach free surface. The basement membrane is a layer of collagen fibrils placed between the epithelium and lamina propria. All epithelial cells reach this membrane; however basal cells are the only ones that are strongly anchored to it by hemidesmosomes. Moreover, the basal cells are believed to help in the adhesion of the other epithelial cells to the basement membrane. Another cell type of the surface epithelium is the goblet cell, whose function is to secrete mucin, which absorbs water and forms nasal mucus. These glandular cells contribute in part to the production of the mucus layer but little is known about their release mechanisms. Probably, physical and chemical irritants of the environment and biochemical mediators may stimulate goblet cell secretion (Alsarra et al., 2010; Gizurarson, 2012; Mygind and Dahl, 1998; Pires et al., 2009).

The columnar cells correspond to the majority of the epithelium cells; their apical surface contains microvilli that increase the surface area of the respiratory epithelium (available for drug absorption) and prevent it from drying, retaining moisture essential for the ciliary function. The major part of this type of cells also presents slender cytoplasmatic projections – the cilia – that are involved in the transport of mucus. Both, cilia and nasal mucus, form the nasal mucociliary apparatus which has the important function of protecting the respiratory tract. Under physiological conditions, synchronized beating of cilia propels nasal mucus across the surface towards nasopharynx, where it is swallowed or expectorated. This mechanism is known as the MCC, a phenomenon that will be discussed in the *section I.2.1.3.* The presence of cilia starts just behind the front edge of the inferior turbinate and extends to the posterior part of the nasal cavity. The distribution pattern of the ciliated columnar cells is inversely proportional to the velocity of the inspired air; therefore the inferior part of the nasal cavity is more densely covered with cilia than the upper part. In addition, it is assumed that low temperature and low humidity reduce the number of ciliated cells. This argument explains the fact that the anterior part of the nasal cavity has less cilia than the posterior one, since the former receives strong streams of cold, dry and polluted air (Gizurarson, 2012; Mygind and Dahl, 1998). All the epithelial cells are bound together by tight junctions, desmosomes and gap junctions; these junctions confer resistance to stress and enable cells to communicate with each other, forming a dynamic, adjustable, semi-permeable diffusion barrier between epithelial cells.

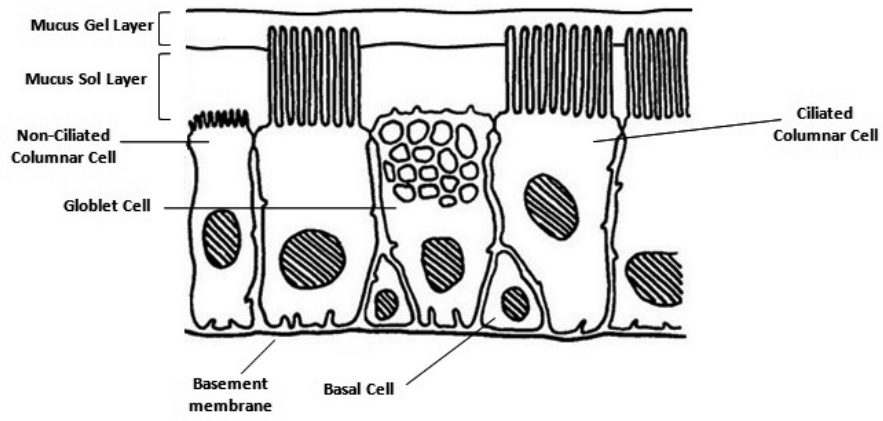

Figure I.2.2. Schematic representation of nasal respiratory epithelium (Adapted from Jorissen and Jaspers, 2013).

The lamina propria of the respiratory epithelium contains several glands, nerves, lymphatic tissue, immune cells and large blood vessels that are embedded in connective tissue. There are two layers of glands, the superficial layer situated just under the epithelium and the deep layer separated by a vascular layer. These glands, connected to the epithelium by an excretory canal, produce secretions; those originated in the anterior part of the nose have lower viscoelasticity than those coming from the posterior part. The blood vessels of the nose differ from the vasculature of the remaining respiratory tract in some peculiar characteristics. There are arterio-venous anastomoses where the blood bypasses the capillary bed and flow from an artery directly into a vein. These vessels are important in water and temperature control because they reduce water and heat loss by avoiding blood to flow through the very permeable fenestrated capillaries of the nasal mucosa. The inferior turbinates have large venous sinusoids which make up the major component of the mucosa thickness, namely the venous erectile tissue. These venous sinusoids are normally in a semi-contracted condition; when they distend with blood, the nasal mucosa swells, causing congestion and increased resistance to airflow. Blood flow through these nasal vessels is controlled by autonomic innervations of the nasal mucosa. Sympathetic stimulation causes reduction in the nasal blood flow and contraction of the vessels with decongestion of venous erectile tissue. This sympathetic stimulation is cyclic giving rise to the nasal cycle. Another type of nerves found in lamina propria is the parasympathetic innervations of the glands. Finally, it is also possible to detect immunocompetent cells in the lamina propria of nasal mucosa, namely mast cells and lymphocytes, able to protect the nose from inhaled pathogens (Mygind and Dahl, 1998; Watelet and Cauwenberge, 1999).

Nasal olfactory mucosa

The nasal olfactory mucosa is also a pseudostratified columnar epithelium lying on a basement membrane adjacent to the lamina propria (*Figure I.2.3.*). It is a modified form of the nasal respiratory epithelium with three different types of cell: olfactory receptor cells, supporting (or sustentacular) cells and basal cells. The olfactory receptor cells are bipolar neurons projecting a single dendrite to the surface of the olfactory mucosa and a single axon to the olfactory bulb. Through these specialized cells, the central nervous

system (CNS) is directly exposed/connected to the external environment. At the epithelial surface, dendrites of olfactory receptor cells terminate in a small knob containing non-motile cilia with membrane chemical receptors where odorant molecules bind for smell perception. Axons cross the basement membrane of olfactory mucosa, progress into the lamina propria, gathering in bundles and passing through the cribriform plate of the ethmoid bone towards the olfactory bulb. In lamina propria, axons of these bundles are covered by glial cells (Schwann cells) which form fluid-filled perineural channels essential for the action potential propagation, creating also an extracellular pathway for the transport of molecules to brain. Both intracellular axonal and extracellular perineural pathways are recognized mechanisms involved in the direct transport of drugs to brain through the olfactory region (Escada et al., 2009; Illum, 2000; Mistry et al., 2009).

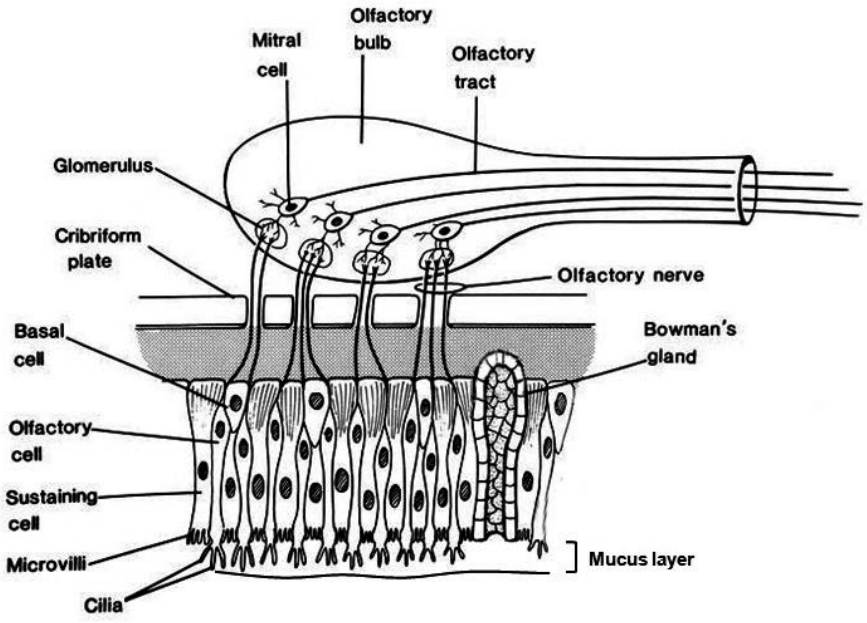

Figure I.2.3. Schematic representation of nasal olfactory mucosa and its connection to olfactory bulb (Adapted from Illum, 2000).

The sustentacular cells are elongated columnar cells interspaced between olfactory receptor cells; they have long microvilli at the apical surface that are entangled with the cilia of the olfactory cells and provide them with mechanical and metabolic support. Basal cells are progenitor cells of the olfactory epithelium, capable of continuously regenerating them along the life span (Escada et al., 2009; Illum, 2000). Lamina propria contains blood

and lymphatic vessels, olfactory axon bundles and the tubuloalveolar serous Bowman's glands, which secrete the mucus that covers the surface of olfactory epithelium. This mucus layer acts not only as a trap but also as a solvent for odorant molecules; its constant flow from the Bowman's glands keeps the mucosa clean so that new scents can be continuously detected as they arise (Gizurarson, 2012).

I.2.1.2. Anatomy, Histology and Physiology of Human Paranasal Sinuses

The nasal cavity is also connected to other small and paired air-containing cavities inside the facial bones – the paranasal sinuses – which are lined with a thin layer of mucosa, similar to the respiratory one. They comprise the maxillary, frontal, sphenoid and ethmoid sinuses. Asymmetries between the two sides of the skeleton and inter-individual variations in shape and size of the paranasal sinuses are frequent and, in some cases, the frontal and sphenoid sinuses are completely absent in one or both sides. The mucosa of paranasal sinuses is ciliated but has low density of glands, is less vascularized and more loosely attached to the bone walls than the respiratory mucosa of nasal cavity. The mucosa of both nasal and sinuses cavities are contiguous and often referred to as sinonasal mucosa (Gizurarson, 2012; Jones, 2001). The paranasal sinuses communicate with the nasal cavity via small openings (ostia) and narrow ducts that allow aeration and sinus mucus drainage. The ostiomeatal complex is an important anatomical area of the middle meatus of the nasal cavity that receives the openings of the maxillary, anterior ethmoidal and frontal sinuses and is involved in the pathophysiology of those sinuses diseases. The posterior ethmoidal and sphenoidal sinuses drain into the superior meatus and to an opening above the superior turbinate of the nasal cavity, respectively. The functions of the paranasal sinuses are controversial and still have to be established. They may assure harmony in facial growth, make the skull lighter and protect the brain. Other possible functions are a contribution in heating and humidifying the inspired air and the increase of the voice resonance (Jones, 2001; Watelet and Cauwenberge, 1999).

I.2.1.3. Physiology of Human Nose

The nose is the first line of defense of the respiratory system. The two major physiological functions of this organ are breathing and olfaction. Other important

protective and supportive activities of the nose are filtration/cleansing, heating and humidification of the inspired air before it reaches the lower respiratory tract. These basic functions related to nasal breathing make the nose an important system of defense of the delicate tissues of the lower respiratory tract. The anatomical and histological characteristics of the nose control airflow dynamics and are determinant for the optimal performance of all those nasal functions (Bitter et al., 2011; Jones, 2001; Pires et al., 2009).

The air flows in the nasal cavity at different velocities, according to the width of the nasal airway passages. The speed at the entrance of the nasal cavity is 2-3 m/s, but in its narrowest portion – nasal valve – is high and reaches 12-18 m/s, returning to 2-3 m/s in the region of the turbinates where the airflow becomes more horizontal. Interestingly, during sniffing the airflow is deviated in the direction of the superior turbinate and the olfactory region; this fact shows that the velocity of inhaled airstream also influences the nasal airflow (Watelet and Cauwenberge, 1999). Nasal resistance to airflow depends on the shape and size of nasal cavity, in which the nasal turbinates play an important role. There are several factors that can modify the size of turbinates and thus nasal resistance, namely exercise, emotions, environment, hormones and drugs. For example, exercise usually decreases nasal airway resistance while dust, smoke and alcohol increase it. The turgescence of turbinates normally varies along the nasal cycle. This physiological phenomenon consists of periodic congestion and decongestion of the nasal venous sinusoids that occurs alternatively in each half of the nasal cavity every 3-7 h (Jones, 2001; Mygind and Dahl, 1998; Watelet and Cauwenberge, 1999). Despite the continuous diameter changes in left and right nasal cavities, the total nasal airway resistance remains almost constant in a healthy nose. The nasal patency (measure of cross-sectional area of nasal cavity) and nasal airway resistance, as well as the nasal delivery device used, influence drug nose distribution/deposition (Hanif et al., 2000; Mygind and Dahl, 1998).

The nose is well suited to its air-conditioning function due to the slit-like shape of nasal cavity and its turbinates that promote the contact between the inhaled air and nasal mucosa, the ability to rapidly change the width of the nasal cavity by alteration of venous sinusoids contraction according to the external environment, the blood flow in the opposite direction of incoming airflow and the high mucus secretion capacity. Unconditioned inspired air produces changes in the epithelium of respiratory system and

may lead to crusting and infections which are two complications of a deficient air conditioning function. Therefore, the nose plays an important role in heating and humidifying the inhaled air. It has been demonstrated that inhaled air at room temperature (20-25 °C) is heated to 34 °C and humidified at 80% by the time it reaches pharynx. After breathing cold air (0 °C), inhaled air is conditioned to 31 °C and 98% relative humidity. These values prove the efficacy of the nose in conditioning inspired air (Mygind and Dahl, 1998; Naftali et al., 2005; Watelet and Cauwenberge, 1999).

Inspired air contains a high concentration of particles (e.g. allergens, dust, pathogens), which can damage the fragile tissues of lower respiratory tract and cause serious consequences. Therefore, deposition of inhaled particles in the nose is indispensable. The nose is the site of entry of inhaled air and is the principal organ of particle deposition in the respiratory airways. Its nostrils present hairs that block larger particles at the entrance and the shape of the nasal cavity provides sufficient air turbulence, causing impaction of particles into the adhesive mucus layer. Inhaled particles, trapped in the "nasal filter" are then cleared from the nose by mucociliary transport. The efficacy of the nose as a filter depends on the diameter of the particles. During normal breathing, almost all particles larger than 10 µm are retained in the nose, while particles smaller than 2 µm can reach the lower airways. The nose is also a protective filter against water-soluble and irritant gases (Jones, 2001; Mygind and Dahl, 1998; Watelet and Cauwenberge, 1999).

Nasal mucus and mucociliary clearance

Apart from the above described functions of warming, humidifying and filtering the airflow, nasal mucus plays a fundamental role in human physiology by cleaning the particles trapped in the filtering process, through a mechanism called MCC.

The nasal respiratory mucosa is covered by a watery sticky material called mucus. This substance is composed of water (95%), mucin (2%), other proteins such as albumin, lactoferrin, lysozymes and immunoglobulins (1%), inorganic salts (1%) and lipids ($^{<}$1%) (Mygind and Dahl, 1998). The pH of nasal mucus is slightly acidic and varies between 5.5 and 6.5. Mucins are important components that determine the viscoelastic properties of

nasal mucus; they are glycoproteins produced by globet cells in the epithelium and by secretory glands in the lamina propria (Arora et al., 2002; Chugh et al., 2009).

The nasal mucus blanket consists of two layers: a lower aqueous/sol layer, bathing the cilia (the periciliar fluid) and an upper viscous/gel layer, resting on the tips of the cilia (*Figure I.2.2.*) This blanket of mucus is propelled by synchronized beating of epithelial cilia towards the nasopharynx, oropharynx and then swallowed to gastrointestinal tract for digestive elimination. This process is called MCC and is dependent on the activity of cilia and on the mucus volume and viscoelasticity. The thickness and composition of the double layer is important for mucociliary transport. If the periciliary layer is too thin, the beating of cilia will be hindered by the surface gel layer. On the other hand, if it is too thick, the surface gel layer loses its contact with cilia and cannot be moved by their tips, impairing the mucociliary transport (Jones, 2001; Mygind and Dahl, 1998; Pires et al., 2009). In healthy humans, the MCC rate is different in different sites of the nose, since it depends on the length, density and beat frequency of cilia as well as mucus properties, with an average value of 5 mm/min. Despite the inter-individual variability of nasal MCC transit time, the nasal mucus blanket is renewed every 15 to 20 min. A duration of 30 min is considered the limit that discriminates healthy subjects from those with impaired MCC (Bitter et al., 2011; Deborah and Prathibha, 2014; Pires et al., 2009). This effective cleansing mechanism is thus required to maintain the health and defense of the nose and paranasal sinuses as the mucus blanket is contiguous across these cavities and sinus MCC propels the mucus from the sinuses to the nasal cavity to be further removed from the nose.

Factors affecting mucociliary clearance

The nose may be vulnerable to high ambient concentrations of toxic substances and to virusal and microbial infections, being afflicted by many diseases that compromise MCC. In fact, viruses and bacteria can disrupt sinonasal epithelium with loss of a continuous cilia distribution, impairing mucus transport. For example, in common cold it was found that viruses can lead to a decreased number of cilia. Moreover, mucus hypersecretion is a common characteristic of sinus and nasal tissues inflammation observed in CRS and allergic rhinitis and is associated with hyperplasia and hypertrophy of epithelial globlet cells and lamina propria glands. In patients with CRS, areas of ciliary denudement have been observed, but whether the ciliary beating frequency is altered is

still a controversial issue. The ciliated surface of polyps in these patients can undergo squamous metaplasia but, when the mucociliary apparatus is preserved, mucus flows normally; however due to the pedunculated swelling mucosa the direction of the mucus flow may be lost. Patients with CRS have an impaired mucociliary function most likely due to loss of cilia, mucus pathway obstructions and increased viscosity of nasal mucus (Arora et al., 2002; Chen et al., 2006; Ferguson et al., 1988; Jones, 2001; Marttin et al., 1998; Sahin-Yilmaz and Naclerio, 2011).

A decrease in MCC function favors a longer contact of sinonasal mucosa with noxious particles and pathogens and may lead to local underlying tissue damage or lower respiratory tract infections.

Enzymatic and immunological protection

Besides the physical barrier provided by adhesive nasal mucus, MCC and intercellular junctions of epithelial cells, the nose plays a fundamental role in enzymatic and immunological protection. The former takes place through the metabolism of endogenous and xenobiotic substances. The immunological barrier is provided by antimicrobial molecules (e.g. lactoferrin, lysozyme, defensin) and immunoglobulins present in nasal mucus and by the activation of innate and adaptive immune response of immunocompetent cells of nasal mucosa (Kern and Decker, 2013; Mygind and Dahl, 1998; Pires et al., 2009; Sarkar, 1992).

I.2.2. INTRANASAL DRUG DELIVERY

I.2.2.1. Rationale of Intranasal Drug Delivery – Therapeutic Applications

Intranasal drug delivery has been the target of intensive research over the past decades, due to the anatomical and histological characteristics of the nose which make it an attractive option for topical (or local) and systemic delivery. The nasal cavity is an easily accessible route with several advantages. Its large surface area, porous epithelial membrane (with a higher molecular mass cut-off than gastrointestinal tract) and highly vascularized mucosa ensure a rapid and good drug permeation and systemic absorption, leading to a quick onset of therapeutic action. In addition, IN drug delivery avoids

gastrointestinal acidic and metabolic drug degradation, as well as hepatic first-pass metabolism; therefore, it is recognized as an alternative route to overcome the low bioavailability of some compounds observed after oral administration (Arora et al., 2002; Bitter et al., 2011; Ghori et al., 2015; Ugwoke et al., 2001). In fact, lipophilic drugs are well absorbed from nasal cavity with identical pharmacokinetic profiles to those obtained after IV administration and bioavailability close to 100% (Bitter et al., 2011; Illum, 2003). Other features of IN administration offer practical advantages: it is non-invasive, essentially painless, needle free and easily performed by patients, thus improving patient convenience and compliance, which is particularly important in the treatment or control of chronic conditions (Costantino et al., 2007; Pires et al., 2009). Given these positive attributes, a large number of drugs, ranging from small molecules (like analgesics, antibiotics and antiepileptics) to large biomacromolecular drugs (e.g. peptides, proteins and vaccines), can be administered by IN route for several therapeutic effects (Chugh et al., 2009; Costantino et al., 2007; Fortuna et al., 2014; Johnson and Quay, 2005; Pardeshi and Belgamwar, 2013; Pires et al., 2009).

Historically, IN drug delivery appeared at first as a logical and natural method to apply drugs into nasal (or paranasal sinuses) mucosa for ***topical (or local) effects***, as will be detailed below (*section I.2.2.3.*) (Alsarra et al., 2010; Costantino et al., 2007).

The widespread interest of this route was triggered by the potential revealed as a promising alternative to the traditional oral and IV routes for the delivery of systemically-acting drugs. The rapid absorption and onset of action of intranasally administered drugs meet the medical needs in crisis resolution and high nasal bioavailability makes this route suitable for treatment of long term disorders. Examples of therapeutic areas, where marketed or under development drugs for IN ***systemic delivery*** may be used, include acute pain relief, migraine, smoking cessation, *angina pectoris*, osteoporosis, cancer, insulin-dependent diabetes and hormonal replacement (Fortuna et al., 2014; Grassin-Delyle et al., 2012; Mathias and Hussain, 2010).

The IN administration has also received great attention as a route for ***immunization***, since nasal mucosa is rich in nasal-associated lymphoid tissue. The majority of infections are caused by pathogens entering the body through mucosal surfaces which form a first-line defense barrier. IN delivery of vaccines is known to produce not only local but also

systemic immune responses mediated by the nasal-associated lymphoid tissue present in the lamina propria, which contains agglomerates of immunocompetent cells. In fact, significant levels of immunoglobulin A have been detected in other mucosal secretions after IN vaccination. Therefore, immune protection by nasal vaccines is not restricted to the upper airway infections (e.g. influenza and measles) but may extend to non-respiratory infections in distant mucosal organs (e.g. cholera and human immunodeficiency virus). In spite of intensive research, only few nasal vaccines are fully developed and commercially available and they are all against the influenza virus (Fortuna et al., 2014; Jadhav et al., 2007; Pires et al., 2009; Slütter et al., 2008).

Recent research has demonstrated the ability of IN route to bypass the blood-brain-barrier through olfactory and trigeminal nerve transport, enabling a direct transport of CNS-acting drugs. Both nerves innervate nasal mucosa, directly connecting nose and brain. However, olfactory nerve pathway is well recognized as the most direct portal for drugs to enter CNS and is commonly reported as the preferential component for IN drug delivery to CNS. In fact, the neuroepithelium of nasal mucosa has the unique anatomical feature of linking the external environment of nasal cavity to the brain. This feature makes IN an advantageous route for targeting drugs to brain. IN administration especially to the olfactory region has been shown to deliver a wide variety of compounds from the nasal cavity directly to CNS within few minutes. Consequently, a rapid and preferential drug uptake into the brain may be obtained, improving the therapeutic efficacy with less systemic exposure and enhanced tolerability. *CNS delivery* by IN route has emerged as a new approach and attracted extensive research to treat neurological disorders, such as epilepsy, Alzheimer´s and Parkinson's diseases (Barnett and Perlman, 1993; Chugh et al., 2009; Dhuria et al., 2010; Illum, 2003; Pardeshi and Belgamwar, 2013; Serralheiro et al., 2013; Vyas et al., 2005).

Despite the multiple advantages of IN drug delivery, there are some limitations. For example, the limited capacity of nasal cavity restricts the volume of the formulation to be administered. In order to avoid anterior or posterior loss of the formulation from the nasal cavity, the total volume should be lower than 200 µL. For that reason, IN drug delivery is more appropriate for drugs which are effective at low doses or for those with high water solubility. Frequent IN administration may also result in mucosal damage (with epistaxis

and burning sensation); inter- and intra-individual variability associated with the method of delivery is another disadvantage (Alagusundaram et al., 2010; Grassin-Delyle et al., 2012; Ugwoke et al., 2001). Moreover, nasal physiological barriers must be overcome (*section I.2.2.2.*) to get the highest possible benefit of IN delivery positive characteristics. Further research work in this area is expected to contribute to new therapeutics or to extend the life cycle of existing and well-known drugs.

I.2.2.2. Processes and Factors Conditioning Intranasal Drug Delivery

In order to achieve the goals of IN drug delivery for either topical, systemic or CNS effects, it is important to consider the drug pathway from the administration point to the target area. Drug availability/disposition via IN administration is governed by nasal physiological factors and by the physicochemical properties of the drug. The processes combining both aspects will now be briefly discussed, also in view of their role in the choice or development of drug formulations and delivery devices. Understanding these issues is the key for optimizing drug concentration at the target site and for designing new and successful nasal products for a specific therapeutic purpose.

Nasal permeability and nasal metabolism

The first step following IN administration is the passage of therapeutic molecules through the mucus layer. The transport of drug molecules across this mucus blanket is mainly based on diffusion mechanisms which are governed by several physicochemical factors and mucus composition. Small uncharged particles can easily cross the network of mucus glycoproteins but this is more difficult for larger or charged particles. The potential binding of drugs to these mucus glycoproteins can hinder the drug diffusion process (Chand et al., 2010; Fortuna et al., 2014; Jadhav et al., 2007). Drugs need to be solubilized in nasal mucus before they reach nasal epithelium because only their molecularly disperse form can penetrate biomembranes. Therefore, in the case of powders and suspensions, a rapid drug dissolution rate is crucial since otherwise solid particles will be removed by MCC (Alsarra et al., 2010). After diffusion through the mucus layer, drugs at the apical surface of nasal epithelium penetrate this membrane into the underlying tissue (where systemic absorption can take place) or into the directly connected CNS tissue. The passage across

the nasal epithelium may occur by two types of transport, one involving a paracellular pathway and the other a transcellular route.

The paracellular transport takes place via gaps and pores between epithelial cells. It is an aqueous pathway favorable for more hydrophilic drugs. Thigh junctions observed in both respiratory and olfactory regions are determinant for this type of transport because their size is comprised between 3.9 and 8.4 Å, restricting the transport of larger molecules. Hence, there is an inverse relationship between nasal epithelium permeation and molecular weight of hydrophilic compounds (Fortuna et al., 2014; Illum, 2003; Jadhav et al., 2007; Pires et al., 2009).

In transcellular transport, drugs may cross the epithelial cell by concentration-dependent passive diffusion, as well as by active carrier-mediated or vesicular (transcytosis) transport processes. The first mechanism involves a lipoidal pathway through the phospholipidic bilayer cell membrane and is responsible for the transport of lipophilic drugs; therefore, the rate and extent of nasal permeation will be dependent on their degree of lipophilicity. Moreover, several transporters are expressed at the membrane of epithelial cells that are responsible for the influx and efflux of a wide variety of drugs, thus regulating their uptake/excretion in the nasal mucosa (Costantino et al., 2007; Fortuna et al., 2014; Jadhav et al., 2007; Oliveira et al., 2016).

The permeation through nasal epithelium of drugs with molecular weight < 300 Da is not significantly influenced by their physicochemical properties, since these drugs will easily diffuse through the aqueous channels of the membrane. However, the rate and extent of permeation is sensitive to molecular size and lipophilicity (measured by partition coefficient octanol/water) if the molecular weight is > 300 Da. Drugs with molecular weight higher than 1000 Da have been reported to exhibit poor bioavailability after IN administration. Besides these physicochemical properties, the pKa is also important because it determines the ionized/unionized fraction which affects the permeation since unionized (or zwitterionic) species are more liable to transcellularly transverse the epithelial membrane (Arora et al., 2002).

Nasal MCC may act as a barrier to nasal bioavailability (i.e. drug absorption into systemic circulation) by limiting drug contact time with the nasal epithelium. A rapid diffusion through mucus layer together with a rapid penetration across epithelial membrane circumvent the effects of nasal MCC (Gizurarson, 1993; Ugwoke et al., 2001).

After IN administration, drugs may still be metabolized in lumen of nasal cavity or during the passage across nasal mucosa, due to the presence of a broad range of enzymes that have been identified in human nose. These include oxidative phase I enzymes – such as cytochrome P450 (CYP) isoenzymes, carboxylesterases and aldehyde dehydrogenases – conjugative phase II enzymes – like glucuronyltranferases and glutathione *S*-transferases – as well as proteases and peptidases (Oliveira et al., 2016; Pires et al., 2009). Although IN drug delivery avoids gastrointestinal and hepatic first-pass metabolism, a nasal pseudo first-pass effect cannot be overlooked. Drug metabolizing enzymes are present in nasal mucus, in nasal epithelial cells and in lamina propria, with a trend for a higher expression in olfactory mucosa compared with respiratory mucosa. In particular, although CYP content in nasal mucosa expressed per gram of tissue, is the second most abundant after the liver, its catalytic activity in nasal mucosa was found to be comparable or even higher than in any other tissue (Illum, 1996; Oliveira et al., 2016; Sarkar, 1992). Notwithstanding, nasal enzymatic activity does not appear to have a significant effect on the extent of systemic absorption of most compounds, except for proteins and peptides. Compared to gastrointestinal mucosa and liver metabolism (determinant of drug bioavailability after oral administration), the nose is considered to offer a more favorable metabolic environment for drug delivery. Further research is needed to fully explore the effect of nasal enzymatic barrier, thus increasing the potential of this route (Bhise et al., 2008; George, 1981; Hussain, 1998; Sarkar, 1992; Wong and Zuo, 2010). In addition, after nasal application a portion of the drug might be swallowed (due to the nasal MCC) and subsequently subjected to gastrointestinal absorption and metabolism, reducing the amount of drug available in nasal cavity for the intended therapeutic propose. To avoid this drug loss, several strategies have been used, ranging from reducing the volume used, increasing the nasal residence time and dividing the dose to both nostrils (Gizurarson, 1993; Gonda and Gipps, 1990; Wong and Zuo, 2010).

Formulation and device factors

Apart from nasal physiological factors and drug physicochemical properties, the nasal formulation and administration device must be considered in IN drug delivery, as they are also important factors for drug permeation across nasal tissue and they

determine the site and pattern of nasal drug deposition/distribution (Arora et al., 2002; Kublik and Vidgren, 1998).

The pH of IN formulation affects drug permeation; it should be adjusted in the range of 4.5-6.5, a pH similar to that found in nasal cavity, to avoid mucosa irritation. High viscosity of the formulation increases drug permeation by increasing the contact time between drug and nasal mucosa and hindering normal ciliary beating and MCC. The addition of polymers confers viscosity to the formulation, leading to longer drug residence time, which results in higher concentration gradient and subsequent increased rate of absorption (Alsarra et al., 2010; Jadhav et al., 2007).

The dosage form of IN formulation, the administration device and the administration technique are important features that influence the site and deposition area of the drug. Deposition at nasal vestibule, a non-ciliated keratinized and poorly vascularized region, may prolong the residence time of drug but absorption is low. On the contrary, the respiratory region is a preferred deposition area for absorption to occur, but its rate and extension may be compromised due to elimination of the formulation by MCC. The anterior part of this region is a better option since the ciliary density is lower and the distance before reaching nasopharynx is longer. Deposition in the olfactory epithelium may facilitate the direct transport of the drug into the brain but for susceptible compounds it is liable to increase enzymatic degradation (Bhise et al., 2008; Ugwoke et al., 2001). To achieve a specific therapeutic effect, dose precision and drug deposition should be as accurate and reproducible as possible; therefore the administration device and type of formulation should be carefully designed. Several basic types of formulation are available for IN administration, namely solutions, suspensions, powders, ointments, creams and gels. Devices should be compatible with the formulation to be delivered and examples include catheters, droppers (or pipettes), squeeze bottles, insufflators, (metered-dose) pump sprays and nebulizers (Kublik and Vidgren, 1998; Ugwoke et al., 2001).

Nasal drops are one of the simplest and convenient delivery methods; however, the delivered volume cannot be clearly controlled and thus the main disadvantage of nasal drops is the lack of dose precision. In addition, there is a great risk of contamination during use, microbiological growth and drug instability. Moreover, nasal rapid drainage occurs with this delivery method (Ghori et al., 2015; Jadhav et al., 2007). Solution and suspension nasal sprays are preferred over powder sprays because powders induce more nasal

mucosa irritation. Metered-dose spray pumps dominate the IN delivery market and are comparatively more accurate than drops, generating precise and defined doses with a typical spray pattern. Dose volumes between 25-200 µL are available as standard (Djupesland, 2013; Kublik and Vidgren, 1998; Pires et al., 2009). Nasal powders are less frequently used in IN drug delivery; they are preferred when drug is instable in liquid formulations and present the extra advantage of not requiring preservatives (Jadhav et al., 2007). Nasal gels are semi-solid preparations with increased viscosity; they have the ability to prolong the contact time of drug with mucosa and they reduce the post-nasal drip, the anterior leakage of the formulation and the irritation potential of mucosa by using soothing agents. Recently, metered-dose gel devices have been developed for accurate dosing; however, there is a limit to the viscosity of the formulated nasal gel to be appropriately administered. The deposition of nasal gels in the nasal cavity depends on the mode of administration. In fact, due to their viscosity, nasal gels have poor spreading abilities and only reach a smaller distribution area in nasal cavity, near the place where they are directly applied (Bhise et al., 2008; Kublik and Vidgren, 1998). In the last decade, *in situ* nasal gels have emerged as a novel promising approach for IN drug delivery. *In situ* gels gather the advantages of both solutions and gels, as they have the ability to undergo transition from a solution, which can be more easily administered, to a gel at the physiological condition of nasal cavity, preventing the rapid clearance from the nose. The so called smart polymers used (with varying mucoadhesive properties) have paved way for scientific investigation on topical (or local), systemic and CNS therapeutic effects (Karavasili and Fatouros, 2016; Singh et al., 2013).

To optimize IN drug delivery, it is important to understand both physiological and pharmaceutical factors that influence nasal drug permeability and metabolism. From a pharmacokinetic point of view, the nose is a complex organ since several processes occur concurrently at nasal mucosa after deposition of formulation – drug release, absorption, local excretion and enzymatic degradation, removal of formulation by MCC – which determine the overall concentration of drug at the nasal mucosa (Gizurarson, 1993; Grassin-Delyle et al., 2012; Jogani et al., 2008).

I.2.2.3. Intranasal Delivery of Topical Drugs

IN drug delivery is an intuitive choice for topical treatment of local diseases of nose and paranasal sinuses and has been widely used for a long time. Typical examples of topically-acting intranasally administered drugs are decongestants, antihistamines, corticosteroids and antimicrobials. They are mainly used in the treatment of nasal congestion, rhinitis and rhinosinusitis inflammation and infection, as well as related allergic conditions. Topical IN therapies enable direct drug delivery to the target organ (biophase), leading to a quick pharmacologic action and relief of symptoms. Moreover, due to the fact that relatively low doses are effective when administered topically, potential systemic adverse effects, which are commonly associated with conventional administration routes (oral and IV), are minimized (Bitter et al., 2011; Costantino et al., 2007; Pires et al., 2009; Serralheiro et al., 2013). Nasal products for topical effects constitute a very significant fraction of the total nasal products available in the market; representative examples are shown in *Table I.2.1.* together with their approved indications.

Table I.2.1. Examples of marketed nasal products for topical (or local) delivery (Pires et al., 2009; Kumar et al., 2016; Rudmik et al. 2013; Drugs@FDA: FDA Approved Drug Products; EMA: European Assessment Reports; Infomed; electronic Medicines Compendium).

Drug	Delivery Method	Posology	Trade Name Manufacturer	Therapeutic Indication
DECONGESTANTS				
Oxymethazoline hydrochloride	Nasal Drops (0.5 mg/mL)	2-4 drops/nostril BID	Nasex® Janssen-Cilag	Nasal congestion (common cold, rhinitis, sinusitis)[3]
	Nasal Spray	1-2 actuations/nostril BID or TID	Sinexsensi® Vicks Sinex® Procter & Gamble	Nasal congestion (common cold, rhinitis, sinusitis)[3]
Phenylephrine	Nasal Drops (5 mg/mL)	2-3 drops/nostril	Neo-Sinefrina® Omega Pharma Portuguesa	Nasal congestion (common cold, rhinitis, sinusitis)[3]
Xylometazoline hydrochloride	Nasal Drops (1 mg/mL)	2-3 drops/nostril BID, TID or QID	Otrivin® GlaxoSmithKline	Nasal congestion (common cold, rhinitis, sinusitis)[3]
ANTIHISTAMINES				
Olopatadine hydrochloride	Nasal spray (metered-dose: 665 µg/actuation)	2 actuations/nostril (2660 µg) BID Total daily dose: 5.32 mg	Patanase® Alcon	Seasonal allergic rhinitis[1]
Azelastine hydrochloride	Nasal spray (metered-dose: 137 µg/actuation)	2 actuations/nostril (548 µg) BID Total daily dose: 1.096 mg	Astelin® Meda Pharmaceuticals	Seasonal allergic rhinitis[1] Vasomotor rhinitis[1]

ANTIHISTAMINES				
Azelastine hydrochloride	Nasal spray (metered-dose: 140 µg/actuation)	1 actuation/nostril (280 µg) BID Total daily dose: 0.56 mg	Rhinolast®/Allergodil® Meda Pharmaceuticals	Seasonal and perennial allergic rhinitis[3]
CORTICOSTEROIDS				
Beclomethasone dipropionate monohydrate	Nasal spray (metered-dose: 42 µg/actuation)	1-2 actuations/nostril BID Total daily dose: 0.168–0.336 mg	Beconase AQ® GlaxoSmithKline	Seasonal and perennial allergic rhinitis[1] Vasomotor rhinitis[1] Prevention of CRSwNP after surgery[1]
Beclomethasone dipropionate	Nasal aerosol (metered dose: 50 µg/actuation)	2 actuations/nostril BID 1 actuation/nostril TID or QID Total daily dose: 0.4 mg	Neo-Sinefrina®Alergo Omega Pharma Portuguesa	Seasonal and perennial allergic rhinitis[3]
Budesonide	Nasal spray (metered-dose: 32 or 64 µg/actuation)	1-2 actuation(s)/nostril OD or BID Total daily dose: 0.128–0.256 mg	Rhinocort®/Pulmicort® Astrazeneca Pharms	Seasonal and perennial allergic rhinitis[1,3] Vasomotor rhinitis[3] CRSwNP[3]
Ciclesonide	Nasal spray (metered-dose: 50 µg/actuation)	2 actuations/nostril OD Total daily dose: 0.2 mg	Omnaris® Sunovion Pharmaceuticals	Seasonal and perennial allergic rhinitis[1]
Flunisolide	Nasal spray (metered-dose: 25 µg/actuation)	2 actuations/nostril BID Total daily dose: 0.2 mg	Flunisolide Bausch & Lomb Inc	Seasonal and perennial allergic rhinitis[1]
Fluticasone furoate	Nasal spray (metered-dose: 27.5 µg/actuation)	2 actuations/nostril OD Total daily dose: 0.110 mg	Avamys® GlaxoSmithKline	Allergic rhinitis[2,3]
Fluticasone propionate	Nasal spray (metered-dose: 50 µg/actuation)	2 actuations/nostril OD Total daily dose: 0.2 mg	Flonase® GlaxoSmithKline Flixonase® GlaxoSmithKline UK	Seasonal and perennial allergic rhinitis[1,3]
Mometasone furoate monohydrate	Nasal spray (metered-dose: 50 µg/actuation)	2 actuations/nostril OD or BID Total daily dose: 0.2 or 0.4 mg	Nasonex®/Nasomet® Merck Sharp & Dohme	Seasonal and perennial allergic rhinitis[1,3] CRSwNP[1,3]
Triamcinolone acetonide	Nasal spray (metered-dose: 55 µg/actuation)	2 actuations/nostril OD Total daily dose: 0.22 mg	Nasacort® Sanofi Aventis	Seasonal and perennial allergic rhinitis[1,3]
ANTIMICROBIALS				
Mupirocin calcium	Nasal ointment (2 % w/w)	single-use tube or small amount (match head size) BID or TID	Bactroban® GlaxoSmithKline	Eradication of nasal colonization of Staphylococci[1,3]

[1]FDA approved indication.

[2]EMA approved indication.

[3]Approved indication in European countries (e.g. Portugal and United Kingdom).

CRSwNP, chronic rhinosinusitis with nasal polyps; OD, once daily; BID, twice daily; TID, three times daily; QID, four times daily.

Decongestants

Topical IN decongestants are widely prescribed for the symptomatic relief of nasal congestion in common cold, allergic and nonallergic/vasomotor rhinitis, acute and chronic rhinosinusitis.

Nasal congestion, described as a feeling of blockage, fullness or restricted airflow, is the primary symptom of upper respiratory tract diseases. The principal cause of nasal congestion is inflammation which is characterized by venous engorgement, increased nasal secretion and tissue swelling/edema leading to the nasal blockage sensation. Thus, there are two types of pharmacologic strategies, one that targets inflammation mechanisms (use of corticosteroids and antihistamines) and another targeting venous engorgement by the use of decongestants (Meltzer et al., 2010).

IN decongestants include sympathomimetic amines (e.g. phenylephrine and pseudoephedrine) and imidazoline derivatives (e.g. oxymetazoline, xylometazoline and tramazoline). Their pharmacologic effect results from direct or indirect activation of post-synaptic α-adrenergic receptors of the nasal mucosa vasculature; this produces vasoconstriction and subsequent decrease of mucosa swelling and nasal resistance to airflow which leads to decongestion (Corboz et al., 2008). Moreover, imidazolines which predominantly address α_2-adrenoceptors, also cause a reduction in the nasal mucosal blood flow due to their activity on the resistance vessels (α_2-adrenoceptors) (Caenen et al., 2005; Hochban et al., 1999). This reduction has been correlated to the long duration of decongestant effect of imidazolines as compared to sympathomimetic amines (Bende and Löth, 1986). Due to the non-selective and preferential affinity to α_2-adrenoceptors of imidazolines, less and transient cardiovascular adverse effects were observed after IN administration when compared to sympathomimetic amines (Corboz et al., 2008).

The most common problem associated with overuse of topical decongestants is rebound nasal congestion, reduction in efficacy (tachyphylaxis) and nonspecific nasal hyper-reactivity. This clinical condition is defined as rhinitis medicamentosa and it limits the practical utility of topical decongestants to short-term therapies (less than 10 days) (Akpinar et al., 2012). Topical IN decongestants have proven to be an effective treatment for nasal congestion in allergic rhinitis. Moreover, evidence supporting the usefulness of IN decongestants in nonallergic/vasomotor rhinitis or rhinosinusitis is very limited. These

drugs are, in fact, more appropriate for relief of congestion associated with common cold due to the shorter duration of this disease (Meltzer et al., 2010).

Antihistamines

Topical IN antihistamines are efficacious topical drugs used in the treatment of allergic rhinitis which is often associated with comorbid conditions such as asthma, rhinosinusitis, otitis media, polyposis and allergic conjunctivitis. This pathology is defined as an inflammation of the nasal mucosa caused by a hyperactive immune system response to benign, non-infectious environmental aeroallergens (e.g. pollens, mites, animal danders). The allergic airway inflammation process initiates with the recognition of allergens, causing degranulation and release of mediators, such as histamine (Dykewicz and Hamilos, 2010). Antihistamines, the commonly known H_1 receptor antagonists, are particularly effective at reducing the symptoms of sneezing, nasal itching and rhinorrhea in allergic rhinitis. *In vitro* investigations revealed that some antihistamines have also the potential to modify the inflammatory process, in addition to their H_1 histamine receptor blocking action (Howarth, 2000). However, for these effects to be fully evident *in vivo* antihistamine doses must be higher than those usually tolerated. Thus the topical IN delivery of antihistamines appears as an advantageous strategy to produce the additional benefit of reducing the inflammatory process by directly targeting the organ with high therapeutic drug concentrations and minimizing the risk of systemic sedative adverse effects (Howarth, 2000; Meltzer et al., 2010; Salib and Howarth, 2003).

IN antihistamines include levocabastine, azelastine, and olopatadine and their efficacy is equal or superior to that of second-generation oral antihistamines. These topical agents have a rapid onset of action, which makes them an appropriate “as required” therapy for episodic allergic symptoms relief (Dykewicz and Hamilos, 2010; Kaliner et al., 2009). Although levocabastine is a second-generation antihistamine, it causes some sedation when administered orally. For this reason and because of its remarkable potency, levocabastine was subsequently developed for IN delivery. Topical levocabastine has an onset of action of 10-15 min with a systemic bioavailability that ranges from 60-80% (Salib and Howarth, 2003). Topical azelastine is another second-generation antihistamine that has been developed to overcome the sedation effects of oral administration. This drug offers an onset of action of 15 min and has a systemic bioavailability of 40% following IN

administration. IN azelastine exhibits superior efficacy compared to IN levocabastine. Olopatadine is the most recent topical nasal antihistamine introduced in the market. Firstly it was approved as an ophthalmic solution, but in 2008 olopatadine appeared as a nasal spray indicated for the treatment of seasonal allergic rhinosinusitis. Clinical trials of olopatadine nasal spray have shown an onset of action within 30 min, a significant efficacy in relieving nasal allergy symptoms, including nasal congestion and an absolute bioavalability of 57% (Roland et al., 2010).

Corticosteroids

Topical IN corticosteroids are recognized as "the gold standard" of therapeutic choice in allergic rhinitis and the efficacy and safety of some of them is also well established for the treatment of rhinosinusitis. Compared with oral or local antihistamines, IN corticosteroids are more effective in what concerns the relief of nasal congestion symptom. This pharmacological class acts very early in the inflammatory pathway, modifying the ability of pro-inflammatory transcription factors to up-regulate gene expression (Howarth, 2000; Suh and Kennedy, 2011). This mechanism of action implies a time delay between administration and clinical activity. Hence, IN corticosteroids have a slower onset of action (several hours) than IN antihistamines with maximum efficacy developing over a period of days or weeks (Salib and Howarth, 2003).

Since oral corticosteroids have systemic adverse effects (*section I.1.6.1.*) which increase with dose and duration of treatment, IN administration of corticosteroids emerges as a promising alternative route to enhance the safety profile of these agents, especially in chronic conditions where courses of long duration are needed. Nevertheless, one should be aware of the possibility of these topical agents reaching the systemic circulation in sufficient concentration to produce adverse effects (Salib and Howarth, 2003; Sastre and Mosges, 2012). The systemic bioavailability of IN corticosteroids varies from <1% up to 40–50%. The newer IN corticosteroid agents (e.g. fluticasone propionate, mometasone furoate, ciclesonide, fluticasone furoate) have some characteristics that minimize their systemic bioavailability compared to older IN corticosteroids (e.g. beclomethasone, flunisolide, triamcinolone acetonide, budesonide). The systemic bioavailability of the newer IN corticosteroid drugs is negligible (<1%), which contributes to minimal risk of systemic adverse effects. This could be attributed to their higher

lipophilicity and metabolism by CYP isoenzymes. Increased lipophilicity is associated with greater binding affinity for corticosteroid receptor and retention in nasal tissue; consequently less free drug is available for systemic absorption and more for nasal metabolism, which results in fewer systemic adverse effects (Sastre and Mosges, 2012). For this reason, safety has been particularly well demonstrated for newer IN corticosteroids in many clinical trials (Salib and Howarth, 2003; Sastre and Mosges, 2012).

Antimicrobials

Topical IN antimicrobials are only commercially available for a particular and poorly defined risk group of individuals carrying staphylococci in the nasal vestibule. Although different antimicrobials have been evaluated to eradicate recurrent staphylococcal carriage, only IN 2% (w/w) mupirocin ointment application has been approved as a specific treatment to reduce/control infection rates in patients of a certain group risk undergoing surgery. In fact, several studies have demonstrated relatively prolonged periods of sterile nares in staphylococcal carriers after a short-course of IN mupirocin as compared to other antimicrobial agents. The above mentioned marketed nasal product for topical delivery of mupirocin is generally limited to the nasal vestibule (Goh and Goode, 2000; Kluytmans et al., 1997).

Topical antimicrobial agents are well established for treatment/control of skin infections as well as of mucosal infections in ocular and otic membranes. Nasal cavity and paranasal sinuses are common sites of infection with quite similar advantages for easy application through the nose. However, few studies have been conducted to exploit the feasibility of IN administration of antimicrobials in rhinosinusitis (Bremond-Gignac et al., 2011; Chaplin, 2016; Goh and Goode, 2000).

Even though acute rhinosinusitis has an infectious etiology, it has not attracted as much attention as CRS in the last two decades. One of the few studies reported is that conducted by Sahin-Yilmaz (2008); the effect of CIP otic drops (0.3%, w/v) in the context of acute rhinosinusitis induced in mice was evaluated by administering 50 µL in each nostril twice daily. No significant decrease in the number of bacteria was observed in nasal lavage after IN treatment. This lack of improvement on the bacterial infection was attributed to rapid nasal clearance of formulation or inability of drops to reach the site of infection in the sinuses (Sahin-Yilmaz et al., 2008). Bacterial and fungal biofilms have been associated

with chronic infections and are difficult to eradicate. Topical antimicrobial treatment presents a promising option for delivering the high concentrations needed at the target site to eradicate these biofilms with minimal systemic exposure and adverse effects. This has placed CRS in the centre of topical IN antimicrobial investigation as already discussed in *section I.1.6.2.*. An important systematic review of IN antimicrobials in the management of CRS reported that no definite conclusion could be made regarding the use of antifungals. However, topical IN antibacterials, although not recommended as first line management, may be successfully used in a subgroup of patients refractory to the recommended topical corticosteroids and oral antibiotics (Lim et al., 2008; Serralheiro et al., 2013).

I.2.2.4. Intranasal Delivery Methods Applied to Chronic Rhinosinusitis

To achieve the desirable effect of drugs intended to topical (or local) action, a broad distribution on the mucosal surfaces is required. Moreover, it is important to target the delivery to paranasal sinuses and to the middle and superior meatus, where most of the sinuses openings are located and may be obstructed by polyps in patients with CRS. Delivery devices are commonly divided into two groups, namely low volume and large volume devices. The former include droppers, sprays pumps or nebulizers, whereas the latter are intended to deliver at least 50 mL of solution (ranging up to 240 mL) and include neti pots, squeeze bottles, bulb syringes and powered devices (Djupesland, 2013; Thomas et al., 2013; Varshney and Lee, 2017).

Wormald et al. (2004) compared three methods of IN delivery, namely metered-dose nasal spray, nasal nebulization and nasal irrigation in patients with CRS who had previously undergone ESS which increases the access between the nasal cavity and the paranasal sinuses and removes hindering anatomical obstacles. They found that nasal irrigation was significantly more effective in penetrating the maxillary sinuses than the other two methods, whereas the sphenoid and frontal sinuses were poorly irrigated by all three methods (Wormald et al., 2004). This is in accordance with the recommendation, based on a systematic review by Thomas et al. (2013) for high-volume devices (for nasal irrigations) in post-surgical patients as opposed to low-volume devices, as they do not reliably reach the paranasal sinuses (Thomas et al., 2013). Singhal et al. (2010) investigated

the extent of sinus penetration achieved with nasal irrigation by varying the ostium size and head position in cadavers, concluding that as sinus ostium dimension increased, penetration by irrigant improved and that an ostium of at least 4.7 mm allowed maximal penetration in the maxillary and sphenoid sinuses. However, the same was not observed in the frontal sinus, but the effect of head position was only relevant in this case; most favorable penetration was observed with the head in a forward angle position (Singhal et al., 2010).

Conventional nasal pump sprays are commonly used mainly because of their ease of use and diverse available formulations. The majority of spray deposition occurs in nasal vestibule with some distribution in the inferior turbinate and less in the middle turbinate and middle meatus. Notwithstanding this limited distribution beyond the nasal vestibule, deposition in the middle meatus can be improved if the spray angle is optimized (Albu, 2012; Charlton et al., 2007; Sanan et al., 2017; Thomas et al., 2013; Weber et al., 1999).

Aerosol delivery devices such as nebulizers have the ability to deliver smaller diameter particles. In fact, nebulization covers a larger deposition surface area, reaching more posterior regions in nasal cavity than nasal pump sprays, with good access to the ostiometal complex. To achieve this, particles should have an optimal size of < 5 µm (Albu, 2012; Lim et al., 2008; Möller et al., 2011; Saijo et al., 2004). Although standard nasal nebulizers do not penetrate paranasal sinuses as efficiently as nasal irrigations, new emerging nebulizers (with pulsating airflow delivery) are evolving and some are already marketed. This may represent an important advance that will foster new nasal products with the specific approved indications for CRS (Djupesland, 2013; Möller et al., 2011).

Besides the dynamics of IN delivery methods, a major factor that impacts the success of topical therapies is patients' anatomy. The wide variability between individuals in shape and caliber of nasal cavity makes it difficult to give uniform recommendations for IN delivery (Homer et al., 2003).

I.3. FLUOROQUINOLONES

I.3.1. QUINOLONE GENERATIONS

Fluoroquinolones are one of the most promising and intensively studied drugs of contemporary anti-infective chemotherapy. They are members of a group of synthetic and structurally related compounds, known as the quinolone class. The first quinolone, nalidixic acid, was accidentally discovered in 1962 as a by-product of the synthesis of the antimalarial drug, chloroquine. The introduction of nalidixic acid for clinical use was limited to the treatment of uncomplicated urinary tract infections and its usefulness was hindered by its short half-life. Since then, several modifications have been made in its chemical structure in an attempt to improve the antibacterial activity and pharmacokinetic properties, leading to the development of numerous quinolone compounds. This class of antibacterial agents is currently classified into four generations (*TableI.3.1.*).

Table I.3.1. Classification of the quinolone class of antibacterials (Sousa et al., 2014).

Quinolone Generations		
First generation	**Third generation**	**Fourth generation**
Nalidixic acid	Levofloxacin	Trovafloxacin
Cinoxacin	Pazufloxacin	Clinafloxacin
Pipemidic acid	Sparfloxacin	Sitafloxacin
Second generation	Temafloxacin	Moxifloxacin
Norfloxacin	Gatifloxacin	Gemifloxacin
Lomefloxacin	Grepafloxacin	Ulifloxacin
Ciprofloxacin	Tosufloxacin	
Ofloxacin		

The first-generation quinolones (e.g. nalidixic acid, cinoxacin and pipemidic acid) have moderate activity against aerobic gram-negative bacteria and very little or no activity against aerobic gram-positive, anaerobes or atypical pathogens. These agents also present minimal systemic distribution and are less used nowadays (Andriole, 2005; Emmerson and Jones, 2003; Peterson, 2001; Sharma et al., 2009). The major breakthrough was the fluorination of the quinolone molecule at the position 6 of its basic structure (*Figure I.3.1.*),

which gave rise to the second-generation quinolones – the original FQs – with expanded activity against gram-negative bacteria, atypical pathogen coverage and moderately improved gram-positive activity, but still lacking activity against anaerobes (e.g. norfloxacin, lomefloxacin, CIP and ofloxacin). With the appearance of FQs, this class of antibacterial agents became an important and effective group of drugs with considerable interest in clinical practice. Amongst the second-generation quinolones, CIP became the benchmark against which all the new-generation analogues would be compared with, remaining the most potent quinolone of the class against *P. aeruginosa*. Third-generation agents (e.g. levofloxacin, sparfloxacin, grepafloxacin and gatifloxacin) have improved activity against gram-positive bacteria, in particular pneumococci, and good activity against anaerobes, while retaining the expanded gram-negative and atypical pathogen activity. Finally, the fourth-generation agents (e.g. trovafloxacin, gemifloxacin and MOX) are FQs similar to the previous generation but with superior coverage and increased activity against pneumococci and anaerobes. These compounds of the third- and fourth-generations are known as the respiratory FQs because they are characterized by great activity against pathogens that cause respiratory infections, such as *S. aureus*, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (Andriole, 2005; Bolon, 2009; Emmerson and Jones, 2003; Oliphant and Green, 2002; Sharma et al., 2009; Sousa et al., 2012).

During the development of this quinolone class, substantial progress has been made leading not only to antibacterial agents with a broader spectrum of antibacterial activity but also with improved pharmacokinetic properties, such as high oral bioavailability, enhanced elimination half-lives and good tissue penetration that provides, in some circumstances, higher tissue drug concentrations than those attained in plasma. This evolution is particularly evident in the case of the last two generations of quinolones and has directed the attention of researchers and clinicians to this class as important therapeutic tools in the management of infectious diseases (Andriole, 2005; Oliphant and Green, 2002; Sharma et al., 2009).

I.3.2. STRUCTURE AND PHYSICOCHEMICAL PROPERTIES

Fluoroquinolones are bicyclic heterocyclic aromatic compounds with a ketone group at position 4, a carboxylic group at position 3 and a fluorine atom at position 6. This

basic structure of FQ molecules (pharmacophore) is represented in *Figure I.3.1.*. The 3-carboxyl and 4-ketone groups are essential for the antibacterial activity; they bind to and subsequently inhibit two bacterial enzymes involved in DNA replication, transcription, repair and recombination, resulting in rapid bacterial cell death. Therefore, it is important to keep the molecule integrity at positions 3 and 4 to maintain the antibacterial activity (Andersson and MacGowan, 2003; Appelbaum and Hunter, 2000; Bolon, 2009; Mehlhorn and Brown, 2007; Sharma et al., 2009). Structural modifications at specific sites of FQ nucleus may explain the differences observed in the behavior of quinolones. The most common target sites for structural changes have been the position 1 with a cyclopropyl group (e.g. CIP, MOX and gemifloxacin) and the position 7 with the typical amine substituents, piperazine (e.g. levofloxacin, CIP), pyrrolidine (e.g. gemifloxacin) or azabicyclo (e.g. MOX) groups. Moreover, differentiation between FQ molecules is also reinforced by inclusion of small substituents commonly amine, alkyl, methoxy and halogen groups (Appelbaum and Hunter, 2000; Mehlhorn and Brown, 2007; Peterson, 2001; Sousa et al., 2012).

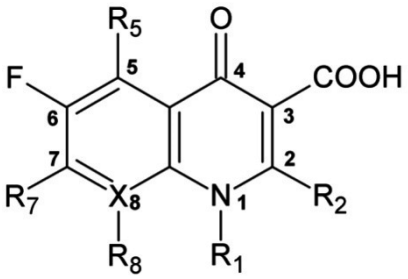

Figure I.3.1. General structure of fluoroquinolones, using the accepted numbering scheme for positions on the molecule. The radicals R1, R2, R5, R7 and R8 indicate possible positions for structural modification; X, usually correspond to a C or N atom (Sousa et al., 2014, 2012).

Substituent groups play an active role in determining the physicochemical properties of FQs. These compounds have a molecular weight usually between 300 and 500 Da. Due to the carboxylic group of the nucleus and the amine substituent at position 7, FQs are amphoteric molecules with dissociation values, pka_1 and pKa_2, in the range 5.5–6.5 and 7.6–9.5, respectively. Consequently, they may exist in solution as four different chemical species, corresponding to positive, zwitterionic, neutral and negative forms; their relative abundance depends on the dissociation constants of the FQ and on the pH of the

solution. These different molecular species have distinct physicochemical properties, for example, with respect to water solubility and lipophilicity. As it is well-known, the ionized forms are usually more water soluble while neutral forms are more lipophilic and have higher membrane permeability. The influence of the pH of the medium on the lipophilicity was also evaluated by measuring the partition coefficient between 1-octanol and water ($P_{octanol/water}$) as a function of pH, the so called distribution coefficients. The data currently available in literature is deviating and contradictory, which makes it a difficult task to compare octanol/water coefficients of FQs obtained from different sources. Despite that, the pH-dependent behavior of FQs is a common and well-established knowledge. The profile of the curve obtained (in a log-scale) has a parabolic shape with a maximum at the isoelectric point, which reflects the fact that the predominant species at isoelectric point are the most lipophilic (zwitterionic and neutral species). The decrease of $P_{octanol/water}$ on either side of the maximum is due to the increased contribution of the charged, less lipophilic, species (Brighty and Gootz, 2000; Ross et al., 1992; Takács-Novák et al., 1992). The structure of FQ molecules influences their intrinsic lipophilicity. For example, the introduction of a second fluorine atom at position 8 or the presence of an oxazine ring attached to the FQ nucleus (e.g. levofloxacin) decrease their lipophilicity, whereas azabicyclo groups at position 7 (e.g. MOX) result in increased lipophilicity (Andersson and MacGowan, 2003; Ross et al., 1992). These physicochemical properties may influence the pharmacokinetics of FQs. Moreover, the development of the quinolone class brought a deeper insight into the structure-activity and structure-adverse effects relationships. For example, modifications at positions 5 and 8 are known to produce adverse effects (cardiotoxicity and phototoxicity) and affect bacterial enzymes affinity (Sousa et al., 2014).

I.3.3. ADVERSE EFFECTS AND CLINICAL USE OF FLUOROQUINOLONES

Decades of FQ development gave rise to several structurally related compounds with some molecular differences in order to enhance their antibacterial activity and pharmacological properties. The increased attractiveness due to these features has been however hampered by concerns of safety and tolerability. Although FQs have proved to be efficient with improved pharmacokinetics and generally safe and well tolerated, they have been associated with some adverse effects following both oral and IV administration.

The most common adverse effects inherent to the quinolone class involve the gastrointestinal tract (e.g. nausea and diarrhea) and the CNS (e.g. headache and dizziness); these adverse effects are usually mild and, hence, do not require discontinuation of therapy. However, uncommon but serious and potentially life-threatening adverse effects have also been reported for some of the third- and fourth-generations agents, which include neurological and psychiatric events, pronounced phototoxicity and skin rash, heart rate-corrected QT interval prolongation, dysglycemia and arthropathy and tendinopathy. Moreover, severe idiosyncratic adverse effects such as hepatotoxicity and hemolytic uremic syndrome have been associated with trovafloxacin (TRO) and temafloxacin, respectively. Therefore, the benefits of third- and fourth-generations FQ therapy must be weighed against the risks of toxicity. Some of the serious adverse effects lead to the halt in the development and to the restriction, suspension or even withdrawal from the market of a number of third- and fourth-generation FQs or some of their pharmaceutical formulations (*Table I.3.2.*) (Andersson and MacGowan, 2003; Bolon, 2009; Oliphant and Green, 2002; Owens and Ambrose, 2005; Paladino, 2001; Stahlmann and Lode, 2013).

Levofloxacin (LEV), MOX and second-generation CIP are examples of relatively safe FQs that are currently available (as oral, IV or eye/ear drops formulations) both in US and Europe and are the most commonly prescribed and widely used in clinical practice. Clinical applications of antibacterial agents of the quinolone class evolved from their unique use in urinary tract infections to a large variety of other infections and, in the current millennium, to the treatment of respiratory tract infections. This progress in the therapeutic role started with the discovery of CIP and since then the value of FQs has extended beyond urinary tract infections into gastrointestinal and gynaecologic infections, sexual transmitted diseases, prostatitis, as well as skin, soft tissue, bone, joint and respiratory infections.

Amongst the latter, the so called respiratory FQs are indicated in the treatment of acute bacterial exacerbations of chronic bronchitis and chronic obstructive pulmonary diseases, community-acquired pneumonia and acute bacterial rhinosinusitis. In the cases where the severity of infection and the presence of underlying risk factors increase the prevalence of gram-negative pathogens, including *P. aeruginosa*, CIP remains the preferred choice. For example, CIP is recommended in hospital-acquired pneumonia and, in Europe, for the treatment of acute exacerbation of CRS caused by gram-negative bacteria. Despite

the broad spectrum of many FQs, no one member of this class will be appropriate for all patients and for all infections; amongst other factors, the choice should be governed by the susceptibility of the pathogen to the antibacterial agent to avoid the development of resistance (Andriole, 2005; Bolon, 2009; Oliphant and Green, 2002; Paladino, 2001; Sharma et al., 2009; Stahlmann and Lode, 2013; Infomed; electronic Medicines Compendium; Drugs@FDA: FDA Approved Drug Products).

Table I.3.2. Some examples of third- and fourth-generation fluoroquinolones withdrawn from the market or whose development was halted (Sousa et al., 2014).

Fluoroquinolone	Serious adverse drug effects	Implications	Available formulations
Third-generation			
Sparfloxacin	QTc interval prolongation Phototoxicity	Withdrawn from the European and US markets	NA
Temafloxacin	Hemolytic uremic syndrome Dysglycaemia Hepatotoxicity	Withdrawn from the European and US market	NA
Gatifloxacin	Dysglycaemia	Discontinued as oral or intravenous formulations from US market Not approved in Europe	Ophthalmic solution
Grepafloxacin	QTc interval prolongation	Withdrawn from the European and US market	NA
Fourth-generation			
Trovafloxacin	Hepatotoxicity	Withdrawn from the US market	NA
Clinafloxacin	Hypoglycemia Phototoxicity	Development halted	NA

QTc, heart rate-corrected QT; NA, Not applied; US, United States.

I.3.4. MECHANISM OF ACTION AND BACTERIAL RESISTANCE

FQs exert their antibacterial action by inhibiting two enzymes involved in the replication and transcription of bacterial DNA and other cellular processes. These enzymes are the DNA topoisomerases – DNA gyrase and topoisomerase IV – which are responsible for maintaining the integrity of the supercoiled DNA double-helix during replication and transcription events. Hence, the interference with their normal action culminates in rapid bacterial death. DNA gyrase is the target of FQ agents in gram-negative bacteria, whereas topoisomerase IV is typically the primary target in gram-positive bacteria. It is generally

accepted that the fourth-generation FQs are likely to have a dual-activity, inhibiting both DNA gyrase and topoisomerase IV, thus limiting the emergence of FQ resistance.

Bacterial resistance to FQs develops through two main mechanisms: chromosomal mutation of genes encoding the target bacterial enzymes and the reduction of FQ concentration in the interior of the bacterial cell by expression of efflux pumps or alterations in porin channels. The development of bacterial resistance depends on the bacterial species, antibacterial potency and antibacterial concentrations. Therefore, bacterial resistance to various FQs emerges at different rates; for example, CIP (with great potency against gram-negative bacteria) demonstrates a higher frequency of resistance in gram-positive pathogens than third- and fourth-generation FQs. With the widespread use of FQs, the risk of bacterial resistance is increasing and constitutes a major concern. To minimize this problem it is important to continuously monitor changes in local resistance patterns and choose the appropriate antibacterial agent to act against the susceptible planktonic bacteria (Blondeau, 2004; Bolon, 2009; Paladino, 2001).

I.3.5. PHARMACOKINETICS AND PHARMACODYNAMICS

Fluoroquinolones are well absorbed following oral administration showing high bioavailability (≥ 70%), with the exception of norfloxacin (NOR). For some of them, systemic drug exposure achieved after oral administration is comparable to that attained by IV route. These antibacterial agents are also quickly absorbed from gastrointestinal tract, with the maximum concentration (C_{max}) in plasma being usually achieved within 1 to 2 h. (Oliphant and Green, 2002; Paladino, 2001; Turnidge, 1999; Zhanel and Noreddin, 2001). FQs vary to some extent with respect to their plasma protein binding ability, ranging from 10 to 70%. This pharmacokinetic parameter may have a significant impact in clinical efficacy, since only the unbound fraction diffuses across capillary membranes, reaching the site of infection where it exerts its antibacterial activity. These antibacterial agents are well distributed throughout the body, although their distribution is not uniform in all tissues and fluids. Remarkable levels of FQs are achieved in renal tissue, prostate gland, lungs and in neutrophils and macrophages, while penetration into cerebrospinal fluid is poor and therefore drug concentrations are not above plasma drug levels. As a class, FQs do not easily cross the blood-brain barrier and infected body sites have increased tissue to plasma

drug concentration ratios. The two newer generations of FQs have generally increased tissue penetration, as reflected by the values of their apparent volume of distribution, with tissue and fluid concentrations usually higher than plasma concentrations. Aminimanizani et al. (2001) and Zhanel and Noreddin (2001) tabulated some data related to the penetration characteristics of several FQs into selected tissues and fluids of the body. According to this published information, FQs of third- and fourth-generations, as well as CIP, show excellent distribution to respiratory tissues and fluids with concentrations exceeding those found in plasma, which accounts for the success of these drugs in the treatment of respiratory infections (Aminimanizani et al., 2001; Oliphant and Green, 2002; Paladino, 2001; Sharma et al., 2009; Sörgel et al., 1989; Zhanel and Noreddin, 2001). Another important advantage of third- and fourth-generation FQs is their long elimination half-lives, allowing once-daily dosing. FQs are eliminated via renal and hepatobiliary routes. The degree to which they are eliminated by hepatic metabolism or by renal excretion differs greatly amongst FQ agents. The majority of FQs are primarily excreted in urine as the parent compound, through glomerular filtration or tubular secretion (e.g. gatifloxacin, LEV and lomefloxacin). In contrast, some agents are eliminated predominantly by metabolism in liver (e.g. MOX and TRO) while others undergo modest metabolism and significant renal elimination (e.g. CIP and NOR). The pharmacokinetic parameters of selected FQs after the conventional routes of administration are shown in *Table I.3.3.*. Based on the fact that FQs are not highly protein-bound and their metabolism by cytochrome P450 (CYP) system is limited to the CYP1A2 isoenzyme, FQ interactions with other drugs are somewhat minimized (Blondeau, 1999; Oliphant and Green, 2002; Sharma et al., 2009; Stein, 1987; Turnidge, 1999; Vance-Bryan et al., 1990; Zhanel and Noreddin, 2001).

Table I.3.3. Main pharmacokinetic parameters of selected fluoroquinolones.

FQ / References	Dose (mg)	F (%)	C_{max} (mg/L)	t_{max} (h)	AUC_{∞} (mg/L.h)	$t_{1/2\ el}$ (h)	Vd (L/kg)	PPB (%)	Metabolites	UE (%)
CIP										
Zhanel et al., 2001	500 PO od	70	2.3	1.2	10.1	3.5	3.5	30	Oxo-CIP, Sulfo-CIP (major),	34
Lettieri et al., 1992	400 IV od	–	4.0	–	11.4	3.53	–	NR	Desethylene-CIP, Formyl-CIP [a]	NR
LOM										
Morrison et al., 1988	400 PO od	NR	3.0	1.3	27.3	7.8	NR	NR	Not described	76.3
Gross et al., 1989	400 PO od	NR	3.8	2.0	34.0	6.4	1.8	NR		54.7
NOR										
Stein, 1987	400 PO od	30-40	1.5	1.5	6.4	3.7	1.7	15	3-oxo-1-piperazinyl-NOR (major)	30
GAT										
Nakashima et al., 1995	400 PO od	NR	3.3	1.97	32.4	8.4	2.2	20	GAT glucuronide	83
Aminimanizani et al., 2001	400 PO od	96	3.8	1.0	33.0	7.8	NR	NR	Open-ring metabolites [b]	72
	400 IV	–	5.5	NR	35.1	7.4	1.5	NR		62
GEM										
Aminimanizani et al., 2001	320 PO od	NR	2.0	0.8	9.3	8.2	NR	NR	*E*-isomer	33
Zhanel et al., 2001	320 PO od	70	1.19	1.2	7.3	8.0	3.5	60	GEM acyl glucuronide *N*-acetyl-GEM [c]	27
LEV										
Aminimanizani et al., 2001	500 IV	–	6.3	NR	55.3	6.6	1.2	NR	Desmethyl-LEV	61
Zhanel et al., 2001	500 PO od	99	5.08	1.7	48.0	6.9	1.1	31	LEV-*N*-oxide [d]	83
MOX										
Aminimanizani et al., 2001	400 IV	–	3.6	NR	34.6	15.4	2.1	NR	*N*-sulphate-MOX	22.1
Zhanel et al.,2001	400 PO od	86	3.34	1.7	33.8	12.1	3.3	48	MOX acyl glucuronide [e]	19

Table I.3.3. *(Continued)* Main pharmacokinetic parameters of selected fluoroquinolones.

FQ / References	Dose (mg)	F (%)	C_{max} (mg/L)	t_{max} (h)	AUC_{∞} (mg/L.h)	$t_{1/2\ el}$ (h)	Vd (L/kg)	PPB (%)	Metabolites	UE (%)
PAZ										
Yamaki et al., 1997	200 PO od	≈ 70	2.98	< 1.0	NR	NR	≈ 1.43	20	PAZ glucuronide	> 80
PAZFLO leaflet 2010	500 IV	–	11.0	0.5	21.7	1.88	NR	NR		90
TRO										
Teng et al., 1997	200 PO od	87.6	2.2	2.3	30.4	11.3	NR	73	TRO glucuronide	NR
Vicent et al., 1997	200 IV (ALA)	–	2.3	1.0	31.2	12.3	1.3	NR	*N*-sulphate-TRO *N*-acetyl-TRO [f]	≈ 10
ULI										
Keam et al., 2004	600 PO od (PRU)	NR	1.6	1.0	7.3	10.7	≈ 17.58	≈ 45	ULI (active metabolite) ULI acyl glucuronide, Ethylenediamino, diol, amino and oxo forms [g]	17-23

[a] Vance-Bryan et al., 1990; [b] Nakashima et al., 1995; [c] Yoo et al. 2004; [d] Martin et al. 1998; [e] Aminimanizani et al. 2001; [f] Vicent et al., 1998; [g] Matera et al., 2006

ALA, Alatrofloxacin; CIP, Ciprofloxacin; GAT, Gatifloxacin; GEM, Gemifloxacin; LEV, Levofloxacin; LOM, Lomefloxacin; MOX, Moxifloxacin; NOR, Norfloxacin; PAZ, Pazufloxacin; PRU, Prulifloxacin; TRO, Trovafloxacin; ULI, Ulifloxacin.

AUC, Area under the concentration-time curve; C_{max}, Maximum plasma concentration; F, Oral bioavailability; FQ, Fluoroquinolone; od, Once daily; IV, Intravenous administration; NR, Not reported; PO, Oral administration; PPB, Plasma protein binding; t_{max}, Time to reach maximum plasma concentration; $t_{1/2\ el}$, Elimination half-life time; UE, Urinary excretion of unchanged drug; Vd, Apparent volume of distribution.

Two important pharmacodynamic properties of FQs are concentration-dependent antibacterial activity and post-antibiotic effect (PAE). MIC is an *in vitro* parameter that measures the potency of antibacterial agents to a specific bacterial species and is the most widely used pharmacodynamic parameter. Increasing the concentration of FQs results in more rapid and more extensive killing of bacteria; therefore it is necessary to take into consideration the pharmacokinetic properties that describe the fluctuation of drug concentration in the body and correlate them with pharmacodynamic parameters.

The parameters used to predict FQ efficacy are the C_{max}/MIC ratio and the 24 h area under drug concentration versus time curve (AUC_{0-24}) to MIC ratio (AUC_{0-24}/MIC). These parameters are also important in preventing the development of bacterial resistance to FQ agents. Several *in vitro*, animal models or human studies have been performed in order to correlate these parameters with bacteriological and clinical outcomes. Clinical data indicate that a C_{max}/MIC ratio ≥ 10 and AUC_{0-24}/MIC ratios in the range of 100-125 were associated with maximum bacterial eradication and clinical cure in critically ill patients with nosocomial lower respiratory tract infection caused by gram-negative bacteria, such as *P. aeruginosa*. Other studies have not validated the same thresholds for C_{max}/MIC and AUC_{0-24}/MIC, suggesting that these values depend on the disease state and on the target pathogen. For example, in outpatients with community-acquired respiratory infections caused by the gram-positive bacteria, *S. pneumoniae*, an AUC_{0-24}/MIC ratio > 25 produced best rates of bacterial eradication *in vitro*, as well as in animal and clinical studies (Bolon, 2009; Gonzalez et al., 2013; Turnidge, 1999; Zhanel and Noreddin, 2001).

FQs exhibit PAE which consists of continued suppression of bacterial growth after antibacterial exposure. This effect has a duration of 1 to 6 h (or longer), depending on the drug and pathogen involved. The probability of resumption of bacterial growth during the periods of plasma or tissue drug concentrations inferior to MIC decreases with increasing magnitude of PAE. The degree of this persistent effect is also dose-dependent and may have an important impact on the design of dosing regimens (Blondeau, 1999; Bolon, 2009; Zhanel and Noreddin, 2001).

Bearing in mind the above considerations it is of the utmost importance to fully understand the pharmacokinetics/pharmacodynamics of FQs to avoid therapeutic failure

and development of antibacterial resistance. In the case of biofilm infections reliable predictions of therapeutic success are still to be established and further data from *in vivo* and clinical trials is therefore required to shed light on this topic.

I.4. AIMS

This thesis addresses a difficult to treat and multifactorial disease of the upper respiratory tract, where the implication of bacterial biofilms in nasal cavity and paranasal sinuses mucosa is increasingly recognized and responsible for its chronic nature. Traditional antibiotherapies have proven to be inefficient to achieve the required but difficult compromise between efficacy and absence of adverse/toxic systemic effects due the high antibacterial resistance of biofilms. Due to the paucity of novel and potent antibacterial agents, one of the possible strategies is to use the existing ones and investigate their administration for an additional indication through alternative routes. This is particularly important in the present situation where the overuse of antibiotics has led to the worrying emergence of antibacterial resistance.

The aim of the present thesis is to evaluate the potential of IN administration of topical-acting FQs – a class of antibiotics with a wide range of clinical applications and a broad spectrum of antibacterial activity – in the treatment of CRS by assessing the advantages and limitations of this delivery strategy with respect to efficacy and safety. The progression of the experimental work led to the study of CIP and LEV, which are two of the most commonly used and well-known FQs of two different generations. This work exploits the pharmacokinetics of both drugs after IN delivery, to gain information about their drug exposure at the biophase and their value for systemic and brain drug exposure, in comparison with a conventional route of administration.

The following specific aims were outlined for the implementation of this work:

- Development and validation of a liquid chromatography (LC) method for the simultaneous determination of NOR, CIP and lomefloxacin (LOM) and another LC method for the simultaneous determination of LEV, pazufloxacin (PAZ), gatifloxacin (GAT), MOX and TRO, both in human plasma.
- Development and implementation of a bioanalytical method for the determination of LEV, NOR, CIP and LOM in rat matrices (plasma, nasal mucosa and olfactory bulb) by LC.

- *In vivo* comparison of the pharmacokinetic behavior of CIP in nasal mucosa, plasma and olfactory bulb, following IN and IV administration to rats.
- *In vivo* comparison of the pharmacokinetic behavior of LEV in nasal mucosa, plasma and olfactory bulb, following IN and IV administration to rats.

CHAPTER II

BIOANALYSIS OF FLUOROQUINOLONES

II.1. GENERAL CONSIDERATIONS

Bioanalysis is defined as the quantitative measurement of biomarkers, drugs and their metabolites in biological fluids (e.g. blood, plasma, urine, saliva, tears) or tissues (e.g. kidney, liver, lung, brain). Method validation is a crucial step in bioanalysis as it demonstrates the ability of developed methods in providing reliable and reproducible data and thus their suitability for the intended use (González et al., 2014; Li and Bartlett, 2014; Pandey et al., 2010). In fact, concentration of target analytes in biological samples gives valuable information that is used to understand, evaluate and interpret pharmacokinetic, pharmacodynamic, toxicology, bioavailability and bioequivalence findings as well as to perform routine therapeutic drug monitoring (TDM) in order to establish appropriate dosage schemes in clinical practice (Kollipara et al., 2011; Li and Bartlett, 2014; Nováková and Vlčková, 2009; Shah et al., 2000). The validation of any bioanalytical method plays an essential role in ensuring the high quality of data obtained in research, drug discovery and development and in drug monitoring fields. Therefore, guiding principles for the validation of these bioanalytical methods must be established and followed by the pharmaceutical community. Several workshops and conferences have been organized since 1990 aiming to harmonize the method validation requirements and served as the basis for the elaboration of the US FDA guidelines on bioanalytical method validation (US Food and Drug Administration, 2001). To account for the advances in science and technology these guidelines were revised and reissued as a draft in 2013 (US Food and Drug Administration, 2013). Apart from this FDA guidance, other guidelines have come into force from various regulatory agencies to facilitate regulatory submissions in their countries. One of these is the European Medicines Agency (EMA) guidelines agreed in 2011 (European Medicines Agency, 2011), which also provide recommendations for the validation of bioanalytical methods applied to quantitative analysis of biological matrices obtained in animal and human studies (Kollipara et al., 2011; Shah et al., 2000; Viswanathan et al., 2007). Validation establishes whether the performance characteristics of a particular method are appropriate and reliable for a specified analytical application. Three different types/levels

of validation can be used. Full validation of bioanalytical methods is mandatory in the course of development and implementation of a novel bioanalytical method (e.g. for the analysis of a new drug entity), where fundamental parameters like selectivity, sensitivity, linearity, accuracy, precision and stability are evaluated. In cases where minor modifications of an already fully validated bioanalytical method, partial validation can be performed ranging from one intra-assay accuracy and precision determination to a nearly full validation. Typical alterations allowing a partial validation include, but are not limited to: change in equipment, in species within matrix (e.g. human plasma to rat plasma), in matrix within species (e.g. rat plasma to rat brain), modifications in sample processing or in storage conditions and in case of rare matrices. Cross-validation is another type of validation consisting of a comparison of validation parameters when two or more analytical techniques or laboratories are involved (European Medicines Agency, 2011; Shah et al., 2000; US Food and Drug Administration, 2001).

Liquid chromatography is a powerful analytical technique able to separate, identify and quantify a compound in complex mixtures such as biological samples. High performance liquid chromatography (HPLC) remains the analytical method of choice in bioanalysis. In particular, the reversed-phase high performance liquid chromatography (RP-HPLC) is the most frequent approach found in literature for the determination of FQs (Carlucci, 1998; Nováková and Vlčková, 2009; Sousa et al., 2012). Taking advantage of their fluorescence properties, this chapter presents three RP-HPLC methods coupled with fluorescence detection (FD) that were developed, validated and implemented in our laboratory: two fully validated RP-HPLC-FD methods in human plasma (Sousa et al., 2013, 2011) and one partially validated RP-HPLC-FD method in rat biological matrices. The former two can be clinically applied for routine use in therapeutic monitoring of FQs, but also support the subsequent implementation and validation of the last presented method in rat biological matrices which will be used for the bioanalysis of samples obtained from the animal studies contemplated in this thesis.

II.2. DEVELOPMENT AND VALIDATION OF A FAST ISOCRATIC LIQUID CHROMATOGRAPHY METHOD FOR THE SIMULTANEOUS DETERMINATION OF NORFLOXACIN, CIPROFLOXACIN AND LOMEFLOXACIN IN HUMAN PLASMA

II.2.1. INTRODUCTION

NOR, CIP, and LOM (*Figure II.2.1.*) are synthetic antibiotics developed from fluorination of the nalidixic acid, the first quinolone introduced for clinical use (Oliphant and Green, 2002; Wise, 2000). Since the discovery of nalidixic acid in the early 1960s, numerous quinolone derivatives have emerged, which are currently classified into four generations based on their spectrum of activity and pharmacokinetic properties. From a pharmacological point of view, the latter generations of quinolones have several advantages over the earlier ones, mainly a broader spectrum of activity and improved pharmacokinetics with an appropriate tissue penetration that allows tissue drug concentrations equal to or greater than those attained in plasma (Ball, 2000; Emmerson and Jones, 2003; Zhanel and Noreddin, 2001). However, despite the better pharmacokinetic and pharmacodynamic profiles of the newer FQs, it should be noted that those of the second-generation, such as NOR, CIP, and LOM remain in clinical use as valuable antimicrobial agents (Adriaenssens et al., 2011; Ferech et al., 2006; Oliphant and Green, 2002; Rafalsky et al., 2006). In particular, CIP is extensively used in outpatients and in hospitalized patients with severe infections. In addition, CIP is commonly used as part of antibiotherapy regimens prescribed to critically ill patients in intensive care units (Bellmann *et al.*, 2002; Spooner *et al.*, 2011; van Zanten *et al.*, 2008; Wallis *et al.*, 2001). Taking into account the concentration-dependent antibacterial activity characteristic of the FQ antibiotics, it is evident that intra- and inter-individual pharmacokinetic variability may result in inadequate antibiotic concentrations and therapeutic inefficacy. Nowadays, two relevant parameters are considered as good predictors of treatment success, namely $AUC_{0\text{-}24}$/MIC ratio and C_{max}/MIC ratio. Therefore, there is a growing body of evidence suggesting the usefulness of TDM of FQs in critically ill hospitalized patients. Target values

for AUC_{0-24}/MIC and C_{max}/MIC ratios have been established for CIP (Conil *et al.*, 2008; Pea *et al.*, 2006; van Zanten *et al.*, 2008). Thus, the availability of a simple, fast and selective bioanalytical method is required to enable routine TDM and dosage individualization of FQs.

Until now, a lot of analytical methods have been reported in the literature for the simultaneous determination of two or more FQs in a wide variety of samples. Amongst these quantification methods, LC is one of the most used, although capillary electrophoresis, spectrophotometry and microbiological assay are also employed (Chierentin and Salgado, 2016; El-Kommos *et al.*, 2003; Ferdig *et al.*, 2004). Due to widespread use and/or misuse of FQs not only in human but also in veterinary medicine, several chromatographic methods have been developed to detect and monitor the levels of these drug residues in foodstuffs of animal origin and environmental matrices (such as natural and wastewaters) which constitute a potential health risk of developing bacterial resistance. The majority of these methods are validated for a large number (up to 10-20) of (fluoro)quinolones, including the second-generation quinolones of human use, NOR, CIP and LOM, as the most commonly found (Kumar *et al.*, 2008b; Li *et al.*, 2009; Pearce *et al.*, 2009; Pena *et al.*, 2010; Schneider and Donoghue, 2002; Seifrtová *et al.*, 2010; Tamtam *et al.*, 2009; Tang *et al.*, 2009; Wan *et al.*, 2006; Yu *et al.*, 2012; Zhang *et al.*, 2010; Zhao *et al.*, 2007). On the other hand, few LC methods have been developed for the concomitant analysis of two or more FQs of the second-generation in human plasma/serum or urine matrices (De Smet et al., 2009; Kumar et al., 2008a; Nemutlu et al., 2007; Rambla-Alegre et al., 2009; Samanidou et al., 2003). Only one LC method was reported by Cañada-Cañada *et al.* (2007) enabling simultaneous determination of NOR, CIP and LOM in veterinary and human pharmaceutical formulations. Up to date and to the best of our knowledge no method has been reported for the determination of NOR, CIP and LOM in human plasma/serum (Cañada-Cañada et al., 2007).

The proposed research work describes the first LC developed and validated to quantify NOR, CIP and LOM in human plasma. The method was validated in a wide concentration range for each compound (0.02–5.0 μg/mL) and therefore it can be applied to routine TDM and also to other pharmacokinetic-based studies intended to investigate, for instance, the bioavailability/bioequivalence of drug formulations of NOR, CIP and LOM.

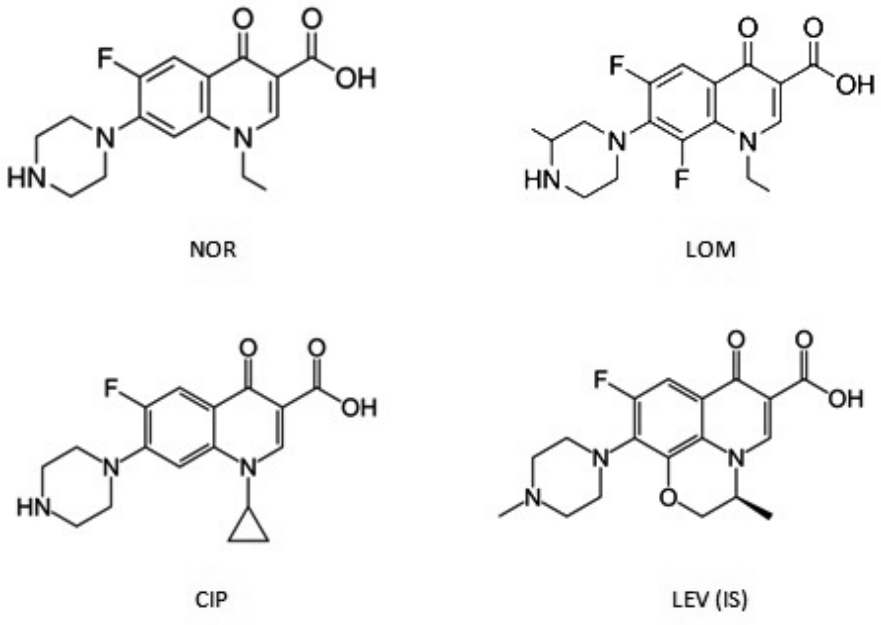

Figure II.2.1. Chemical structures of norfloxacin (NOR), lomefloxacin (LOM), ciprofloxacin (CIP) and levofloxacin (LEV) used as internal standard (IS).

II.2.2. MATERIALS AND METHODS

II.2.2.1. Chemicals, Materials and Reagents

NOR (lot number 028K1480), CIP (lot number 1396107) and LEV (lot number 1395156) used as internal standard (IS) were purchased from Sigma-Aldrich (St Louis, MO, USA). LOM hydrochloride (lot number 62277) was obtained from Molekula (Shaftesbury, Dorset, UK). Methanol (HPLC gradient grade) was purchased from Fisher Scientific (Leicestershire, UK) and ultrapure water (HPLC grade, > 15 MΩ) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Reagents like hydrochloric acid fuming 37%, formic acid (98–100%) and triethylamine (TEA) were acquired from Merck KGaA (Darmstadt, Germany), and trichloroacetic acid was obtained in solid state from Sigma-Aldrich (Steinheim, Germany).

Blank human plasma samples from healthy donors were kindly provided by the Portuguese Blood Institute after written consent of each subject.

II.2.2.2. Apparatus and Chromatographic Conditions

The LC system used for analysis consisted of a pump Model 305 (Gilson Medical Electronics S.A., Villiers-le-Bel, France), a manometric module Model 805 (Gilson Medical Electronics S.A., Villiers-le-Bel, France) and a manual injector Model 7125 (Rheodyne, Cotati, CA, USA) with a 20 µL loop. Detection was performed with an LC 305 Model fluorescence detector (LabAlliance, State College, PA, USA) and data acquisition was controlled by Data Apex Clarity software version nº 2.6.06.574 (Data Apex Ltd., Prague, Czech Republic).

The chromatographic separation of the three analytes (NOR, CIP and LOM) and IS was achieved in 7 min and it was carried out at room temperature, by isocratic elution with 0.1% aqueous formic acid (pH 3.0, TEA)–methanol (82:18, v/v) at a flow rate of 1.2 mL/min, on a reversed-phase LiChroCART® Purospher Star C_{18} column (55 mm x 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). The mobile phase was filtered through a 0.45 µm filter and degassed ultrasonically for 15 min before use. A sample volume of 20 µL was injected and the excitation and emission wavelengths selected for the detection of all compounds were 278 and 450 nm, respectively.

II.2.2.3. Stock Solutions, Calibration Standards and Quality Control Samples

Stock solutions of 1 mg/mL were individually prepared for NOR, CIP, LOM, and IS. The stock solutions of NOR and CIP were prepared by dissolving appropriate amounts of each compound in a mixture of methanol/hydrochloric acid 37% (99.5:0.5, v/v), while stock solutions of LOM and IS were prepared in methanol. Appropriate volumes of each of the stock solutions of NOR, CIP, and LOM were combined and diluted in methanol to obtain two intermediate solutions each one containing the three FQs at the concentrations of 200 µg/mL and 20 µg/mL. Then, the combined intermediate solutions were adequately diluted in methanol to obtain six spiking solutions at 0.5, 1, 4, 15, 50 and 125 µg/mL, which were used to spike aliquots of blank human plasma in order to prepare six plasma calibration standards at 0.02, 0.04, 0.16, 0.6, 2.0 and 5.0 µg/mL. An aqueous IS working solution at 10 µg/mL was also prepared daily by appropriate dilution of the corresponding stock solution. All spiking solutions were stored and protected from light at 4 °C until use, except the IS

working solution which was prepared daily. Quality control (QC) samples were prepared independently in the same matrix (blank human plasma).

II.2.2.4. Sample Preparation

Each aliquot (500 µL) of human plasma samples was mixed with 100 µL of the IS working solution (10 µg/mL) and 100 µL of 20% (w/v) trichloroacetic acid. The final sample was vortex-mixed for 30 s and centrifuged at 5800 rpm for 5 min to precipitate plasma proteins. An aliquot of the clear supernatant (20 µL) was directly injected into the LC system for analysis.

II.2.2.5. Method Validation

The method validation was performed according to the international guidelines for bioanalytical method validation (European Medicines Agency, 2011; US Food and Drug Administration, 2001) as well as other international recommendations (Shah et al., 2000). The method was validated by determination of selectivity, linearity, limits of quantification and detection, precision and accuracy, sample dilution, recovery and stability.

Selectivity

In order to assess method selectivity, the potential chromatographic interference from endogenous compounds at the retention times of NOR, CIP, LOM and IS was investigated by analysing blank human plasma samples obtained from six different subjects. Interference from other commonly prescribed drugs such as paracetamol, salicylic acid, ibuprofen, theophylline, ranitidine, hydrochlorothiazide, furosemide, carbamazepine, amitriptyline, cefoxitin, dexamethasone, enoxaparin, propofol, ketamine, phenytoin, lamotrigine, erythromycin, penicillin-G, trimethoprim, neomycin, azithromycin, streptomycin, bromazepam and omeprazole at a concentration of 10 µg/mL was also tested.

Linearity of calibration curves

The linearity of the analytical method for NOR, CIP, and LOM was assessed in the range of 0.02–5.0 µg/mL by using calibration curves prepared on five separate days (*n* = 5) with spiked calibration standards at six different concentration levels. Calibration curves were prepared by plotting the peak-area ratios (peak area analyte/peak area IS) *versus* concentration and fitted to y = mx + c using $1/x^2$ as weighting factor for all analytes. This weighting factor was selected because it yielded the best fit of peak area ratios *versus* concentration for all compounds (Almeida et al., 2002).

Limits of quantification and detection

The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve which can be measured with precision (expressed as percentage of coefficient of variation, % CV) not exceeding 20% and accuracy (expressed as percentage of deviation from nominal concentration, % bias) within ±20%. The LLOQ was evaluated by analyzing plasma samples which were prepared in five replicates (*n* = 5). The limit of detection (LOD), defined as the lowest concentration that can be distinguished from the noise level, was determined by analysing plasma samples with known concentrations of NOR, CIP, and LOM after successive dilutions, and it was established by visual evaluation of the minimum level at which the analytes can be reliably detected.

Precision and accuracy

Intra and interday precision and accuracy were assessed by using QC samples analysed in replicate (*n* = 5) at three different concentration levels **[**low (QC_1), medium (QC_2) and high (QC_3)**]** representative of the entire range of the calibration curves. The concentrations tested were 0.05, 2.5 and 4.5 µg/mL for all analytes (NOR, CIP and LOM). The acceptance criterion for precision was a CV less or equal to 15% (or 20% in the LLOQ), and for accuracy a bias value within ±15% (or ±20% in the LLOQ).

Sample dilution

The dilution effect (1:5) was also investigated with appropriate plasma QC samples at 10 µg/mL (QC_{Dil}) for NOR, CIP, and LOM to ensure that plasma samples exceeding the highest concentration of the calibration range (0.02–5.0 µg/mL) could be diluted with blank human plasma and still be accurately quantified. The precision and accuracy of diluted plasma QC samples were determined intra and interday by replicated analysis (*n* = 5).

Recovery

The recovery of NOR, CIP and LOM from human plasma samples submitted to protein precipitation was determined at three concentration levels **[**low (QC_1), medium (QC_2) and high (QC_3)**]** representative of the calibration range, namely 0.05, 2.5 and 4.5 µg/mL, by repeated analysis (*n* = 5). The relative recovery of the analytes was calculated by comparing the analyte/IS peak area ratio of processed plasma samples with the corresponding ratio obtained from the processed aqueous solutions at the same concentrations. The absolute recovery of analytes was also determined by direct comparison of analyte peak areas of processed plasma samples with the corresponding areas for processed aqueous solutions at the same concentrations. The absolute recovery of the IS was also similarly evaluated at the concentration used in sample analysis as the peak area ratio of the IS from the spiked processed plasma samples and aqueous solutions at equivalent concentrations.

Stability

Human plasma stability of NOR, CIP and LOM was assessed at low (QC_1 = 0.05 µg/mL) and high (QC_3 = 4.5 µg/mL) concentration levels, at room temperature for 3 h, at 4 °C for 24 h and at -30 °C for 30 days, in order to simulate sample handling and storage time in the freezer before analysis. The effect of three freeze-thaw cycles on the stability of the analytes was also investigated in plasma. Aliquots of spiked plasma samples were stored at -30 °C for 24 h, thawed unassisted at room temperature and refrozen for 24 h under the same conditions until completing the three cycles. The stability of NOR, CIP and LOM was

also studied at 4 °C during 24 h in the supernatant of processed spiked plasma samples, simulating the time that sample may be in the auto-sampler before analysis. The stability was assessed comparing the data of samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). A stability/reference samples ratio of 85–115% was accepted as stability criterion (n = 5).

II.2.3. RESULTS AND DISCUSSION

In this work, a mobile phase composed of an acidic aqueous polar phase of 0.1% formic acid solution adjusted to pH 3.0 with TEA and methanol as the organic modifier was used. The amphoteric character of FQs is responsible for distinct rates of migration of the cationic, neutral, zwitterionic and anionic forms through reverse-phase columns, resulting in different retention times and thus enhancing peak bandwidth. As in most mobile phases of this type of LC methods described in literature, an acidic aqueous polar phase was chosen in order to reverse the ionization of the FQ carboxylic function group, thus reducing peak bandwidth. Another issue associated with FQs analysis is the peak tailing effect caused by interactions between free silanol groups of silica-based columns and amine basic groups of FQs. This problem may be overcome by using amine additives such as TEA that compete with FQs, or by using an acidic mobile phase, reducing the peak tailing effect. A mobile phase with an aqueous solution with the composition described above was found to yield symmetric and sharp peaks and was selected for all the subsequent LC methods developed and presented in this thesis.

Chromatographic detection was performed by selecting excitation and emission wavelengths of 278 and 450 nm, respectively, which are the most commonly used in the quantification of second-generation quinolones and in the case of NOR and CIP are close to their maximum excitation and emission wavelengths (Cañada-Cañada et al., 2007; Herrera-Herrera et al., 2009; Liang et al., 2002; Pena et al., 2010; Seifrtová et al., 2008).

The IS usually used in the determination of FQs by LC methods coupled with FD is a member of the quinolone class, taking advantage of the similarity of physicochemical properties (Galaon et al., 2007; Liang et al., 2002; Sousa et al., 2012). In this case a third-generation quinolone, namely LEV, was used. The specificity of fluorescence results in

sufficient clean chromatograms and therefore allows simple and fast sample preparation involving only plasma protein precipitation.

II.2.3.1. Chromatographic Separation and Selectivity

The chromatographic separation of NOR, CIP, LOM, and LEV (IS) in spiked human plasma samples was successfully achieved using the chromatographic conditions previously described (*Figure II.2.2.*).

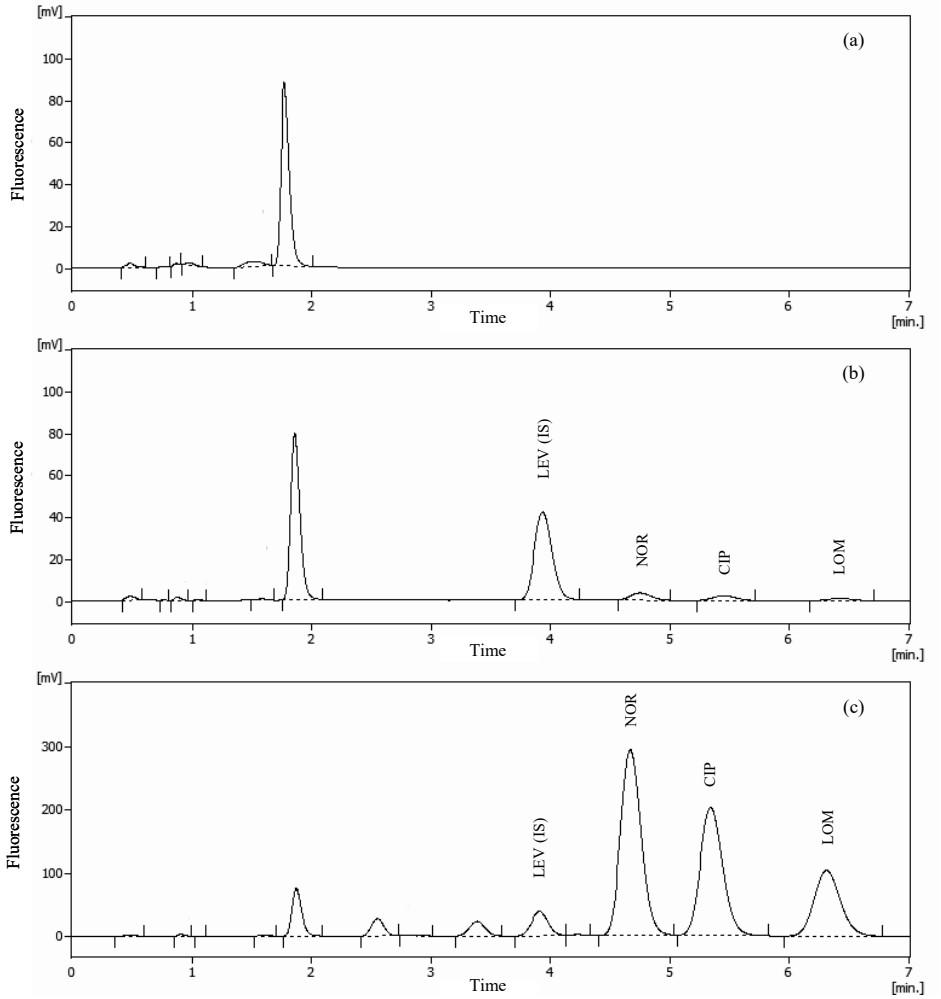

Figure II.2.2. Typical chromatograms of extracted human plasma samples obtained by fluorimetric detection at $\lambda_{ex}/\lambda_{em}$ of 278/450 nm: (a) blank plasma; (b) spiked plasma with levofloxacin (LEV) used as internal standard (IS) and the analytes norfloxacin (NOR), ciprofloxacin (CIP), and lomefloxacin (LOM) at concentrations of the lower limit of quantification (0.02 µg/mL); (c) spiked plasma with LEV (IS) and the analytes NOR, CIP and LOM at concentrations of the upper limit of calibration range (5.0 µg/mL).

Under these analytical conditions the last-eluting analyte was LOM, with a retention time of approximately 6.30 min and the order of elution of the compounds was the following: LEV (IS), NOR, CIP, and LOM. Representative chromatograms of blank and spiked human plasma samples are shown in *Figure II.2.2.*. The analysis of blank human plasma samples showed no interfering peaks in the retention times of NOR, CIP LOM and IS. Similarly, none of the tested drugs commonly prescribed with NOR, CIP and LOM were found to interfere with the peaks of the analytes or of the IS.

II.2.3.2. Linearity of Calibration Curves, LLOQ and LOD

The calibration curves prepared in human plasma for NOR, CIP and LOM were linear ($r^2 \geq 0.994$) over the concentration range 0.02–5.0 μg/mL. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the best-fit weighting factor for all compounds. Typical weighted regression equations (n = 5) of the calibration curves were y = 3.945x + 0.018 (r^2 = 0.994) for NOR, y = 1.739x + 0.007 (r^2 = 0.996) for LOM and y = 2.945x + 0.016 (r^2 = 0.996) for CIP, where y represents the ratios of analyte/IS peak area and x represents the plasma concentration. These results demonstrated a good linearity between peak area ratios and concentrations. The LLOQ of the assay was set at 0.02 μg/mL for all analytes (NOR, CIP and LOM) with good precision (CV ≤ 8.19%) and accuracy (bias ±7.15%) (*Table II.2.1.*). The LOD was established at 0.001 μg/mL for NOR, CIP and LOM.

Table II.2.1. Precision (% CV) and accuracy (% bias) for the determination of norfloxacin (NOR), ciprofloxacin (CIP), and lomefloxacin (LOM) in human plasma samples at the concentration of the lower limit of quantification (LLOQ) (n = 5).

Analyte	Nominal Concentration (μg/mL)	Measured Concentration (mean ± SD, μg/mL)	Precision (% CV)	Accuracy (% bias)
Intraday				
NOR	0.02	0.020 ± 0.002	6.39	3.70
CIP	0.02	0.021 ± 0.002	8.19	2.78
LOM	0.02	0.019 ± 0.002	7.94	-7.15
Interday				
NOR	0.02	0.020 ± 0.001	3.51	0.05
CIP	0.02	0.020 ± 0.001	5.39	0.81
LOM	0.02	0.020 ± 0.002	7.21	1.12

CV, coefficient of variation; SD, standard deviation

II.2.3.3. Precision and Accuracy

The results for intra and interday precision and accuracy obtained from plasma QC samples (QC_1, QC_2 and QC_3) at three different concentrations (0.05, 2.5 and 4.5 µg/mL) representative of the entire calibration range (0.02–5.0 µg/mL) are shown in *Table II.2.2.*.

Table II.2.2. Precision (% CV) and accuracy (% bias) for the determination of norfloxacin (NOR), ciprofloxacin (CIP), and lomefloxacin (LOM) in human plasma samples over the calibration range 0.02–5.0 µg/mL, and following a sample dilution (*) by a 5-fold factor (*n* = 5).

Analyte	Nominal Concentration (µg/mL)	Measured Concentration (mean ± SD, µg/mL)	Precision (% CV)	Accuracy (% bias)
Intraday				
NOR	0.05	0.053 ± 0.004	6.32	5.35
	2.5	2.665 ± 0.083	3.11	6.58
	4.5	4.459 ± 0.292	6.54	-0.91
	*10	10.744 ± 0.692	6.43	7.44
CIP	0.05	0.052 ± 0.004	6.10	4.71
	2.5	2.729 ± 0.074	2.69	9.16
	4.5	4.589 ± 0.321	6.98	1.98
	*10	10.024 ± 0.676	6.74	0.24
LOM	0.05	0.051 ± 0.003	5.67	1.34
	2.5	2.773 ± 0.076	2.74	10.91
	4.5	4.767 ± 0.359	7.53	5.94
	*10	9.317 ± 0.635	6.81	-6,83
Interday				
NOR	0.05	0.051 ± 0.004	6.43	2.71
	2.5	2.573 ± 0.165	6.42	2.93
	4.5	4.383 ± 0.160	3.65	-2.61
	*10	10.459 ± 0.629	6.02	4.59
CIP	0.05	0.051 ± 0.003	5.73	1.71
	2.5	2.646 ± 0.180	6.80	5.83
	4.5	4.549 ± 0.239	5.26	1.10
	*10	10.056 ± 0.610	6.06	0.56
LOM	0.05	0.049 ± 0.003	6.38	-2.23
	2.5	2.640 ± 0.179	6.79	5.58
	4.5	4.701 ± 0.284	6.04	4.46
	*10	9.485 ± 0.652	6.87	-5.15

CV, coefficient of variation; SD, standard deviation

All the data fulfill the acceptance criteria. The intra and interday CV values did not exceed 7.53%. The intra and interday bias values varied between -2.61 and 10.91%. These data indicate that the developed LC method coupled with FD for the quantification of NOR,

CIP and LOM in human plasma is accurate, reliable and reproducible, since neither CV nor bias exceeded 15%, in agreement with literature recommendations. For the dilution of plasma samples (QC_{Dil} = 10 µg/mL) the precision and accuracy values varied from 6.02 to 6.87% and from -6.83 to 7.44%, respectively. These results show that a 5-fold dilution with blank human plasma can be applied, if the concentration of a trial sample exceeds the highest concentration of the calibration curve.

II.2.3.4. Recovery

The recovery of NOR, CIP and LOM from human plasma at three different concentrations (0.05, 2.5 and 4.5 µg/mL) was assessed and the results (for the corresponding QC_1, QC_2 and QC_3 samples) are listed in *Table II.2.3.*. The mean relative recoveries of NOR, CIP and LOM ranged from 90.12 to 111.48% and showed low CV values. Although their absolute recoveries ranged from 58.27 to 76.89% with CV ≤ 8.91%, they do not deviate from the only requirement of international guidelines of a precise and reproducible extent of recovery. In fact, the absolute recovery values obtained at the three assessed concentration levels (*n* = 15) for each analyte are associated with low CV values, namely 6.15%, 9.27% and 9.34% for NOR, CIP and LOM, respectively. The absolute recovery of the IS was also evaluated and a mean value of 68.88% with CV = 11.84% was obtained. These absolute recoveries still give sufficiently low limits of quantification for the intended purpose of bioanalytical analysis of these FQs.

Table II.2.3. Relative and absolute recovery (%) of norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) from human plasma samples (*n* = 5).

Analyte	Nominal Concentration (µg/mL)	*n*	Relative Recovery (%)		Absolute Recovery (%)	
			Mean±SD	CV (%)	Mean±SD	CV (%)
NOR	0.05	5	101.21 ± 8.43	8.33	76.89 ± 4.73	6.15
	2.5	5	101.41 ± 8.68	8.56	71.13 ± 1.66	2.34
	4.5	5	111.48 ± 6.46	5.80	66.81 ± 4.28	6.41
CIP	0.05	5	92.99 ± 8.16	8.78	70.63 ± 4.60	6.51
	2.5	5	91.17 ± 7.71	8.45	63.95 ± 1.63	2.55
	4.5	5	98.49 ± 6.21	6.31	59.03 ± 4.07	6.90
LOM	0.05	5	90.12 ± 10.09	11.20	68.42 ± 6.10	8.91
	2.5	5	90.85 ± 7.66	8.43	63.72 ± 1.69	2.67
	4.5	5	97.23 ± 6.60	6.78	58.27 ± 4.30	7.39

CV, coefficient of variation; SD, standard deviation

II.2.3.5. Stability

The stability of NOR, CIP and LOM was evaluated under different conditions likely to be encountered during the analytical process and sample storage, by analyzing five replicates of low (QC_1 = 0.05 µg/mL) and high (QC_3 = 4.5 µg/mL) QC samples. The results of stability assays showed that no significant degradation occurred for NOR at the studied conditions both in unprocessed and processed plasma samples. For LOM and CIP the stability criteria previously established were not fulfilled in plasma when samples were stored at -30 °C for more than 7 days (in the case of LOM) and for more than 21 days (in the case of CIP). Stability data are shown in *Table II.2.4.*.

Table II.2.4. Stability (%) of norfloxacin (NOR), ciprofloxacin (CIP), and lomefloxacin (LOM) in unprocessed plasma samples left at room temperature for 3 h, at 4 °C for 24 h, after three cycles freeze (-30 °C)/thaw, at -30 °C until 30 days, and in processed plasma samples left at 4 °C for 24 h (*n* = 5).

Analyte	NOR		CIP		LOM	
Nominal Concentration (µg/mL)	0.05	4.5	0.05	4.5	0.05	4.5
Plasma						
Room temperature (3 h)	95.1	98.5	96.0	99.4	85.6	95.2
4 °C (24 h)	99.1	100.1	98.9	100.1	99.1	100.1
Freeze (-30 ºC)/thaw (3 cycles)	107.1	101.4	96.1	102.4	95.7	103.0
-30 °C (7 days)	107.5	106.3	108.8	109.4	107.1	110.9
-30 °C (14 days)	90.4	109.1	92.5	114.4	90.2	117.2
-30 °C (21 days)	92.5	106.6	92.7	113.8	93.8	123.6
-30 °C (30 days)	101.5	107.3	102.3	118.6	92.2	120.3
Processed plasma sample						
4 °C (24 h)	97.5	97.8	97.3	98.2	97.1	98.3

CV, coefficient of variation

II.2.3.6. Application of the Method to Real Plasma Samples

The fully validated method was applied to the quantification of CIP in human plasma of hospitalized patients treated with this drug. These real plasma samples were kindly supplied obtained in accordance with the ethical principles of the Declaration of Helsinki. A representative chromatogram is shown in *Figure II.2.3.*. We can observe that

the peak shape and chromatographic resolution are similar to those obtained from spiked plasma samples.

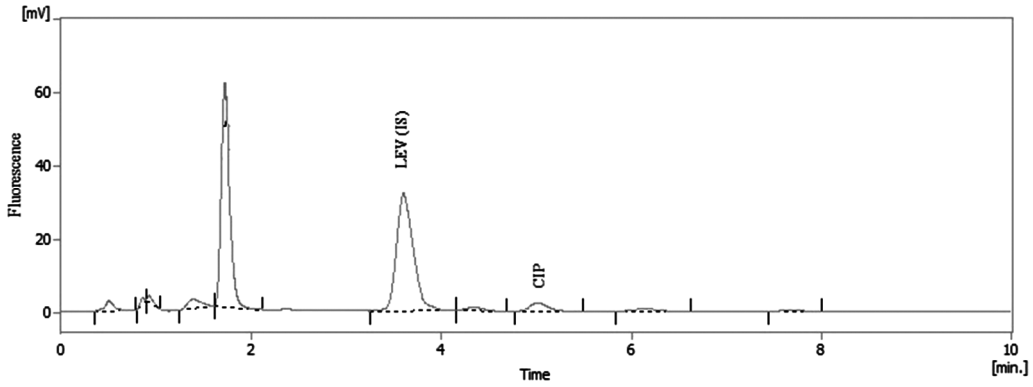

Figure II.2.3. Representative chromatogram of real plasma samples obtained from patients treated with ciprofloxacin (CIP). The measured plasma concentration of CIP was 0.02 µg/mL.

II.2.4. CONCLUSION

To our knowledge there is no reported LC method in the literature that determines simultaneously NOR, CIP, and LOM in human plasma. Therefore, the present work describes the first LC method coupled with FD developed and fully validated for the simultaneous quantification of NOR, CIP, and LOM in the biological matrix of human plasma.

The method we have developed presents several important bioanalytical advantages. First of all, it was shown to be an accurate, precise, highly selective and sensitive LC method for the determination of NOR, CIP, and LOM in human plasma, using very simple and economical chromatographic conditions. Particularly, a good peak resolution of the different FQs was achieved on a reversed-phase column using an isocratically pumped mobile phase essentially composed of water (82%), requiring only a small percentage of methanol as organic modifier (18%). In addition, this method permitted a rapid analysis of samples since the elution of all analytes is achieved within 7 min; typical chromatographic run times for the determination of more than one FQ are usually of 10 min or even longer (De Smet et al., 2009; Kumar et al., 2008a; Samanidou et al., 2003). The coupling of FD to the LC procedure that has been used enhances the sensitivity of the method. Indeed, the value of LLOQ obtained herein (0.02 µg/mL) is lower than those achieved by methods using ultraviolet (UV) detection: 0.05 µg/mL (Helmy,

2013; Maya et al., 2001), 0.1 µg/mL (Sowinski and Kays, 2004) or even 0.2 µg/mL (Davis et al., 1993; Liang et al., 2002). Moreover, the sample preparation consists of a simple one-step deproteinization with trichloroacetic acid. A more complex manipulation involving either liquid-liquid extraction, solid-phase extraction or protein precipitation followed by evaporation and reconstitution of the residue is often referred in literature (De Smet et al., 2009; Kamberi et al., 1999; Nemutlu et al., 2007; Samanidou et al., 2003; Srinivas et al., 2008). With our method minimal sample handling is required reducing time and error sources. In addition, no organic solvents are needed, making the procedure of sample preparation safer and less pollutant.

The validation of this method has demonstrated that it fulfills the international requirements and the linearity was demonstrated in a wide range of concentrations for all analytes (0.02–5.0 µg/mL). The feasibility of sample dilution for human plasma concentrations above the calibration range was also successfully assessed. No interferences were found between NOR, CIP, or LOM, and human plasma endogenous compounds or commonly prescribed drugs.

In conclusion, a selective, accurate, reliable and reproducible new method for the simultaneous quantification of NOR, CIP, and LOM has been developed and fully validated. Hence, it will be suitable to support the routine TDM of NOR, CIP, or LOM, and it can be applied to other pharmacokinetic-based studies involving these FQs of the second-generation.

II.3. DEVELOPMENT AND VALIDATION OF A GRADIENT LIQUID CHROMATOGRAPHY METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEVOFLOXACIN, PAZUFLOXACIN, GATIFLOXACIN, MOXIFLOXACIN AND TROVAFLOXACIN IN HUMAN PLASMA

II.3.1. INTRODUCTION

Fluoroquinolones are an important class of synthetic antibiotics widely used in anti-infective chemotherapy due to their remarkably broad spectrum of activity (Appelbaum and Hunter, 2000; Sharma et al., 2009). They include a large and continuously expanding group of structurally related compounds that are classified into four generations. Third- and fourth-generation FQs (e.g. GAT, LEV, MOX, PAZ, and TRO) have several advantages over the earlier ones; they not only exhibit stronger and expanded activity against both gram-negative and gram-positive bacteria and anaerobes but also have improved pharmacokinetic properties. Particularly, these new FQs present high oral bioavailability with plasma drug concentrations comparable to those after IV administration, long elimination half-lives and good tissue penetration (Andriole, 2005; Bolon, 2009; Oliphant and Green, 2002; Sousa et al., 2012; Watabe et al., 2010). FQs have a concentration-dependent bactericidal activity and their efficacy can be predicted by measuring two important parameters, the C_{max}/MIC and the AUC_{0-24}/MIC. Given the intra- and inter-individual pharmacokinetic variability, particularly significant in hospitalized patients, it is important to monitor plasma drug concentrations to attain the optimal drug dosage regimens and to prevent bacterial resistance (De Smet et al., 2009; Muchohi et al., 2011; Pea and Viale, 2006; Sousa et al., 2011; Watabe et al., 2010). According to published pharmacokinetic studies, typical values of C_{max} range from approximately 3 to 5/6 μg/mL (depending on the dosage), after a single oral or IV dose administration. In the case of PAZ, C_{max} could reach values up to 10 μg/mL after IV infusion (Sousa et al., 2012). This implies the need of simple and adequate analytical methods that can be easily applied in clinical settings for quantification of FQs in human plasma at those expected concentrations.

A large number of HPLC methods with FD or UV detection have been described for the determination of a single FQ of the third- and fourth-generations in biological matrices (Borner et al., 1999; Doyle et al., 2000; Overholser et al., 2003; Phapale et al., 2009; Respaud et al., 2012; Siewert, 2006; Sousa et al., 2012). However, only a few HPLC methods have been reported in literature for the separation and simultaneous quantification of two or more of these new FQs in human plasma or serum (Baietto et al., 2009; D'Angelo et al., 2016; Helmy, 2013; Liang et al., 2002; Nemutlu et al., 2007; Nguyen et al., 2004; Schulte et al., 2006; Srinivas et al., 2008; Watabe et al., 2010). For example, Nemutlu et al. (2007) and Baietto et al. (2009) developed an HPLC-UV method for the measurement of LEV and MOX in human plasma, amongst other drugs and quinolones; whereas Watabe et al. (2010) described an HPLC-FD method for the quantification of LEV and PAZ, and the second-generation fluoroquinolone, CIP, in the same type of matrix. Other publications refer to the simultaneous determination of human plasma concentrations of three and four new FQs, namely GAT, LEV and MOX (Helmy, 2013; Nguyen et al., 2004); GAT, MOX and sparfloxacin (Srinivas et al., 2008); LEV, MOX and ulifloxacin (D'Angelo et al., 2016) and GAT, LEV, MOX and TRO (Liang et al., 2002). Nguyen et al. (2004) developed a fully-automated HPLC with a column-switching technique, allowing direct human serum injection into the chromatographic system without any classical pre-treatment steps; however this technique is not feasible in all analytical laboratories. Liang et al. (2002) developed an HPLC method with FD and UV detection but it presents the disadvantage of using a complex mobile phase with ion-pair reagent; although four new FQs were separated by optimization and adjustment of chromatographic conditions, the HPLC method was only fully validated for the measurement of LEV in human plasma.

To the best of our knowledge, no bioanalytical method has been reported for the simultaneous determination of five of the new FQs in human plasma or serum. The aim of this research work was to develop and validate a simple and reliable RP-HPLC method coupled with FD to quantify LEV, PAZ, GAT, MOX and TRO (*Figure II.3.1.*) in human plasma. The proposed method was validated in a wide concentration range for each compound and therefore can be applied to therapeutic monitoring of these FQs in clinical practice and to support other clinical pharmacokinetic- and toxicokinetic-based studies.

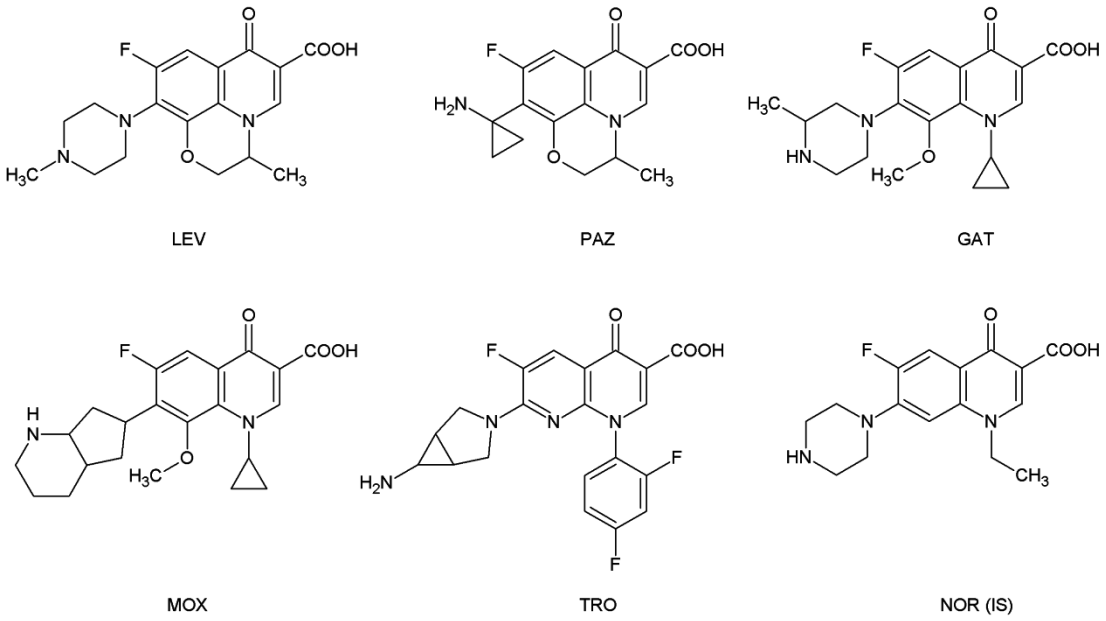

Figure II.3.1. Chemical structures of levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX), trovafloxacin (TRO) and norfloxacin (NOR) used as internal standard (IS).

II.3.2. MATERIALS AND METHODS

II.3.2.1. Chemicals, Materials and Reagents

LEV (lot no. 1395156), TRO (lot no. 020M47081) and NOR (lot no. 028K1480), used as IS, were purchased from Sigma-Aldrich (St Louis, MO, USA). GAT (lot no. M11254/07-10) and PAZ (lot no. M11529/12-10) were obtained from Biokemix (New Mills, Derbyshire, UK) while MOX (lot no. 51587) was acquired from Molekula (Shaftesbury, Dorset, UK). Methanol and acetonitrile (both HPLC gradient grade) were purchased from Fisher Scientific (Leicestershire, UK). Ultrapure water (HPLC grade, >15 MΩ) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Reagents such as fuming hydrochloric acid (37%), formic acid (98–100%) and TEA were acquired from Merck KGaA (Darmstadt, Germany).

Blank human plasma samples from healthy donors were kindly provided by the Portuguese Blood Institute after written consent of each subject.

II.3.2.2. Apparatus and Chromatographic Conditions

The chromatographic analysis was carried out on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a solvent delivery model (LC-20A), a degasser (DGU-20A5), an autosampler (SIL-20AHT), a column oven (CTO-10ASVP) and fluorescence detector (RF-20AXS). The HPLC apparatus and data acquisition were controlled by LCsolution software (Shimadzu Corporation, Kyoto, Japan).

The chromatographic separation of the five analytes (LEV, PAZ, GAT, MOX and TRO) and IS was performed under gradient elution using a reversed-phase LiChroCART® Purospher Star C_{18} column (55 mm x 4 mm, 3 µm particle size; Merck KGaA, Darmstadt, Germany) thermostated at 25 °C. The mobile phase consisted of a mixture of 0.1% aqueous formic acid adjusted to pH 3.0 with TEA (solvent A), acetonitrile (solvent B) and methanol (solvent C) and was pumped at a flow rate of 1.0 mL/min, applying the time gradient elution program described in *Table II.3.1.*. After restoring the initial composition of the mobile phase, the column was re-equilibrated for 5 min, leading to a total run time analysis of 18 min. The fluorescence excitation and emission wavelengths were set at 260 nm and 455 nm, respectively, and the sample injection volume was 20 µL.

Table II.3.1. Gradient elution program for HPLC analysis. Solvents A, B and C correspond to 0.1% aqueous formic acid (pH 3.0, triethylamine), acetonitrile and methanol, respectively.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	80	0	20
2	80	0	20
3	82	2	16
5	82	2	16
7	42	2	56
11	42	2	56
13	80	0	20
18	80	0	20

II.3.2.3. Stock Solutions, Calibration Standards and Quality Control Samples

Stock solutions were individually prepared for LEV, PAZ, GAT, MOX, TRO and IS at the concentration of 1 mg/mL. Stock solutions of PAZ and IS were prepared by dissolving appropriate amounts of each compound in a mixture of methanol and 37% hydrochloric

acid (99.5:0.5, v/v), while stock solutions of LEV, GAT, MOX and TRO were prepared in pure methanol. Appropriate dilutions of stock solutions were made with methanol to obtain two intermediate solutions per analyte at the concentrations of 250 and 20 µg/mL. Each of the stock or diluted solutions were combined and diluted in methanol to prepare six spiking solutions, containing all five analytes with final concentrations of 0.1, 0.2, 1.0, 5.0, 30.0, 100.0 µg/mL for GAT; 0.4, 0.8, 2.8, 10.0, 40.0, 100.0 µg/mL for LEV, PAZ and MOX and 0.8, 1.6, 4.0, 12.0, 40.0, 100.0 µg/mL for TRO. The IS stock solution was also diluted in methanol to a concentration of 250 µg/mL; from this intermediate solution, an aqueous IS working solution of 10 µg/mL was prepared daily by dilution with deionised water. All stock solutions were stored at -30 °C for one month and protected from light, while intermediate and spiking solutions were kept at 4 °C in the dark until use. Indeed, stock solutions of FQs have been prepared in a variety of solvents (water, methanol, acetonitrile and basic or acidic solutions) and are usually stored at 4 °C or -20/-30 °C protected from light for 1 to 3 months (Al-Dgither et al., 2006; Ocaña González et al., 2005; Sousa et al., 2012; Watabe et al., 2010; Zhou et al., 2007).

Calibration standards were daily prepared by spiking blank human plasma aliquots with the same volume (10 µL) of increasing concentrations of the above mentioned spiking solutions in order to obtain the following six concentration levels of calibration curves: 0.005, 0.01, 0.05, 0.25, 1.50 and 5.00 µg/mL for GAT; 0.02, 0.04, 0.14, 0.50, 2.00, 5.00 µg/mL for LEV, PAZ and MOX and 0.04, 0.08, 0.20, 0.60, 2.00, 5.00 µg/mL for TRO.

The QC samples were prepared independently in the same matrix at low (QC_1: 0.015 µg/mL for GAT, 0.06 µg/mL for LEV, PAZ, MOX and 0.12 µg/mL for TRO), medium (QC_2: 2.50 µg/mL) and high (QC_3: 4.50 µg/mL) concentrations representative of the entire range of calibration curves. Two other QC samples were also prepared: one at the concentration of the LLOQ (QC_{LLOQ}) and the other at the concentration of 10 µg/mL in order to evaluate the sample dilution effect (QC_{Dil}). For all QC samples, the preparation procedure was the same as described for the preparation of calibration standards with appropriate spiking solutions.

II.3.2.4. Sample Preparation

Each 200 µL aliquot of human plasma was spiked with 10 µL of IS working solution (10 µg/mL). Then, 600 µL of acetonitrile was added to the sample to precipitate plasma proteins. The mixture was vortex mixed for 30 seconds and centrifuged at 13400 rpm for 5 min. The resulting supernatant (≈ 800 µL) was transferred to a glass tube and evaporated to dryness under a nitrogen stream at 45 °C. The residue was reconstituted with 100 µL of deionised water, vortex mixed for 1 min and placed in an ultrasonic bath at room temperature for 1 min. The reconstituted samples were transferred to 0.22 µm Spin-X centrifugal filters and centrifuged at 13400 rpm for 1 min. The final filtered samples were placed in amber autosampler vials for HPLC analysis.

II.3.2.5. Method Validation

Method validation was performed according to internationally accepted recommendations for bioanalytical method validation (European Medicines Agency, 2011; Shah et al., 2000; US Food and Drug Administration, 2001). The proposed method was validated with respect to selectivity, linearity, limits of quantification and detection, precision and accuracy, sample dilution effect, recovery and stability.

Selectivity

Selectivity of the method was assessed by evaluating potential interference from endogenous compounds and other commonly administered drugs at the same retention times of the analytes and IS. Six different and randomly selected blank human plasma samples were analyzed under the described chromatographic conditions to determine whether any endogenous compounds would interfere at the retention time of each analyte and IS. In addition, interference from other commonly administered drugs namely paracetamol, salicylic acid, ibuprofen, hydrochlorothiazide, cefoxitin, dexamethasone, enoxaparin, propofol, erythromycin, penicillin-G, trimethoprim, neomycin, azithromycin, cloxacillin and omeprazole was also evaluated by injecting standard solutions of these compounds at a concentration of 10 mg/mL.

Linearity of calibration curves

Calibration curves of the five analytes were constructed using six calibration standards over a concentration range of 0.005–5.0 µg/mL for GAT, 0.02–5.0 µg/mL for LEV, PAZ and MOX, and 0.04–5.0 µg/mL for TRO. Five calibration curves were prepared on five different days by plotting the peak area ratios (peak area analyte/peak area IS) against the corresponding nominal plasma concentrations. Weighted linear regression was performed on calibration data using $1/x^2$ for LEV, PAZ, GAT and MOX and $1/y^2$ for TRO as weighting factors. These weighting factors were selected as they yielded the best fit of peak area ratios versus concentration for all FQs under investigation (Almeida et al., 2002).

Limits of quantification and detection

The LLOQ was defined as the lowest concentration on the calibration curve which can be measured with precision (expressed as %CV) not exceeding 20% and accuracy (expressed as %bias) within ± 20%. The LLOQ was evaluated by analyzing plasma samples which were prepared in five replicates (n = 5). The LOD, defined as the lowest concentration that can be distinguished from the noise level, was determined for all the analytes by analyzing plasma samples with known concentrations, after successive dilutions, and it was established as the minimum level at which the analytes can be reliably detected.

Precision and accuracy

Intra and interday precision and accuracy were assessed by analyzing the three QC samples (QC_1, QC_2 and QC_3) described in *section II.3.2.3.*. The intraday precision and accuracy were determined by analyzing five times each QC sample on the same day, while the inter-day precision and accuracy were evaluated by analyzing each QC sample on five different days. The acceptance criterion for precision was a CV lower than or equal to 15%. Accuracy was determined by comparing the estimated concentrations of the three QC samples with their nominal concentrations. The acceptance criterion for accuracy was a bias value within ±15%.

Sample dilution

The dilution effect (1:5) was investigated with appropriate plasma QC samples at 10 µg/mL (QC_{Dil}) to ensure that human plasma samples exceeding the highest concentration of the calibration range (5 µg/mL) could be diluted with blank human plasma and accurately quantified without losing reproducibility. The precision and accuracy of diluted plasma QC samples were determined, according to the intra and interday assays explained above.

Recovery

The recovery of analytes from human plasma samples submitted to protein precipitation was determined at three concentration levels (QC_1, QC_2 and QC_3) by assaying five replicates (*n*=5). The absolute recovery of analytes was calculated by comparing the peak area of the analyte of processed plasma samples with the corresponding peak area obtained from direct injection of aqueous spiked solution at the same concentration. The absolute recovery of the IS was also evaluated at the concentration used in sample analysis.

Stability

The stability was assessed by comparing the data of QC_1 and QC_3 samples (*n*=5) analyzed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). These stability conditions reflect the conditions of sample storage, handling and analysis. Human plasma stability of all five analytes was assessed at room temperature for 3 h, at 4 °C for 24 h and at -30 °C for 5 and 15 days to simulate sample handling and storage time in the freezer before samples analysis. In order to analyze the effect of three freeze–thaw cycles on the stability of the analytes in plasma, aliquots of QC_1 and QC_3 samples were stored at -30 °C for 24 h, thawed unassisted at room temperature and when completely thawed, the samples were refrozen for 24 h under the same conditions until completing the three cycles. Stability of the five analytes was also studied at 4 °C and at room temperature during 24 h in the reconstitution solvent (deionised water) to resemble the resident time in the autosampler before analysis. The

acceptance criterion for stability was a stability/reference samples ratio between 85 and 115%.

Application of the method to real plasma samples

The application of the proposed method was also demonstrated for the identification and quantification of LEV and MOX in adult patients (3 females and 5 males) plasma samples. Seven of these patients were hospitalized at different medical services (cardiology, pneumology, urology, haematology and internal medicine) in Coimbra University Hospital and treated with LEV by oral or intravenous route; another patient received oral MOX in ambulatory care and sample collection was performed with informed consent at two different times after administration. These real plasma samples were obtained in accordance with the ethical principles of the Declaration of Helsinki. The analysis of those patient samples enabled the reassessment of the selectivity of the described HPLC method since LEV and MOX were administered with a wide variety of other prescribed drugs. Tables containing full details concerning these real plasma samples, co-prescribed drugs and results are presented in *section II.3.3.*.

II.3.3. RESULTS AND DISCUSSION

The method was developed in a reversed-phase LiChroCART® Purospher Star C_{18} column and the best chromatographic resolution of LEV, PAZ, GAT, MOX, TRO and IS was achieved by adjusting chromatographic conditions such as mobile phase and FD wavelengths.

Chromatographic conditions were first optimized through several modifications of mobile phase composition and gradient elution program to achieve the best resolution and peak shapes and to minimize run time analysis. Gradient elution was carried out using a mobile phase composed of an aqueous polar phase (A) and two organic modifiers, acetonitrile (B) and methanol (C). The aqueous polar phase chosen for this work was a 0.1% formic acid solution adjusted to pH 3.0 with TEA (A). This acidic aqueous phase was used in order to reverse the ionization of the FQ carboxylic function and thus stabilize the retention times of analytes, LEV (pK_{a1} = 5.5; pK_{a2} = 8.0), PAZ (pK_{a1} = 5.7; pK_{a2} = 8.6), GAT (pK_{a1} = 5.7; pK_{a2} = 9.0), MOX (pK_{a1} = 6.4; pK_{a2} = 9.5) and TRO (pK_{a1} = 5.9; pK_{a2} = 8.1), and IS

(pK_{a1} = 5.8; pK_{a2} = 8.7) and improve peak shape. TEA was also added to the mobile phase to suppress peak tailing effect caused by secondary interactions between free silanol groups of the silica-based column and amine basic groups of FQs. Overall, the gradient program selected for this HPLC method has two important isocratic segments where analytes and IS elute from the column. From 3 to 5 min, the composition of the mobile phase was 82%(A)/2%(B)/16%(C) allowing the elution of LEV and IS. During the following 2 min, the proportion of solvent A was decreased linearly from 82% to 42% and PAZ was detected at the onset of this composition change. During the second isocratic segment established between 7 and 11 min, GAT, MOX and TRO were separately eluted from the column by a mobile phase with 42%(A)/2%(B)/56%(C). This sequence of two groups of FQs, eluted with mobile phases characterized by different polarities, reveals that LEV, IS and PAZ are more polar than GAT, MOX and TRO. Sharpener peaks and consequent improvement of resolution were obtained with the incorporation of 2% (B) in mobile phase. In order to force the elution of potential interfering substances, a mobile phase containing 80%(A)/0%(B)/20%(C) was used in the first 2 min. To optimize FQs detection, different combinations of excitation and emission wavelengths were tested in the range of 260–300 nm and 430–500 nm, respectively. Amongst all five FQs, GAT was the analyte with the strongest intensity of fluorescence emission while TRO provided the lowest response signal. The excitation and emission wavelengths selected for all analytes were 260 nm and 455 nm, respectively, since they allowed the best compromise between sensitivity for all the analytes and high selectivity against endogenous substances.

Moreover, considering that selectivity and sensitivity can be limited by sample clean-up procedures, the plasma sample preparation was also an important step in the development of this method. To obtain higher recovery values of analytes, the number of clean-up steps in a sample preparation procedure should be kept to a minimum. Protein precipitation is the fastest and simplest sample preparation technique and is commonly used for FQs analysis in plasma matrices. Several organic solvents (e.g. acetonitrile and methanol) and acids (e.g. perchloric acid, phosphoric acid and trifluoracetic acid) have been used as plasma protein precipitants (Baietto et al., 2009; Borner et al., 2000; De Smet et al., 2009; Hemanth Kumar and Ramachandran, 2009; Siewert, 2006; Sousa et al., 2011; Stass and Dalhoff, 1997; Watabe et al., 2010). In the present work, acetonitrile, trichloroacetic acid and a mixture of acetonitrile and phosphoric acid were tested for

plasma protein precipitation. Trichloroacetic acid (20%, w/v) caused almost immediate protein precipitation of plasma samples and provided clean chromatograms; however analytes recovery values were much lower than those attained with acetonitrile/phosphoric acid and the pure organic solvent (acetonitrile). This fact was more evident for GAT, MOX and TRO and can be explained by co-precipitation of analytes with the addition of strong acids such as 20% trichloroacetic acid, being trapped in the protein pellet. The combination of acetonitrile and phosphoric acid was expected to be a good option for plasma protein precipitation and analytes extraction; however the highest recovery values were obtained with pure acetonitrile as precipitating agent, particularly for GAT, MOX and TRO. Evaporation and reconstitution steps were included after sample precipitation to concentrate analytes before HPLC analysis.

Another important issue taken into account during the development of this bioanalytical method was the selection of the IS. The addition of this compound to the sample before sample preparation procedures improves the accuracy and precision of the method by correcting for the loss of analytes during those steps. In this work, NOR was used as IS because it has similar molecular structure, physicochemical properties and chromatographic behavior to those exhibited by the analytes. At the conditions established for the sample preparation and chromatographic separation, NOR displayed an appropriate retention time with a good chromatographic resolution and a good extraction recovery.

II.3.3.1. Chromatographic Separation and Selectivity

Under the chromatographic conditions previously described, LEV, PAZ, GAT, MOX, TRO and the IS were successfully separated in less than 11 min and the retention times were approximately 3.6, 6.0, 9.8, 10.3, 10.7 and 4.4 min, respectively. Representative chromatograms of blank and spiked human plasma samples are shown in *Figure II.3.2.*. The analysis of blank human plasma samples showed no endogenous interferences at the retention times of the analytes and IS. In addition, none of the tested drugs which are commonly administered in clinical practice interfered with the peaks of the analytes and IS.

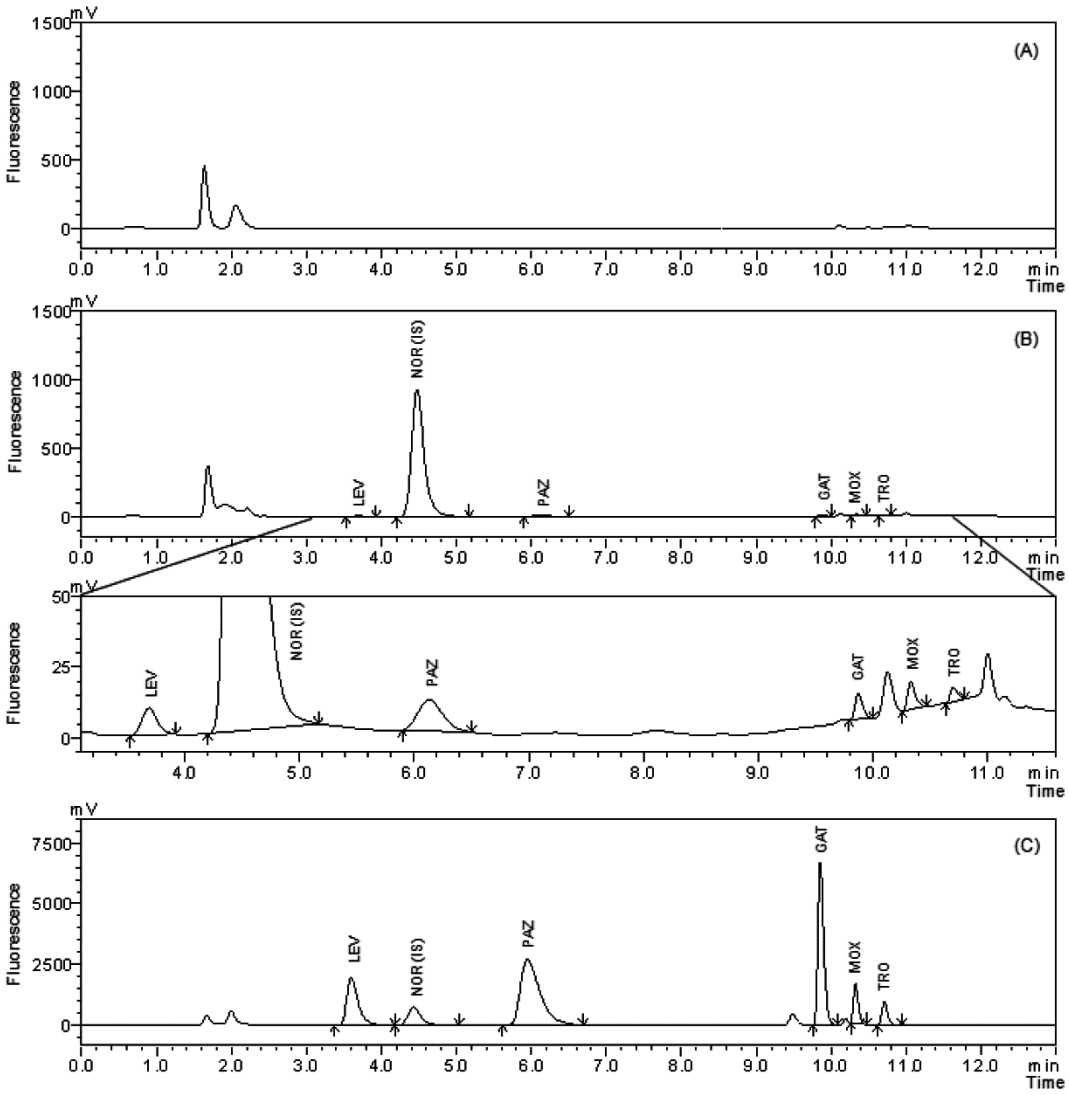

Figure II.3.2. Typical chromatograms of processed human plasma samples obtained by the validated HPLC-FL method: blank plasma (A), spiked plasma with norfloxacin (NOR) used as internal standard (IS) and the analytes levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX) and trovafloxacin (TRO) at concentrations of the lower limit of quantification (B) and at concentrations of the upper limit of the calibration range (C).

II.3.3.2. Linearity of Calibration Curves, LLOQs and LODs

Calibration curves were linear (r^2 ≥ 0.9923) for all analytes over the specified concentration range. The weighted regression equations of calibration curves and the corresponding r^2 values are summarized in *Table II.3.2.*. The LOD was established at 0.0025 µg/mL for GAT, 0.01 µg/mL for LEV, PAZ and MOX and 0.02 µg/mL for TRO. The LLOQ of the assay was set at 0.005 µg/mL for GAT, 0.02 µg/mL for LEV, PAZ and MOX and 0.04

µg/mL for TRO with acceptable precision (CV ≤ 7.58%) and accuracy (bias ± 15.57%) (*Table II.3.3.*).

Table II.3.2. Calibration curve parameters for levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX) and trovafloxacin (TRO) in human plasma (*n*=5).

Analyte	Calibration Range (µg/mL)	Regression Equation	r^2
LEV	0.020–5.0	y = 0.3838x - 0.0012	0.9971
PAZ	0.020–5.0	y = 0.7782x - 0.0044	0.9969
GAT	0.005–5.0	y = 0.7090x - 0.0003	0.9958
MOX	0.020–5.0	y = 0.1465x - 0.0003	0.9923
TRO	0.040–5.0	y = 0.0498x - 0.0003	0.9946

Table II.3.3. Precision (% CV) and accuracy (% bias) for the determination of levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX) and trovafloxacin (TRO) in human plasma samples at the concentration of the lower limit of quantification (LLOQ) (*n* = 5).

Analyte	Nominal Concentration (µg/mL)	Measured Concentration (mean ± SD, µg/mL)	Precision (% CV)	Accuracy (% bias)
Intraday				
LEV	0.020	0.0210 ± 0.0009	4.87	5.24
PAZ	0.020	0.0231 ± 0.0007	4.42	15.57
GAT	0.005	0.0052 ± 0.0001	1.09	4.38
MOX	0.020	0.0200 ± 0.0009	4.70	-0.09
TRO	0.040	0.0418 ± 0.0010	2.90	4.54
Interday				
LEV	0.020	0.0209 ± 0.0013	7.58	4.43
PAZ	0.020	0.0221 ± 0.0008	5.03	10.40
GAT	0.005	0.0052 ± 0.0002	3.22	3.39
MOX	0.020	0.0207 ± 0.0014	7.64	3.61
TRO	0.040	0.0410 ± 0.0007	2.01	2.54

CV, coefficient of variation; SD, standard deviation

II.3.3.3. Precision and Accuracy

The data for intra and interday precision and accuracy obtained from QC plasma samples at three different concentrations representative of the calibration range (QC_1, QC_2 and QC_3) are shown in *Table II.3.4.*. The intra and interday CV values did not exceed 7.32% and the intra and interday bias values ranged from -11.73% to 8.92%. All results fulfill the

acceptance criteria of international guidelines; therefore the developed HPLC-FD method is precise and accurate for the quantification of LEV, PAZ, GAT, MOX and TRO in human plasma. For the dilution integrity (1:5) of plasma samples at the concentration of 10 µg/mL (QC_{Dil}), the precision was lower than or equal to 6.71% and the accuracy values were between 2.77% and 6.71%, clearly demonstrating that a 5-fold dilution with blank plasma can be applied for plasma samples with concentrations of analytes that exceed the highest concentration of the calibration curves.

Table II.3.4. Precision (% CV) and accuracy (% bias) for the determination of levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX) and trovafloxacin (TRO) in human plasma samples at the low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration ranges and following a sample dilution (*) by a 5-fold factor (*n* = 5).

Analyte	Nominal Concentration (µg/mL)	Measured Concentration (mean ± SD, µg/mL)	Precision (% CV)	Accuracy (% bias)
Intraday				
LEV	0.060	0.0654 ± 0.0260	4.16	8.92
	2.5	2.4762 ± 0.0569	2.30	-0.95
	4.5	4.4065 ± 0.1916	4.35	-2.08
	*10	9.6439 ± 0.2798	2.90	-3.56
PAZ	0.060	0.0649 ± 0.0027	4.55	8.25
	2.5	2.4328 ± 0.1333	5.49	-2.69
	4.5	4.3323 ± 0.0651	1.51	-3.73
	*10	9.5468 ± 0.3736	3.92	-4.53
GAT	0.015	0.0158 ± 0.0005	3.38	5.34
	2.5	2.3823 ± 0.0491	2.06	-4.71
	4.5	4.2702 ± 0.1898	4.44	-5.11
	*10	9.5652 ± 0.2650	2.77	-4.35
MOX	0.060	0.0635 ± 0.0022	3.47	5.85
	2.5	2.3863 ± 0.0387	1.62	-4.55
	4.5	3.9722 ± 0.1358	3.42	-11.73
	*10	9.1776 ± 0.3264	3.56	-8.22
TRO	0.12	0.1237 ± 0.0033	2.88	3.08
	2.5	2.4527 ± 0.0315	1.29	-1.89
	4.5	4.3367 ± 0.1155	2.67	-3.63
	*10	10.4967 ± 0.4222	4.03	4.97

Table II.3.4. *(Continued)*

Analyte	Nominal Concentration (μg/mL)	Measured Concentration (mean ± SD, μg/mL)	Precision (% CV)	Accuracy (% bias)
Interday				
LEV	0.060	0.0640 ± 0.0021	3.48	6.75
	2.5	2.4405 ± 0.1235	5.07	-2.38
	4.5	4.6264 ± 0.1979	4.28	2.81
	*10	9.9113 ± 0.5300	5.35	-0.89
PAZ	0.060	0.0640 ± 0.0030	5.11	7.68
	2.5	2.3485 ± 0.0782	3.34	-6.06
	4.5	4.5142 ± 0.2093	4.64	0.32
	*10	9.3431 ± 0.4898	5.25	-6.57
GAT	0.015	0.0156 ± 0.0006	3.76	4.26
	2.5	2.3686 ± 0.1342	5.67	-5.26
	4.5	4.5060 ± 0.1800	3.99	0.13
	*10	9.8492 ± 0.5366	5.45	-1.51
MOX	0.060	0.0629 ± 0.0017	2.85	4.76
	2.5	2.3759 ± 0.1100	4.63	-4.96
	4.5	4.1766 ± 0.1777	4.26	-7.19
	*10	9.4463 ± 0.4340	4.60	-5.54
TRO	0.12	0.1212 ± 0.0076	6.64	1.03
	2.5	2.4372 ± 0.1779	7.32	-2.51
	4.5	4.5138 ± 0.1763	3.91	0.31
	*10	10.4968 ± 0.7040	6.71	4.97

CV, coefficient of variation; SD, standard deviation.

II.3.3.4. Recovery

The mean absolute recovery values from human plasma samples are presented in *Table II.3.5.*. The mean absolute recoveries of LEV, PAZ, GAT, MOX and TRO at the three aforementioned concentration levels were 89.21–109.86%, 78.08–79.76%, 84.61–94.11%, 84.76–104.30% and 73.75–90.44%, respectively, with acceptable CV values (≤ 8.21%). The absolute recovery of each analyte was consistent over the three concentration levels tested (n = 15) as shown by CV values of 10.49%, 5.42%, 6.80%, 10.83% and 10.00% for

LEV, PAZ, GAT, MOX and TRO, respectively. The mean absolute recovery of IS was 85.66% with CV = 1.25%.

Table II.3.5. Absolute recovery (%) of levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX) and trovafloxacin (TRO) from human plasma samples at the low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration ranges.

Analyte	Nominal concentration (µg/mL)	n	Absolute Recovery (%)	
			Mean±SD	CV (%)
LEV	0.060	5	109.86 ± 7.33	6.67
	2.5	5	95.90 ± 6.08	6.34
	4.5	5	89.21 ± 2.01	2.25
PAZ	0.060	5	78.16 ± 6.36	8.14
	2.5	5	79.76 ± 4.05	5.08
	4.5	5	78.08 ± 2.11	2.70
GAT	0.015	5	94.11 ± 6.07	6.45
	2.5	5	90.10 ± 5.73	6.36
	4.5	5	84.61 ± 1.87	2.21
MOX	0.060	5	104.30 ± 8.57	8.21
	2.5	5	91.11 ± 5.77	6.33
	4.5	5	84.76 ± 1.66	1.96
TRO	0.12	5	90.44 ± 5.72	6.33
	2.5	5	82.41 ± 5.22	6.34
	4.5	5	73.75 ± 1.37	1.86

CV, coefficient of variation; SD, standard deviation

II.3.3.5. Stability

The results of stability are shown in *Table II.3.6.* and reveal that no significant degradation occurred in processed plasma samples (reconstitution solvent) at 4 °C and at room temperature for 24 h and in human plasma (unprocessed samples) at room temperature during 3 h, at 4 °C for 24 h and after three freeze/thaw cycles. The analytes, PAZ, GAT and TRO, also demonstrated to be stable in human plasma after storage at -30 °C for 15 days. However, LEV and MOX were only stable in human plasma for 5 days at -30 °C, with stability values near the limit of 85% at QC_1 plasma concentration.

Table II.3.6. Stability (%) of levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX) and trovafloxacin (TRO) in unprocessed plasma samples left at room temperature for 3 h, at 4 °C for 24 h, after three cycles freeze (-30 °C)/thaw, at -30 °C during 5 and 15 days, and in processed plasma samples left at 4 °C and at room temperature for 24 h (*n* = 5).

Analyte	LEV		PAZ		GAT		MOX		TRO	
Nominal Concentration (µg/mL)	0.060	4.5	0.060	4.5	0.015	4.5	0.060	4.5	0.12	4.5
Unprocessed plasma										
Room temperature (3 h)	107.4	96.1	99.7	96.5	107.6	95.6	105.4	94.9	103.3	89.0
4 ºC (24 h)	100.9	96.7	102.9	90.7	101.1	97.3	103.3	96.6	99.3	99.0
Freeze/thaw (3 cycles)	101.4	103.2	111.7	102.1	106.8	102.5	96.0	101.7	109.4	103.3
-30 ºC (5 days)	86.3	104.6	91.4	103.3	88.8	103.5	85.4	104.3	86.1	109.1
-30 ºC (15 days)	82.8	108.4	90.4	102.6	87.6	107.8	82.9	107.2	85.8	113.9
Processed plasma										
4 ºC (24 h)	102.7	98.2	98.8	98.5	101.1	97.6	100.9	98.0	101.6	95.6
Room temperature (24 h)	111.9	92.6	101.8	89.6	110.7	93.0	105.2	94.5	103.2	92.9

II.3.3.6. Application of the Method to Real Plasma Samples

The validated HPLC-FD method was applied to the analysis of LEV and MOX concentrations in plasma samples taken from adult patients. The prescribed regimen of each individual (ID), time of plasma sample collection after drug administration and the results obtained for the plasma concentration of LEV and MOX are summarized in *Table II.3.7.*. Four out of nine concentration values occurred above the upper limit of the calibration range and therefore a 5-fold dilution was applied based on the positive results of the dilution integrity on the precision and accuracy. The results also show variability between individuals with similar prescribed regimens; this is particularly evident for ID_3 and ID_4 and could be related to the influence of the disease state of the patient on the pharmacokinetic profile of the drug. This emphasizes the need for monitoring plasma drug concentrations in hospitalized patients, and the consequent importance of this method to improve therapeutic efficacy and prevent potential bacterial resistance.

Table II.3.7. Plasma concentrations of levofloxacin (LEV) and moxifloxacin (MOX) in real plasma samples obtained from hospitalized (ID_1 to ID_7) and ambulatory (ID_8) patients after oral or intravenous administration of these drugs at different prescribed regimens. Samples were collected at different times after drug administration and analysed by the validated HPLC-FD method.

Patients	FQ	Prescribed Regimen	Post-dosing Sampling Time (h)	Measured Concentration (μg/mL)
ID_1	LEV	750 mg IV injection (id)	12	4.3338
ID_2	LEV	750 mg IV injection (id)	10	6.0554
ID_3	LEV	500 mg IV injection (id)	12	6.0074
ID_4	LEV	500 mg IV injection (id)	12	1.4042
ID_5	LEV	750 mg tablet (id)	12	8.2650
ID_6	LEV	500 mg tablet (id)	12	4.4425
ID_7	LEV	500 mg tablet (id)	12	5.4479
ID_8	MOX	400 mg tablet (id)	3 / 24	3.0652 / 0.2606

FQ, fluoroquinolone; ID, individual; id, once daily; IV, intravenous.

Representative chromatograms of real plasma samples are depicted in *Figure II.3.3.* for LEV (ID_4) and MOX (ID_8 at 3 h after drug administration). No interference from endogenous compounds of patient plasma samples or from a total of forty six (ID_1 to ID_8) co-prescribed drugs (*Table II.3.8.*) were found at the retention time of analytes in any of the samples. It is also clear that the peak shape and chromatographic resolution are similar to those obtained with spiked blank human plasma.

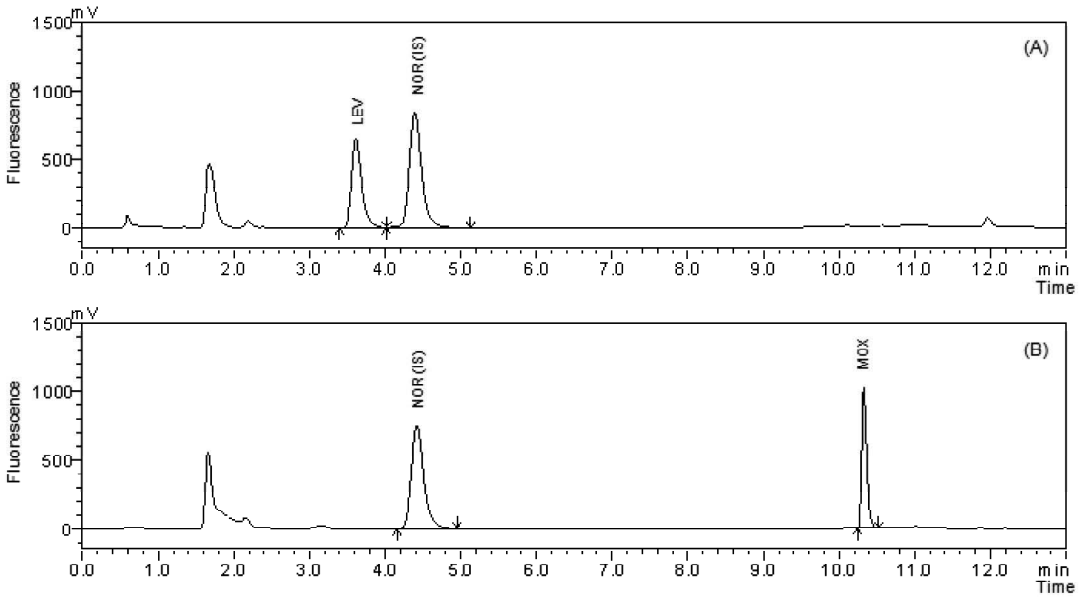

Figure II.3.3. Representative chromatograms of real plasma samples obtained from patients treated with (A) levofloxacin (LEV) or (B) moxifloxacin (MOX). Samples were collected and analysed by the validated HPLC-FD method.

Table II.3.8. Co-prescribed drugs of patients who received levofloxacin or moxifloxacin treatment.

Patients	Co-prescribed Drugs
ID_1	Lactulose, Lisinopril, Naproxen, Oxazepam, Paracetamol, Sennoside, Simvastatin, Tramadol, Warfarin
ID_2	Acetylcysteine, Amitriptyline, Enoxaparin sodium, Lactulose, Mexazolam, Pantoprazole, Paracetamol, Riluzole
ID_3	Acetylsalicylic acid, Aminophiline, Bromhexine, Digoxin, Enoxaparin sodium, Fluticasone, Furosemide, Human Insulin, Ipratropium bromide, Losartan, Salmeterol, Omeprazole, Oxazepam
ID_4	Paracetamol
ID_5	Acetylcysteine, Bisoprolol, Enoxaparin sodium, Furosemide, Ibuprofen, Lactulose, Metamizole magnesium, Omeprazole, Oxazepam, Paracetamol
ID_6	Alprazolam, Betahistine, Clemastine, Filgastrim, Furosemide, Metilprednisolone, Metoclopramide, Omeprazole, Paracetamol, Ranitidine, Rituximab
ID_7	Acetylsalicylic acid, Allopurinol, Alprazolam, Amiodarone, Atorvastatin, Clopidogrel, Digoxin, Furosemide, Pantoprazole, Sertraline, Spironolactone, Warfarin
ID_8	Dienogest and ethinylestradiol

ID, individual.

II.3.4. CONCLUSION

This work represents the first HPLC method developed and fully validated for the simultaneous determination of LEV, PAZ, GAT, MOX and TRO in human plasma. The method presents several important bioanalytical advantages. It is simple, highly selective and sensitive and only requires low cost technology which makes it economically attractive for most clinical laboratories.

The sample preparation consists of a simple deproteinization step with acetonitrile, thereby avoiding a more complex multiple step manipulation as involved in conventional liquid-liquid extraction and solid-phase extraction, reducing time and error sources. Although evaporation and reconstitution steps are included, the sample preparation procedure remains simple and allows high throughput analysis, while requiring a small volume of human plasma (200 µL). The coupling of FD to HPLC enhances the sensitivity and selectivity of the method as shown by the lower LLOQs obtained (0.005, 0.02 and 0.04 µg/mL) as compared to those achieved by methods using UV detection (Baietto et al., 2009; D'Angelo et al., 2016; Helmy, 2013; Liang et al., 2002; Srinivas et al., 2008).

The validation of this method has demonstrated that it fulfills the international requirements for the reliable quantification of all analytes in a wide plasma concentration range. The feasibility of sample dilution for human plasma concentrations above the upper limit of the calibration range and the application of the method to real plasma samples were also successfully assessed.

Hence, a simple, selective, sensitive, accurate and reproducible new method has been developed and validated for the simultaneous quantification of LEV, PAZ, GAT, MOX and TRO in human plasma. It is suitable for TDM of these FQs and can be applied to other pharmacokinetic and toxicological studies.

II.4. DETERMINATION OF LEVOFLOXACIN, NORFLOXACIN, CIPROFLOXACIN AND LOMEFLOXACIN IN RAT MATRICES BY LIQUID CHROMATOGRAPHY

II.4.1. INTRODUCTION

Achievement of the ultimate objectives defined for this thesis required the development and validation of appropriate analytical methods for the quantitative determination of FQs in rat biological samples, namely plasma, nasal mucosa and olfactory bulb. In fact these matrices can probe not only efficacy but also safety of IN administration as compared to traditional/conventional routes.

The LC methods previously described in this chapter for the quantification of second-generation (NOR, CIP and LOM) and third- and fourth-generations (LEV, PAZ, GAT, MOX and TRO) FQs in human plasma (Sousa et al., 2013, 2011) were developed not only as a useful tool for TDM in clinical practice, but also as a basis and starting point for the development of similar methods to quantify FQs in a different species. This approach is particularly crucial to minimize the number of animals needed, reduce time consumption and acquire expertise, thus presenting clear ethical and economical advantages.

Following these considerations, attempts were made to use the LC method validated in human plasma for the new generation FQs, LEV, PAZ, GAT, MOX and TRO, to quantify the same antibiotics in rat plasma. However, several difficulties were encountered, namely the appearance of interfering peaks, that in spite of multiple attempts, could not be overcome, ruling out the possibility of obtaining reliable data. Therefore the next step was to abandon this gradient method in favor of the isocratic method previously developed and validated for NOR, CIP and LOM in human plasma.

Taking into account that LEV, PAZ, GAT, MOX and TRO are more promising antibiotics due to their broader spectrum of antibacterial activity and improved pharmacokinetic properties in comparison with NOR, CIP and LOM, LEV was included as an additional analyte as a compromise option.

The bioanalytical method described in the next section is based on the isocratic LC method above mentioned (*section II.2.*) with a few modifications to adapt it to different

chromatographic equipment and different species/types of matrix. Impact of any changes made in an already validated method should be evaluated and therefore a partial validation must be performed. Due to the small dimensions of rat nasal mucosa and olfactory bulb, their availability is limited and this reinforces the performance of a partial validation.

The next sections provide a description of analytical procedures and results of the partially validated LC method for quantification of LEV, NOR, CIP and LOM (*Figure II.4.1.*) in rat plasma, nasal mucosa and olfactory bulb that will be used to analyze the samples collected from the *in vivo* pharmacokinetic studies.

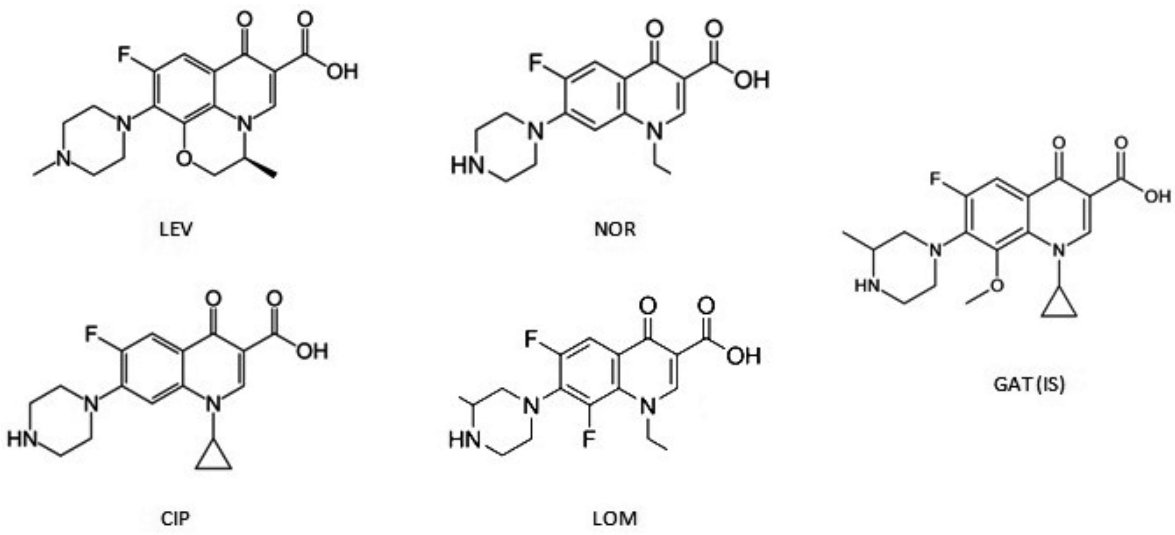

Figure II.4.1. Chemical structures of levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP), lomefloxacin (LOM) and gatifloxacin (GAT) used as internal standard (IS).

II.4.2. MATERIALS AND METHODS

II.4.2.1. Chemicals, Materials and Reagents

LEV (lot no. 1395156), NOR (lot number 028K1480) and CIP (lot number 1396107) were purchased from Sigma-Aldrich (St Louis, MO, USA), whereas LOM hydrochloride (lot number 62277) and GAT (lot no. M11254/07-10) used as IS, were obtained from Molekula (Shaftesbury, Dorset, UK) and from Biokemix (New Mills, Derbyshire, UK), respectively. Methanol and acetonitrile (both HPLC gradient grade) were purchased from Fisher Scientific (Leicestershire, UK) and ultrapure water (HPLC grade, > 15 MΩ) was prepared by

means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Other reagents, fuming hydrochloric acid (37%), formic acid (98–100%) and TEA were acquired from Merck KGaA (Darmstadt, Germany), and trichloroacetic acid was obtained in solid state from Sigma-Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate and ortho-phosphoric acid (85%), used to prepare 25mM phosphate buffer pH 3.0 solution, were obtained from Merck KGaA (Darmstadt, Germany) and Panreac (Barcelona, Spain), respectively.

II.4.2.2. Apparatus and Chromatographic Conditions

The chromatographic analysis was carried out on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a solvent delivery model (LC-20A), a degasser (DGU-20A5), an autosampler (SIL-20AHT), a column oven (CTO-10ASVP) and fluorescence detector (RF-20AXS). The HPLC apparatus and data acquisition were controlled by LCsolution software (Shimadzu Corporation, Kyoto, Japan).

The chromatographic separation of the four analytes (LEV, NOR, CIP and LOM) and IS was performed on a reversed-phase LiChroCART® Purospher Star C_{18} column (55 mm x 4 mm, 3 µm particle size; Merck KGaA, Darmstadt, Germany) at 25 °C by isocratic elution. The mobile phase consisted of a mixture of 0.1% aqueous formic acid adjusted to pH 3.0 with TEA, acetonitrile and methanol (84:1:15, v/v/v) and was pumped at a flow rate of 1.0 mL/min. The excitation and emission wavelengths selected for detection of all analytes were set at 278 nm and 470 nm, respectively, and the total run time was 25 min. The sample injection volume was 20 µL for processed rat plasma samples and 40 µL for processed tissue samples.

II.4.2.3. Blank Biological Samples

Healthy adult male Wistar rats that were not administered with FQs or not submitted to any other pharmacologically active compound or if so with long washout periods were used as the source of drug-free plasma, nasal mucosa and olfactory bulb matrices for the development and validation of this method. Rats were kept in local animal facilities under controlled environmental conditions (12h light/dark cycle, at 20 ± 2 °C and 55 ± 5% relative humidity) with free access to tap water and standard rodent diet (4RF21,

Mucedola®, Italy). To obtain blank matrices, the animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and decapitated. Blood samples were immediately collected into heparinized tubes and plasma samples were obtained after centrifugation at 1514 *g* for 10 min at 4 °C and stored at -24 °C. Nasal mucosa and olfactory bulb tissues were removed, weighed (40 mg) and stored frozen (-24 °C) in Eppendorf® tubes until use.

All animal handling procedures were conducted in accordance with the European Directive (2010/63/EU) (European Parliament and Council of the European Union, 2010) regarding the protection of laboratory animals used for scientific purposes.

II.4.2.4. Stock Solutions, Calibration Standards and Quality Control Samples

Stock solutions of LEV, NOR, CIP and LOM were individually prepared at the concentration of 1 mg/mL. In the case of NOR and CIP, appropriate amounts of each compound were dissolved in a mixture of methanol and hydrochloric acid 37% (99.5:0.5, v/v), while LEV and LOM stock solutions were prepared in pure methanol. Then, these stock solutions were diluted in methanol to obtain intermediate solutions. Each of the stock and intermediate solutions were combined and diluted to obtain six working solutions containing all four analytes that were used to spike rat biological matrices. For plasma matrix, these six spiking solutions were prepared at final concentrations of 0.3, 0.6, 1.8, 6.0, 20.0, 50.0 µg/mL for NOR and CIP and 0.4, 0.8, 2.0, 6.0, 20.0, 50.0 µg/mL for LEV and LOM to yield six calibration standards by adding the same volume (10 µL) of each spiking solution to aliquots of blank plasma. In the case of nasal mucosa matrix, spiking solutions of 0.625, 1.25, 7.50, 50.0, 125 and 200 µg/mL for NOR and CIP and 0.90, 1.80, 7.50, 50.0, 125 and 200 µg/mL for LEV and LOM were prepared and used to spike nasal mucosa samples with the same volume (20 µL) of this spiking solutions and thus generate the appropriate calibration standards. Similarly, another set of spiking solutions with NOR and CIP concentrations of 0.625, 1.25, 3.75, 7.50, 15.0 and 25.0 µg/mL and with LEV and LOM concentrations of 0.90, 1.80, 3.75 7.50, 15.0 and 25.0 µg/mL were used to prepare another set of six calibration standards in olfactory bulb tissue matrix. The calibration ranges were defined as 0.03–5.0 µg/mL (for NOR and CIP) and 0.04–5.0 µg/mL (for LEV and LOM) in plasma, while in tissue matrices of nasal mucosa calibration ranges were 12.5–

4000 ng (for NOR and CIP) and 18.0–4000 ng (for LEV and LOM) and in olfactory bulb 12.5–500 ng (for NOR and CIP) and 18–500 ng (for LEV and LOM).

The QC samples were also prepared in the same biological matrices at LLOQ and at three different levels – low (QC_1), medium (QC_2) and high (QC_3) – representative of the entire calibration ranges. In the case of plasma, another QC sample was prepared at the concentration of 10 µg/mL (QC_{Dil}) in order to evaluate the sample dilution effect (1:5). The nominal values of each QC sample can be seen in *Tables II.4.2.*, *II.4.3.* and *II.4.4.*.

A stock solution of IS was prepared in methanol at 1 mg/mL and diluted to obtain an intermediate solution of 400 µg/mL. From this intermediate solution, two aqueous IS working solutions were daily prepared in deionized water at concentrations of 48 µg/mL and 16 µg/mL, the first one used for nasal mucosa analysis and second one used for plasma and olfactory bulb analysis.

II.4.2.5. Sample Preparation

Each aliquot of rat plasma (100 µL) was spiked with 30 µL of IS working solution (16 µg/mL) and mixed with 30 µL of 20% (w/v) trichloroacetic acid. The final plasma sample was vortex-mixed for 30 s and centrifuged at 13400 rpm (12100 *g*) for 5 min at room temperature to precipitate plasma proteins and obtain a clear supernatant that was directly injected (20 µL) into the HPLC system. Nasal mucosa and olfactory bulb tissues, weighing 40.0 mg, were extracted and homogenized with 1 mL of 25mM phosphate buffer solution pH 3.0 in an Ultra-Turrax device (Ystral® GmbH, Germany) for 2-5 min at room temperature, after addition of 10 µL of IS working solution (48 µg/mL for nasal mucosa and 16 µg/mL for olfactory bulb). The homogenate was centrifuged at 14000 rpm (17530 *g*) for 10 min at 4 °C and 400 µL of the resultant supernatant were mixed with 120 µL of 20% (w/v) trichloroacetic acid. The following vortex-mixing and centrifugation steps were the same as for plasma samples, leading to cleaner and transparent samples, ready to be directly injected (40 µL) into the HPLC system.

II.4.2.6. Partial Validation

Following the international guidelines (European Medicines Agency, 2011; US Food and Drug Administration, 2001) and in the absence of established protocols, partial validation was performed as will now be described.

Selectivity

The method should be selective, i.e., able to differentiate the analytes and IS from endogenous components of biological matrix. In the present work, selectivity was assessed by evaluating potential interference of endogenous compounds present in rat plasma, nasal mucosa and olfactory bulb matrices. Six blank matrices of different rats were analyzed under the described chromatographic conditions and chromatograms were compared with those of spiked samples to determine whether any endogenous compounds interfered at the retention time of each analyte and IS. In addition, possible interference of pentobarbital, the specific anesthetic to be used during the subsequent *in vivo* pharmacokinetic studies was also evaluated.

Linearity of calibration curves

Linearity was assessed in each biological matrix using six calibration standards over the defined concentration ranges prepared in three different days. Three calibration curves (n = 3) were obtained by plotting the peak area analyte/peak area IS ratios against the corresponding nominal values, expressed in µg/mL for rat plasma matrix and in ng for rat nasal mucosa and olfactory bulb matrices homogenized in 1 mL. Linear regression analysis was performed on this data using $1/x^2$ as best weighting factor that yielded the best fit to peak area ratios.

Limits of quantification

The LLOQ was defined has the lowest concentration of the calibration curve which can be measured with precision (expressed as %CV) not exceeding 20% and accuracy (expressed as %bias) within ±20%. In the present work, precision and accuracy of LLOQ

were determined by intra and interday analysis of three replicates for each biological matrix.

Precision and accuracy

Intra and interday precision and accuracy were assessed by analysing three replicates (n = 3) of each QC sample (QC_1, QC_2 and QC_3) for each biological matrix. The intraday precision and accuracy were determined by analysing three times each QC sample in the same day, whereas the interday precision and accuracy were evaluated by analysing each QC sample in three different days. The acceptance criterion for precision was a CV ≤ 15% and for accuracy a bias value within ±15%.

Sample dilution

Dilution effect (1:5) was also investigated in the particular case of rat plasma matrix using QC_{Dil} samples to ensure that plasma samples exceeding the highest concentration of the calibration range (5 µg/mL) could be accurately quantified after dilution with blank rat plasma. Precision and accuracy of diluted QC_{Dil} plasma samples were determined intra and interday by replicated analysis (n = 3) as explained above and the same acceptance criteria was adopted.

Recovery

The absolute recovery of analytes extracted from rat plasma and tissue matrices was determined at three different levels of the calibration range (QC_1, QC_2 and QC_3) by triplicate analysis and comparing the peak area of the analyte in processed samples (submitted to the sample preparation procedures described in *section II.4.2.5.*) with the corresponding peak area of the analyte obtained from non-extracted solutions at the same nominal values. The absolute recovery of IS was also evaluated in a similar way at the concentration used in sample analysis.

Stability

The stability of analytes was assessed by comparing the data of low and high QC samples (QC_1 and QC_3, respectively). Samples were analyzed before and after being exposed to the conditions for stability assessment. These conditions reflect the effect of sample storage and residence time in the autosampler before chromatographic analysis. A stability/reference samples ratio of 85–115% was accepted as stability criterion.

II.4.3. RESULTS AND DISCUSSION

The bioanalytical method in rat matrices was based on isocratic LC method developed and fully validated as described in *section II.2*. with a few modifications. In particular, the composition of the mobile phase was slightly adjusted to enable good resolution of LEV, NOR, CIP and LOM and avoid interfering peaks which were most commonly observed in rat plasma. Due to the fact that a different apparatus was used, the flow rate of the mobile phase was reduced to 1.0 mL/min. Moreover the emission wavelength was altered to 470 nm with the purpose of hindering the detection of interferents and improving the sensitivity to LEV.

Another important issue was the selection of IS; a number of different compounds were tested with respect to their fluorescence properties, co-elution with analytes and recovery. Notwithstanding its high retention time, GAT was chosen as the best candidate fulfilling the above conditions.

Concerning sample preparation, a common step for all matrices was protein precipitation with 20% (w/v) of trichloroacetic acid as precipitating agent in the same sample volume/precipitating agent volume proportion. In all matrices, a clear and transparent supernatant was obtained and easily aspirated without disturbance of the precipitate pellet. To quantify analytes in biological tissues, the solid samples must be disrupted by a mechanical/physical method for the complete liberation of analytes from all cellular structures. With this purpose, the homogenization of nasal mucosa and olfactory bulb (detailed in *section II.4.2.5.*) was accomplished using an Ultra-turrax homogenizer. This type of device has been successfully used by some authors to quantify drugs in nasal mucosa specimens (Gehanno et al., 2002; Kuehnel et al., 2005); the miniaturized version

used in this work was found to be an adequate motorized homogenizing device for the small size tissue samples herein studied. The use of phosphate buffer at pH 3.0 was a good option to enhance FQ solubility and to improve analyte extraction to the aqueous solvent providing also a first mild chemical extraction. Due to the small size of nasal mucosa and olfactory bulb tissues and the concomitant need of a reasonable volume of homogenization, a high dilution of the drug inevitably occurs in this step. The resultant supernatants were treated in a similar way to plasma matrix. This is supported by studies reported by Li and Bartlett (2014), where a highly diluted brain homogenate was prepared, undergoing a similar sample preparation to plasma. Spiking and addition of IS were carried out on the solid samples before homogenization to mimic real samples preparation. The major role of the IS is to compensate for the loss of the analytes during sample preparation and homogenization, the most liable step for this to occur.

II.4.3.1. Chromatographic Separation and Selectivity

Chromatographic separation of LEV, NOR, CIP, LOM and GAT (IS) was achieved under the chromatographic conditions described, with a retention time of approximately 22 min for the last-eluting compound. The absence of interference of endogenous compounds at the retention times of analytes and IS was confirmed for all rat biological matrices herein studied. Representative chromatograms of blank and spiked plasma and tissue matrices of Wistar rats are depicted in *Figure II.4.2.*. Pentobarbital that will be used in experimental protocols of *in vivo* pharmacokinetic studies was not detected and therefore did not interfere with the peaks of analytes and IS.

II.4.3.2. Linearity of Calibration Curves and LLOQs

The linearity of the analytical method was tested over the calibration range defined for each analyte. The calibration curves were subjected to a weighted linear regression analysis using the weighting factor of $1/x^2$. The regression equations and their coefficients of determination (r^2) are summarized in *Table II.4.1.*. The results show good linearity for all analytes ($r^2 > 0.9925$) in all rat matrices. The values of LLOQ set for LEV, NOR, CIP and LOM in rat plasma, nasal mucosa and olfactory bulb indicated in *Table II.4.2.* showed acceptable precision (CV ≤ 16.58%) and accuracy (bias ± 13.00%).

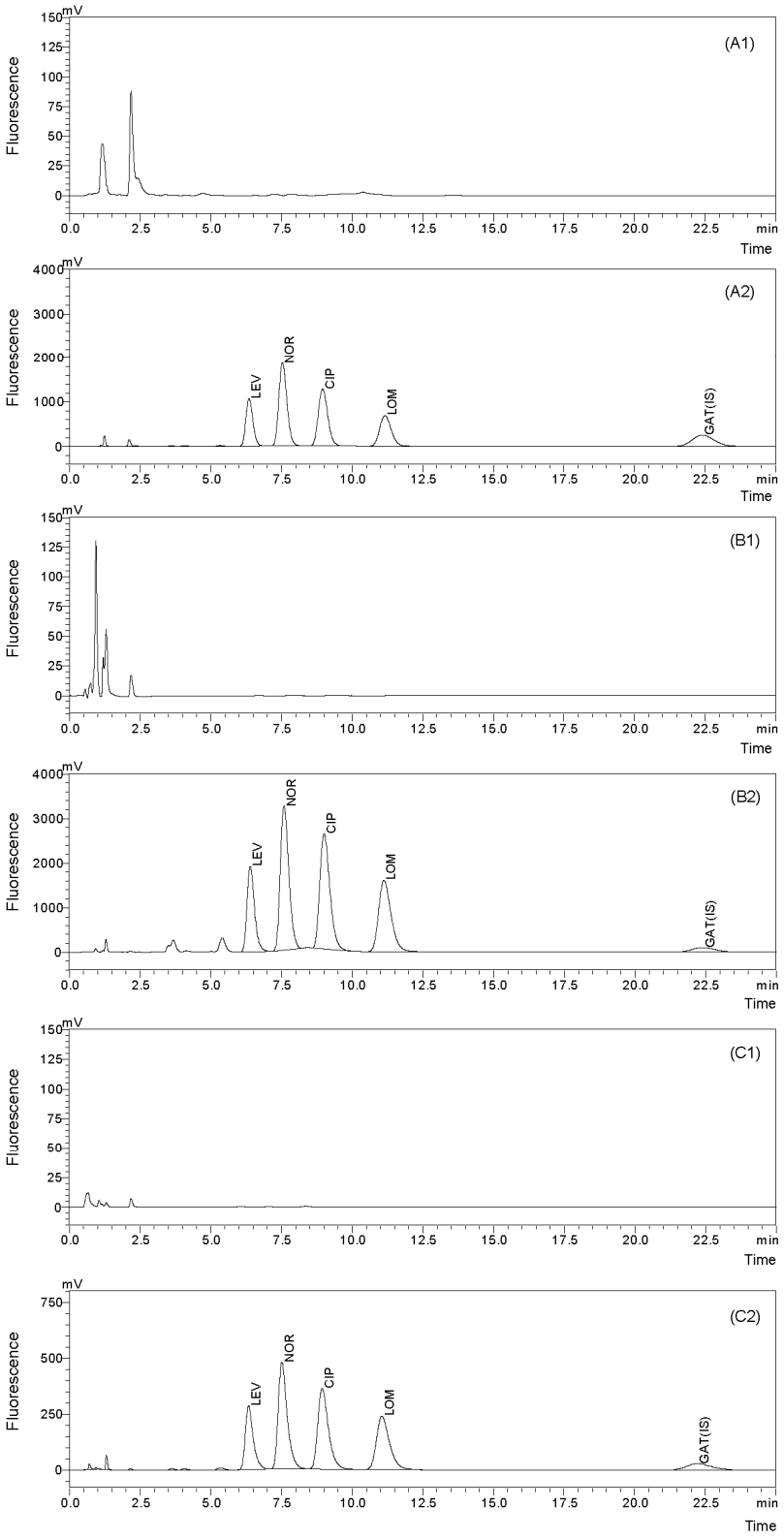

Figure II.4.2. Representative chromatograms of processed rat biological matrices: blank plasma sample (A1); plasma spiked with IS and analytes at the level of the upper limit of quantification of the calibration range (A2); blank nasal mucosa sample (B1); nasal mucosa spiked with IS and analytes at the level of the upper limit of quantification of the calibration range (B2); blank olfactory bulb sample (C1) and olfactory bulb spiked with IS and analytes at the level of the upper limit of quantification of the calibration range (C2).

Table II.4.1. Calibration curve parameters for levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) in rat plasma, nasal mucosa and olfactory bulb matrices (*n* = 3).

Analyte	Calibration Range (µg/mL)	Regression Equation	r^2
Plasma			
LEV	0.040–5.0	y = 0.275333x + 0.002563	0.9966
NOR	0.030–5.0	y = 0.553916x - 0.000709	0.9979
CIP	0.030–5.0	y = 0.445204x - 0.001606	0.9981
LOM	0.040–5.0	y = 0.295450x + 0.000424	0.9941
Analyte	**Calibration Range (ng)**	**Regression Equation**	r^2
Nasal Mucosa			
LEV	18.0–4000	y = 0.001708x + 0.006749	0.9971
NOR	12.5–4000	y = 0.003279x - 0.020387	0.9954
CIP	12.5–4000	y = 0.003069x + 0.011912	0.9961
LOM	18.0–4000	y = 0.002355x + 0.002631	0.9966
Olfactory Bulb			
LEV	18.0–500	y = 0.005796 x - 0.003754	0.9925
NOR	12.5–500	y = 0.011197x - 0.002960	0.9965
CIP	12.5–500	y = 0.010121 x + 0.005623	0.9964
LOM	18.0–500	y = 0.007879 x + 0.004647	0.9981

y represents analyte/IS peak area ratio; *x* represents analyte concentration (expressed in µg/mL for plasma or ng for nasal mucosa and olfactory bulb matrices).

Table II.4.2. Precision (% CV) and accuracy (% bias) for the determination of levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP), and lomefloxacin (LOM) in rat plasma, nasal mucosa and olfactory bulb matrices at the concentration of the lower limit of quantification (LLOQ) (*n* = 3).

Analyte	Nominal Value	Intraday			Interday		
		Measured Value[a]	CV (%)	Bias (%)	Measured Value[a]	CV (%)	Bias (%)
Plasma							
LEV	0.040	0.03480 ± 0.00394	8.75	-13.00	0.03782 ± 0.00730	15.49	-5.45
NOR	0.030	0.03134 ± 0.00158	5.18	4.47	0.02951 ± 0.00202	7.17	-1.62
CIP	0.030	0.03091 ± 0.00086	2.85	3.03	0.03034 ± 0.00443	16.58	1.13
LOM	0.040	0.03923 ± 0.00056	1.49	-1.93	0.03867 ± 0.00217	5.40	-3.32

Table II.4.2. *(Continued)*

Analyte	Nominal Value	Intraday			Interday		
		Measured Value[a]	CV (%)	Bias (%)	Measured Value[a]	CV (%)	Bias (%)
Nasal Mucosa							
LEV	18.0	16.55 ± 1.18	5.42	-8.05	17.84 ± 1.82	8.36	-0.90
NOR	12.5	10.91 ± 1.94	9.24	-12.76	12.8874 ± 2.43	12.74	3.10
CIP	12.5	10.98 ± 1.88	10.77	-12.17	13.91 ± 1.76	10.03	11.32
LOM	18.0	18.30 ± 2.75	13.08	1.69	19.78 ± 1.45	6.95	9.87
Olfactory Bulb							
LEV	18.0	18.22 ± 0.89	4.98	1.21	18.83 ± 1.56	8.57	4.62
NOR	12.5	13.15 ± 0.66	5.19	5.21	13.34 ± 1.10	8.41	6.75
CIP	12.5	12.84 ± 0.53	4.02	2.69	13.06 ± 1.05	7.70	4.49
LOM	18.0	17.61 ± 0.88	4.88	-2.15	18.37 ± 1.86	9.80	2.06

[a] Mean ± standard deviation, *n* = 3.

CV, coefficient of variation.

Nominal and measured values expressed in μg/mL for plasma matrix and in ng for nasal mucosa and olfactory bulb matrices.

II.4.3.3. Precision and Accuracy

The results shown in *Table II.4.3.* and *Table II.4.4.* correspond to the intra and interday precision and accuracy values determined at three different concentration levels (QC_1, QC_2 and QC_3) representative of the calibration range for all the analytes in rat plasma and tissue – nasal mucosa and olfactory bulb – samples, respectively. The intra and interday CV values were ≤ 9.71% and the intraday and interday bias values varied between -12.27% and 11.61% for all biological matrices and analytes. All these data fulfill the acceptance criteria of international guidelines. Therefore, the method developed for the determination of LEV, NOR, CIP and LOM in rat plasma, nasal mucosa and olfactory bulb is precise and accurate. In the particular case of rat plasma, the dilution effect of this matrix was evaluated with another QC sample (QC_{Dil} = 10 μg/mL). The intra and interday precision of the diluted plasma QC_{Dil} samples showed CV values lower or equal to 3.07% and for intra and interday accuracy bias ranged from -3.36% to -6.49% (*Table II.4.3.*). These results demonstrate that a 5-fold dilution with blank rat plasma did not affect precision and accuracy and could be applied to trial samples with analyte concentrations exceeding the upper limit of quantification (5 μg/mL).

Table II.4.3. Precision (% CV) and accuracy (% bias) for the determination of levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP), and lomefloxacin (LOM) in rat plasma at the low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration ranges and following a sample dilution (*) by a 5-fold factor (*n* = 3).

Analyte	Nominal Value	Intraday			Interday		
		Measured Value[a]	CV (%)	Bias (%)	Measured Value[a]	CV (%)	Bias (%)
Plasma							
LEV	0.12	0.11363 ± 0.00337	2.72	-5.31	0.12183 ± 0.00937	7.14	1.53
	2.5	2.49901 ± 0.03411	1.36	-0.04	2.47552 ± 0.04358	1.75	-0.98
	4.5	4.40331 ± 0.11017	2.50	-2.15	4.36841 ± 0.10447	2.05	-2.94
	10*	9.66398 ± 0.30753	3.18	-3.36	9.62012 ± 0.18880	1.96	-3.80
NOR	0.09	0.09412 ± 0.00201	5.18	4.58	0.09632 ± 0.00328	3.45	7.02
	2.5	2.44454 ± 0.02569	1.05	-2.22	2.45453 ± 0.03712	1.51	-1.82
	4.5	4.44424 ± 0.11235	2.53	-1.24	4.36615 ± 0.11827	2.06	-2.97
	10*	9.35107 ± 0.26861	2.87	-6.49	9.44815 ± 0.14743	1.56	-5.52
CIP	0.09	0.09254 ± 0.00341	3.72	2.82	0.09326 ± 0.00586	6.54	3.62
	2.5	2.51409 ± 0.03101	1.23	0.56	2.49126 ± 0.04942	1.99	-0.35
	4.5	4.39820 ± 0.11271	2.57	-2.26	4.39972 ± 0.10597	2.07	-2.23
	10*	9.49568 ± 0.29193	3.07	-5.04	9.46351 ± 0.21419	2.26	-5.36
LOM	0.12	0.12160 ± 0.00364	3.03	-1.33	0.12527 ± 0.00374	2.95	4.40
	2.5	2.47744 ± 0.02352	0.95	-0.90	2.44992 ± 0.02242	0.91	-2.00
	4.5	4.40074 ± 0.10962	2.49	-2.21	4.35853 ± 0.12076	1.53	-3.14
	10*	9.48810 ± 0.28461	3.00	-5.12	9.42002 ± 0.13421	1.42	-5.80

[a] Mean ± standard deviation, *n* = 3.

CV, coefficient of variation.

Nominal and measured values expressed in µg/mL.

Table II.4.4. Precision (% CV) and accuracy (% bias) for the determination of levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP), and lomefloxacin (LOM) in rat nasal mucosa and olfactory bulb matrices at the low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration ranges (*n* = 3).

Analyte	Nominal Value	Intra day			Inter day		
		Measured Value[a]	CV (%)	Bias (%)	Measured Value[a]	CV (%)	Bias (%)
Nasal Mucosa							
LEV	54.0	52.55 ± 3.37	5.83	-2.69	53.21 ± 2.87	5.02	-1.47
	2000	2232.22 ± 196.39	8.78	11.61	2105.60 ± 39.81	1.89	5.28
	3600	3218.78 ± 69.85	2.17	-10.59	3348.10 ± 303.45	2.46	-7.00
NOR	37.5	33.06 ± 2.80	6.50	-11.84	35.42 ± 2.97	7.14	-5.55
	2000	2236.65 ± 204.94	9.14	11.83	2117.35 ± 75.38	3.55	5.87
	3600	3158.36 ± 121.62	3.85	-12.27	3315.95 ± 393.76	3.66	-7.89
CIP	37.5	34.30 ± 2.42	5.94	-8.54	35.98 ± 2.75	6.93	-4.05
	2000	2192.45 ± 213.22	9.71	9.62	2090.93 ± 70.18	3.35	4.55
	3600	3268.42 ± 124.06	3.79	-9.21	3453.85 ± 428.75	2.33	-4.06
LOM	54.0	49.65 ± 2.20	4.20	-8.05	51.07 ± 3.71	7.11	-5.43
	2000	2189.33 ± 189.34	8.66	9.22	2070.07 ± 65.96	3.18	3.50
	3600	3260.50 ± 72.00	2.21	-9.43	3395.68 ± 328.82	1.62	-5.68
Olfactory Bulb							
LEV	54.0	51.83 ± 2.11	4.10	-4.03	53.44 ± 1.01	1.92	-1.04
	250	265.64 ± 2.27	0.85	6.28	267.63 ± 22.98	8.61	7.05
	450	440.00 ± 11.95	2.72	-2.22	449.43 ± 11.31	3.45	-0.13
NOR	37.5	39.25 ± 1.52	3.90	4.67	40.40 ± 0.54	1.35	7.73
	250	257.45 ± 1.86	0.72	3.00	259.37 ± 21.61	8.34	3.75
	450	431.78 ± 13.50	3.13	-4.05	446.03 ± 15.59	4.29	-0.88
CIP	37.5	36.97 ± 1.55	4.14	-1.42	37.92 ± 0.80	2.09	1.11
	250	263.61 ± 1.88	0.71	5.44	265.01 ± 21.67	8.16	6.00
	450	426.98 ± 12.78	2.99	-5.12	441.31 ± 16.46	4.58	-1.93
LOM	54.0	55.00 ± 2.06	3.70	1.86	57.24 ± 0.19	0.32	6.00
	250	259.62 ± 2.15	0.83	3.85	261.46 ± 23.30	8.89	4.59
	450	436.90 ± 12.04	2.76	-2.91	447.20 ± 9.75	3.04	-0.62

[a] Mean ± standard deviation, *n* = 3.

CV, coefficient of variation.

Nominal and measured values expressed in ng.

II.4.3.4. Recovery

Mean absolute recoveries of LEV, NOR, CIP and LOM from rat plasma, nasal mucosa and olfactory bulb samples at the concentration levels tested ranged from 51.47 to 67.78%, 65.26 to 80.64% and 63.13 to 86.29%, respectively, and showed low CV values (≤ 8.92% (*Table II.4.5.*). The extent of recovery of each analyte was consistent over the representative concentrations within the calibration range (*n* = 9) in all matrices with overall CV values lower than 5.19% in plasma, 5.22% in nasal mucosa and 2.58% in olfactory bulb. In the case of IS, absolute mean recovery values of 50.71% in plasma, 82.22% in nasal mucosa and 88.13% in olfactory bulb were obtained, exhibiting CV values lower than 7.05%. The results obtained for CV (presented in the text above and in *Table II.4.5.*) show that sample preparation procedures are precise and reproducible in extraction of the analyte from the biological matrices and fulfill the requirements of international guidelines. For each of the four analytes the extraction efficiency evidences a clear trend to increase from plasma to olfactory bulb samples. Despite the moderate extraction efficiency of analytes and IS in some matrices, this did not affect linearity, accuracy and precision of the method.

II.4.3.5. Stability

The results of stability reveal that no degradation occurred for all analytes in unprocessed samples (plasma and tissue homogenate supernatant samples) stored at -24 °C during 30 days. The analytes also demonstrated to be stable in processed samples at room temperature for 8 h, testing the effect of time of the sample in the autosampler before injection. Stability data are reported in *Table II.4.6.*.

Table II.4.5. Absolute recovery (%) of levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) from rat plasma samples and from olfactory bulb and nasal mucosa tissue samples at the low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration ranges.

Analyte	Nominal Value[a]	n	Absolute Recovery (%)	
			Mean ± SD	CV (%)
Plasma				
LEV	0.12	3	61.63 ± 1.56	2.53
	2.5	3	67.78 ± 0.55	0.81
	4.5	3	67.22 ± 0.78	1.16
NOR	0.09	3	61.74 ± 1.00	1.61
	2.5	3	64.87 ± 0.70	1.07
	4.5	3	64.60 ± 0.56	0.86
CIP	0.09	3	55.89 ± 1.80	3.27
	2.5	3	57.60 ± 0.59	1.02
	4.5	3	57.72 ± 0.46	0.79
LOM	0.12	3	51.47 ± 1.32	2.57
	2.5	3	56.73 ± 0.79	1.39
	4.5	3	57.17 ± 0.45	0.78
Nasal Mucosa				
LEV	54	3	75.15 ± 6.70	8.92
	2000	3	78.75 ± 1.10	1.40
	3600	3	79.57 ± 1.81	2.28
NOR	37.5	3	70.58 ± 4.14	5.86
	2000	3	68.89 ± 1.91	1.91
	3600	3	68.90 ± 4.42	4.42
CIP	37.5	3	65.26 ± 3.62	5.55
	2000	3	66.09 ± 1.51	2.28
	3600	3	66.61 ± 2.56	3.84
LOM	54	3	75.26 ± 3.91	5.19
	2000	3	80.64 ± 1.03	1.28
	3600	3	80.36 ± 2.00	2.49
Brain (Olfactory Bulb)				
LEV	54	3	85.46 ± 1.86	2.18
	250	3	84.81 ± 2.80	3.31
	450	3	86.29 ± 2.16	2.50
NOR	37.5	3	74.46 ± 1.61	2.16
	250	3	75.59 ± 2.43	3.21
	450	3	75.74 ± 1.80	2.37
CIP	37.5	3	73.13 ± 1.89	2.59
	250	3	73.70 ± 2.38	3.22
	450	3	73.97 ± 1.71	2.31
LOM	54	3	83.76 ± 1.56	1.86
	250	3	84.17 ± 2.81	3.34
	450	3	86.04 ± 2.07	2.40

Nominal values expressed in µg/mL for plasma matrix and in ng for nasal mucosa and olfactory bulb matrices.

Table II.4.6. Stability (%) of levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP) and lomefloxacin (LOM) in unprocessed rat plasma samples and in supernatant of extracted rat tissue homogenates left at 4 °C for 30 days and in final processed rat plasma and tissue samples left at room temperature for 8 h.

Analyte	LEV		NOR		CIP		LOM	
	Nominal Val.[a]		Nominal Val.[a]		Nominal Val.[a]		Nominal Val.[a]	
Plasma (*n* = 5)	**0.12**	**4.5**	**0.09**	**4.5**	**0.09**	**4.5**	**0.12**	**4.5**
Unprocessed plasma								
-24 °C (30 days)	92.8	92.1	94.1	92.3	98.2	91.3	89.5	89.8
Processed plasma								
Room temperature (8h)	99.7	100.1	101.4	100.1	104.0	100.3	99.1	100.0
Nasal Mucosa (*n* = 3)	**54**	**3600**	**37.5**	**3600**	**37.5**	**3600**	**54**	**3600**
Homogenate Supernatant								
-24 °C (30 days)	101.6	100.1	98.1	102.1	97.7	107.0	98.3	102.8
Processed Homogenate Supernatant								
Room temperature (8h)	100.6	102.4	100.4	103.9	100.8	100.9	100.8	102.2
Olfactory Bulb (*n* = 3)	**54**	**450**	**37.5**	**450**	**37.5**	**450**	**54**	**450**
Homogenate Supernatant								
-24 °C (30 days)	100.6	99.3	98.4	97.6	98.3	97.4	100.1	97.7
Processed Homogenate Supernatant								
Room temperature (8h)	99.8	100.0	99.7	99.6	99.5	100.0	100.1	99.9

[a] Nominal values in bold and expressed in *μg/mL* for plasma matrix and in *ng* for nasal mucosa and brain (olfactory bulb) matrices.

The bioanalytical method was partially validated for rat tissue samples with a mass of 40 mg. Although ignoring at this stage the exact weight of future samples collected from the *in vivo* pharmacokinetic studies, it was found that the bioanalytical method is also precise and accurate at QC_1, QC_2 and QC_3 levels using 20 mg and 60 mg of nasal mucosa and 30 and 60 mg of olfactory bulb (minimum and maximum values that would presumably be collected). Therefore, the bioanalytical method can be applied within these limits, because the weight did not affect the performance of the method with respect to the above mentioned parameters.

II.4.4. CONCLUSION

The HPLC method herein presented was successfully employed for the quantitative determination of LEV, NOR, CIP and LOM in rat matrices: plasma, nasal mucosa and olfactory bulb. It was based on the isocratic LC method developed and fully validated for

human plasma samples (*section II.2.).* The modifications that were introduced justified a partial validation. The results demonstrated that the bioanalytical method was linear over the calibration range, accurate, precise and selective in all rat matrices, fulfilling the international guidelines requirements. The method has proven to be suitable to reliably quantify LEV, NOR, CIP and LOM in rat plasma, nasal mucosa and olfactory bulb samples and can be applied in the bioanalysis of samples, supporting subsequent *in vivo* pharmacokinetic studies.

CHAPTER III

IN VIVO PHARMACOKINETIC STUDIES: INTRANASAL DELIVERY OF CIPROFLOXACIN AND LEVOFLOXACIN

III.1. PRELIMINARY STUDIES

III.1.1. EXPERIMENTAL CONSIDERATIONS

Rodents, particularly rats, are the most widely used animals for IN drug delivery *in vivo* experiments (Illum, 1996; Wong and Zuo, 2013). Different IN methodologies have been used in animal studies that can significantly influence the results and must be taken into account in their interpretation. In fact, various aspects such as delivery volume, body position, drug formulation, anesthesia and delivery devices are determinant in the deposition and distribution of drugs in the nasal cavity (Gizurarson et al., 2006; Illum, 1996; Kuboyama et al., 1996; Southam et al., 2002).

In this work, adult male Wistar rats were used and several approaches for IN administration were attempted in preliminary experiments. The most natural form of IN administration would be to place drops by a micropipette at the entrance of the nose (opening of nostrils), allowing the awaken animal to sniff them into the nasal cavity. This procedure was tested using a normal saline solution (0.9% sodium chloride). However, due to the difficulty in keeping the animals unstressed and steady in the same position during administration, some loss of the solution administered was observed around rat snout; consequently, the exact amount delivered into nasal tissue could not be known with the desired accuracy and precision. Thus this strategy was abandoned and rats had to be anesthetized; although this does not mimic the real conditions, the majority of preclinical published work has been carried out in anesthetized rodents (Dhuria et al., 2010; Turner et al., 2011).

Taking advantage of the ability of sprays to achieve a more uniform and widespread distribution and to access difficult-to-reach areas within the nose, simple solutions were delivered in the interior of the nasal cavity of anesthetized rats with an atomizer needle attached to a pressurized syringe to generate an aerosol (Penn-Century MicroSprayer Aerosolizer® Model IA-IC & FMJ-250 High Pressure Syringe, *Figure III.1.1.*).

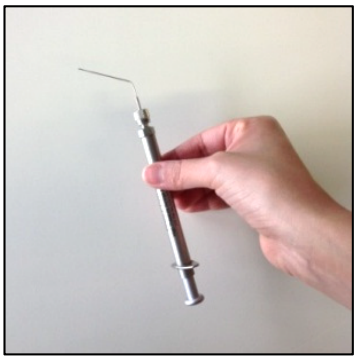

Figure III.1.1. Penn-Century MicroSprayer Aerosolizer® Model IA-IC & FMJ-250 High Pressure Syringe. Designed for precise administration of aerosol doses from 25 µL to 250 µL, enabling accurate measurements of 25 µL or 50 µL or combinations of these volumes.

Rats were placed in recumbent lateral position, as this was found to be the simplest way to precisely insert the needle into the nasal cavity and manipulate the syringe for administration. A set of tests were carried out under these conditions:

(i) When a volume of 50 µL of saline solution was administered bilaterally through each nostril by inserting 1.5 cm of the needle, suffocation occurred and the rat died a few minutes after IN administration. This procedure was repeated inserting the needle 1.0 cm into each nostril in order to increase the nasal airway volume available for the same injected volume and thus avoid rat suffocation. However, a similar outcome was observed.

(ii) The next step was the reduction of the volume of IN administration to 25 µL and unilateral delivery through the right nostril at 1.0 cm depth. Notwithstanding these most favorable conditions, the rat showed respiratory distress and did not survive longer than 10 minutes after IN administration.

After animals death, tests were performed by injecting a die (Trypan Blue) to observe the nasal area exposed to the aerosol right after administration, as depicted in *Figure III.1.2.*.

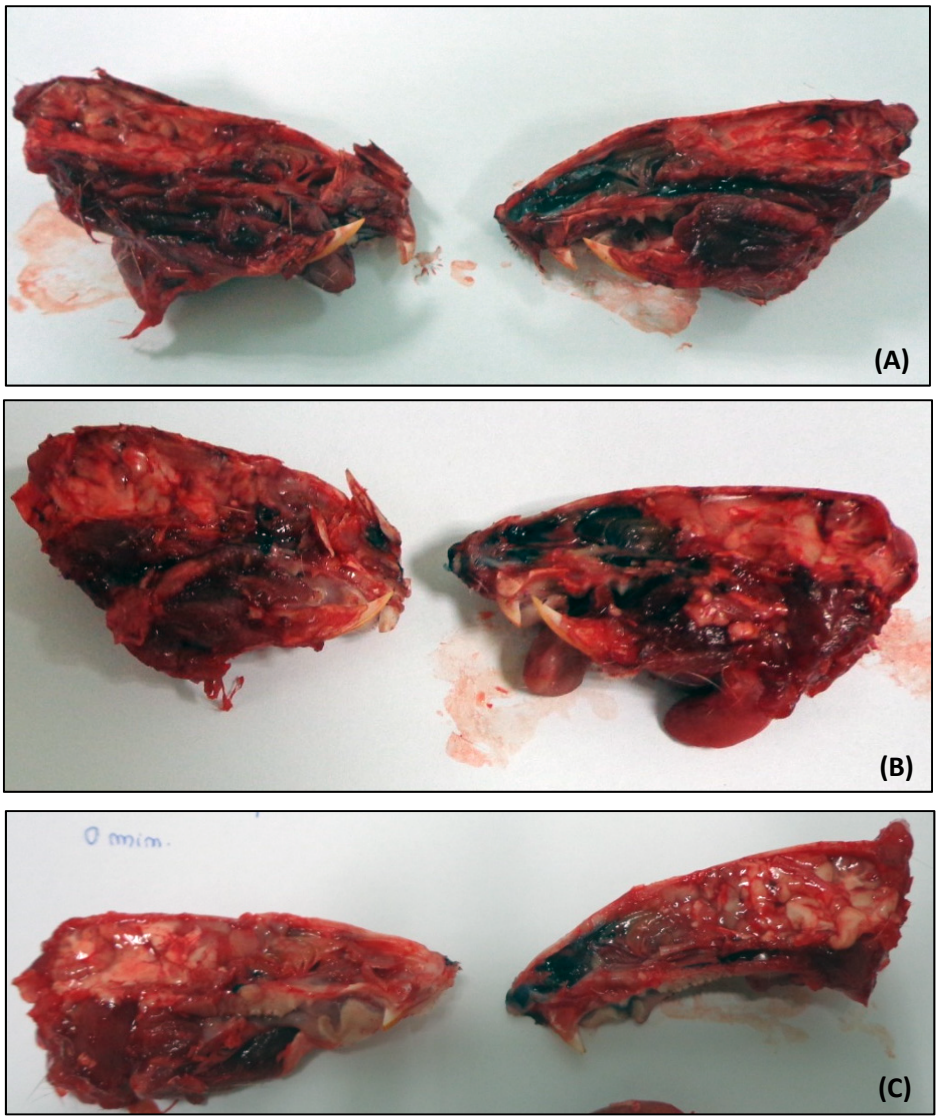

Figure III.1.2. Aerosol deposition in nasal cavity of Wistar rats. (A) Injected volume of Trypan Blue: 50 µL in right nostril; Insertion length: 1.5 cm; t = 0 min; (B) Injected volume of Trypan Blue: 50 µL in right nostril; Insertion length: 1.0 cm; t = 0 min; (C) Injected volume of Trypan Blue: 25 µL in right nostril; Insertion length: 1.0 cm; t = 0 min.

(iii) Following this negative outcome and in the absence of alternative approaches using the aerosolizing system, an attempt was made to understand the deposition and distribution of the aqueous solution in the respiratory tract. The procedure was repeated with another animal by administration of 25 µL of Trypan Blue delivered unilaterally through the right nostril at 1.0 cm depth and it was found that 10 minutes after administration (time of death) the colored aqueous solution had already reached the nasopharynx, pointing also to a short residence time of the aerosolized aqueous solution (*Figure III.1.3.*).

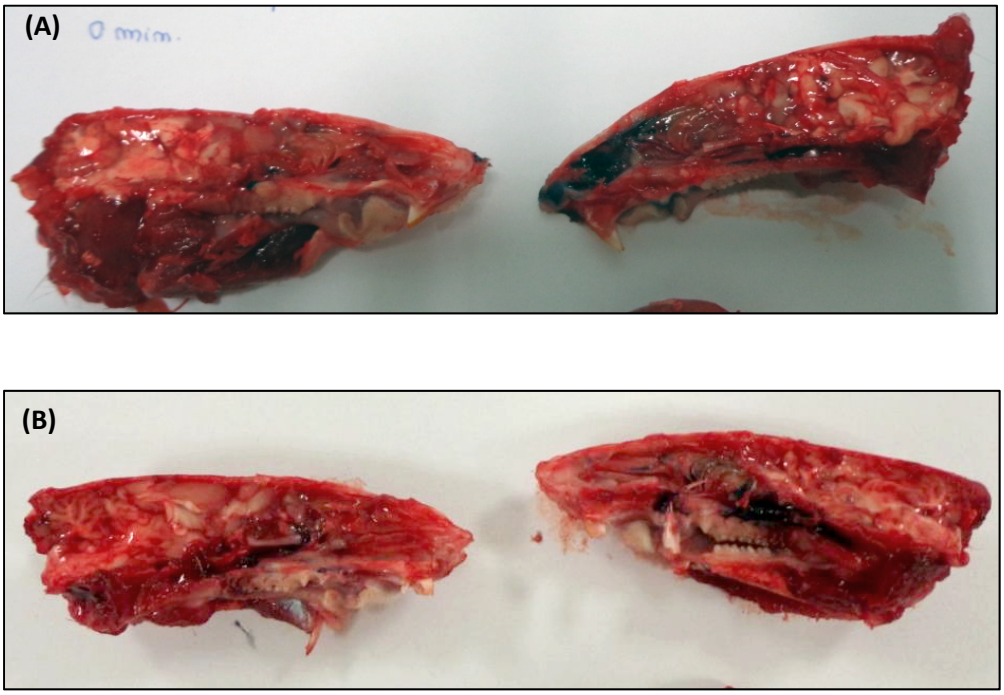

Figure III.1.3. Aerosol deposition and distribution pattern in nasal cavity of Wistar rats. (A) Injected volume of Trypan Blue: 25 µL in right nostril; Insertion length: 1.0 cm; t = 0 min (B) Injected volume of Trypan Blue: 25 µL in right nostril; Insertion length: 1.0 cm; t = 10 min (time of death).

(iv) This suggested that the rapid progression of the aqueous solution to the nasopharynx may cause the occlusion of respiratory airway and subsequent dyspnea. Therefore, this conducted to the use of 25 µL of more viscous solutions liable to be aerosolized, namely 30% (v/v) of propilenoglycol and 0.2% (w/v) of carbopol 974P. In the first case the rat died 30 minutes after aerosolization and in the second case the rat survived, but after 30 minutes the viscous solution was already near the nasopharynx when testing with Trypan Blue die incorporated in 0.2% (w/v) of carbopol 974P.

The fact that animals died together with the reduced residence time of the drug formulation in nasal cavity, showed that this procedure was not viable for *in vivo* pharmacokinetic studies and a different administration technique should be followed. Based on pre-existent experience in the research group the use of a thermoreversible *in situ* gel appeared to be the best solution (*Figure III.1.4.*).

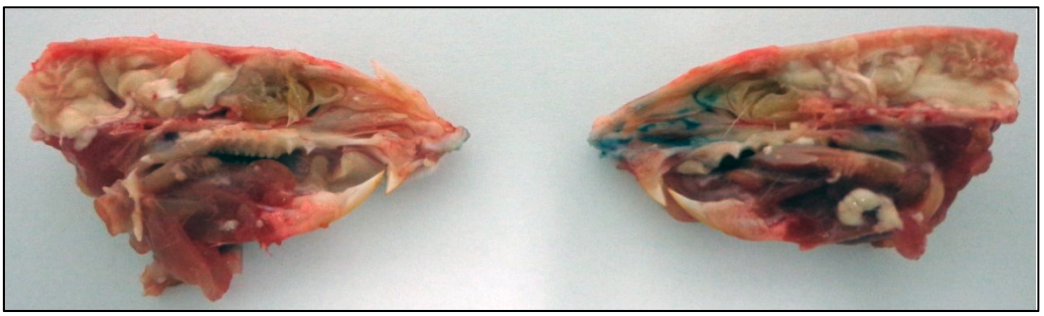

Figure III.1.4. Wistar rat nasal cavity exposure to *in situ* thermoreversible mucoadhesive gel composed of 18.0% (w/v) pluronic F-127 and 0.2% (w/v) carbopol 974P and used to incorporate 10% (w/w) of Trypan Blue die. Injection volume of colored *in situ* gel in right nostril; t = 30 min.

The administration of the thermoreversible *in situ* gel was performed by introducing 1.0 cm of a flexible polyurethane tube attached to a microliter syringe inside the nasal cavity with rats in recumbent lateral position whose convenience was mentioned above. Moreover the *in situ* gel can be more firmly delivered than the aerosolized solution, since in this case the force that must be rapidly applied to create the aerosol may cause slight random movements that hinder a steady administration into the nasal cavity. After the IN administration rats were kept in the lateral recumbent position to facilitate normal breathing of the anesthetized animals (with partially occluded nostrils by the gel) which would not be favored in the "natural" prone position.

Semi-solid dosage forms, such as gels, reduce anterior leakage and enhance the drug nasal residence time by preventing a rapid MCC, thus maintaining the drug formulation in nasal mucosa over a longer period of time than solutions. *In situ* gels have recently attracted great attention as favorable delivery systems due to their ability to undergo transition into a gel just at the delivery site. They present the advantage of easy handling as a liquid form during administration and high viscosity after delivery at the site of interest. Using the know-how of the research group on the development of *in situ* gels with sol-gel transition induced by nasal physiological temperature, a preliminary attempt was made to use pluronic F-127 (PF-127) and carbopol 974P as polymer components of the thermoreversible *in situ* gel.

The preparation of the drug-free thermoreversible *in situ* gels was made using the method described by Schmolka (1972) and detailed in the next sections (*III.2. and III.3.*).

The most economic and easiest way to incorporate the drug into the thermoreversible *in situ* gel was to dissolve it in a concentrated stock solution prior to incorporation. The water solubility of some FQs is too small to achieve an appropriate concentration for a stock solution; for example, water solubility of CIP is 0.09 mg/mL and 0.07 mg/mL at pH 6.8 and 7.5, respectively (Breda et al., 2009; Caço et al., 2008), while for LEV a much higher water solubility at similar pHs is reported (50 mg/mL) (Frick et al., 1998; Kassab et al., 2010). Since FQs are known to have pH dependent solubility, increasing as pH diverges from the neutral value, an acidic solvent – 1% (v/v) of fuming hydrochloric acid (37%) – was used to prepare a 30 mg/mL stock solution for both FQs (CIP and LEV) investigated in the present work. This was dictated by the maximum possible concentration of (the less soluble) CIP in the acidic solution to achieve complete solubilization.

The next step consisted in incorporating this stock solution in the thermoreversible *in situ* gel, in such volume proportions that a 10-fold dilution of CIP or LEV was attained. This final concentration of 3 mg/mL is consistent with that reported in literature (0.3% w/v or w/w) for several *in situ* ophthalmic gels containing PF-127 that are being designed for topical delivery of some FQs (Abdelkader and Mansour, 2015; Ahmed et al., 2014; Mansour et al., 2008; Nesseem, 2011; Sawant et al., 2016; Varshosaz et al., 2008).

The same nasal *in situ* gel prepared by the in-house research team (Serralheiro et al., 2015, 2014) – 18% (w/v) PF-127 and 0.2% (w/v) carbopol 974P – was used to incorporate CIP and LEV stock solutions. In the first case, a turbid white fluid with dispersed particles was obtained, while in the second an opaque yellowish fluid was observed. Therefore it was decided to use a simpler *in situ* gel and the option was to remove the carbopol 974P and maintain the PF-127 responsible for thermosensitive properties of the *in situ* gel, since this was the main objective to achieve for the formulation. To compensate for the absence of carbopol 974P and the consequent effect on viscosity (and also on the gelation temperature of PF-127), the starting percentage of PF-127 was increased from 18% to 20% (w/v). Both CIP and LEV revealed to be soluble after incorporation of their stock solutions into 20% (w/v) PF-127. This solubility persisted after adjustment of pH with minor amounts of 0.5 M sodium hydroxide, an important step to avoid nasal irritation, by using a pH formulation within the range of 4.5-6.5 (Alagusundaram et al., 2010; Chand et al., 2010).

The percentage of PF-127 was optimized to ensure gelation of the thermoreversible gel at nasal physiological temperature. Three different concentrations of PF-127, namely 20%, 22% and 24% (w/v) were tested. To mimic the incorporation of the drug, the same volume of the plain acidic solvent used for drug stock solution was added to the prepared 20%, 22% and 24% (w/v) PF-127 gels under investigation, leading to PF-127 final concentrations of 18%, 19.8% and 21.6% (w/v; prior to the subsequent adjustment of pH), respectively. Gelation temperatures were measured by visual inspection according to the test tube inverting method described by Gilbert et al. (1987) and Mansour et al. (2008) as will be detailed in *section III.2.2.3.*. A starting 24% (w/v) PF-127 was selected as it led to a final concentration of 21.6% (w/v) with a gelation temperature lower than the nasal physiological temperature (i.e. 34 °C). As a result of the optimization procedure, a 24% (w/v) PF-127 gel was used to incorporate CIP or LEV and thus prepare the thermoreversible *in situ* gel to be used as the IN formulation in the *in vivo* studies, with the final PF-127 and drug concentration of 21.6% (w/v) and 3 mg/mL, respectively, and pH subsequently adjusted to 5.3-5.5 (*Figure III.1.5.*).

Figure III.1.5. Thermoreversible *in situ* gel for intranasal administration. Example of ciprofloxacin intranasal formulation used in the *in vivo* pharmacokinetic study.

For the *in vivo* pharmacokinetic studies subsequently conducted, Wistar rats received the IN formulation bilaterally to obtain equal access of both nasal cavity halves to the drug enhancing the surface contact area. The total volume administered was adjusted to the weight of each animal to achieve a constant volume per weight (80 µL/kg), since there is evidence of a correlation between rats body weight and nasal cavity volume in rats 5 to 14 weeks old (Kuboyama et al., 1996).

Intranasal delivery studies reported in literature for drug pharmacokinetic evaluation are performed in animal models, particularly rats (Illum, 1996; Wong and Zuo, 2013). Most of them are focused on drug systemic delivery via nasal absorption and more recently on the assessment of a direct nose-to-brain transport. To date, few studies have quantified drugs locally in nasal mucosa after nasal application, which is the main aim of the present work. Before proceeding with nasal mucosa drug quantification, it is necessary to remove the *in situ* thermoreversible gel. As a first attempt, the excised nasal mucosa was submitted to a lavage with 5 mL of a normal saline solution (0.9% sodium chloride) in a Petri dish with manual agitation; this caused some loss of the fragile nasal tissue and favored drug diffusion mechanisms. Therefore, an alternative procedure was adopted consisting of a nasal lavage in the intact rat nose after decapitation. The volume used to flush the nose of rats (20 mL) was 50 times larger than the rat nasal volume (0.4 mL) (Gizurarson, 1993; Kaur and Kim, 2008); this has the advantage of applying an internal pressure which helps to remove the formulation without damaging the tissue.

III.1.2. BRIEF INTERSPECIES COMPARISON

Anatomical differences between animal species and humans are another aspect to consider in the interpretation of *in vivo* non-clinical studies, since they also influence drug deposition and distribution of IN drug formulations. The diversity among animal species can complicate the comparison of results and in particular their extrapolation to the human situation. Commonly used animals are rats, mice, rabbits, sheep, dogs and monkeys and some of their anatomical and physiological differences are listed in *Table III.1.1.* (Harkema et al., 2006; Ugwoke et al., 2001).

Table III.1.1. Interspecies comparison of nasal cavity characteristics (Gizurarson, 1990; Illum, 1996; Ugwoke et al., 2001).

Animal	Weight (kg)	Length (cm)	Surface Area (cm^2)	Volume (mL)	Turbinate Complexity	MCC time (min)
Man	70	7.5	150-160	15-20	Simple scroll	15-20
Mouse	0.03	0.5	2.8	0.03	Complex scroll	1
Rat	0.25	2.3	14.0	0.4	Complex scroll	5-10
Sheep	60	18	327.0	114	Double scroll	42
Rabbit	3	5.2	61.0	6	Complex scroll	10
Dogs	10	10	221.0	20	Complex scroll	20
Monkeys	7	5.3	62.0	8	Simple scroll	10

MCC, mucociliary clearance

In the context of this thesis, a comparison between human and rat noses should be kept in mind. Humans have a relatively simple nose with breathing as the primary function, while rats have more complex noses with olfaction as the primary function. Major anatomical differences are found in the number and shape of nasal turbinates. The human nose has three simple shaped turbinates in each half (*Chapter I, section I.2.1.1.*), whereas these structures are more complex in rats, with folding and branching patterns and different spatial organization within the nasal cavity. In the anterior part of each half of the main chamber of rats' nose, a nasoturbinate and a maxilloturbinate can be found, while six ethmoid turbinates are located in the posterior region (*Figure III.1.6.*). The absolute surface of rats' nasal cavity is much smaller than in humans; however, due to the greater complexity of rats' nasal turbinates, relative nasal surface area (nasal surface area/nasal volume) is approximately 4 times higher than in humans (*Tabel III.1.1.*). Moreover, the impact on drug systemic exposure is greater in rats, as shown by the comparison of nasal surface area/body weight. Another anatomical difference concerns the nasal septum that in rats is incomplete in its ventral posterior portion enabling free communication between the two halves of the nasal cavity (Harkema, 2015; Harkema et al., 2006; Illum, 1996; Jacob and Chole, 2006; Mery et al., 1994).

Histologically, humans and rats have similar types of nasal epithelium with minor differences in constituent cells. Major differences occur in the regional distribution of squamous, respiratory and olfactory epithelia in both species (*Figure III.1.6.*). The relative proportions of the total surface area of the nasal cavity they cover are 3%, 47% and 50%, respectively, which reflects the importance of olfaction in rats. These proportions can be compared with the corresponding ones for humans of 0.4%, 89.3% and 10.3%,

respectively, with an obvious predominance of the respiratory epithelium. A common histological feature of both species is the presence of a transitional epithelium between squamous and respiratory epithelia and of a similar mucociliary apparatus. Moreover, a physiological nasal cycle resembling that of humans is also found in rats, with rhythmic alternations in tonus of nasal mucosa (Bitter et al., 2011; Bojsen-Möller and Fahrenkrug, 1971; Harkema, 1990; Ugwoke et al., 2001).

Although true paranasal sinuses enclosed by skull and facial bones do not exist in rats as they do in humans, internal cavities shaped by folded turbinates are present, particularly in the posterior portion of the main chamber; this is the case of maxillary and ethmoid recesses (Phillips et al., 2009). For this reason, in the *in vivo* studies described in *sections III.2.* and *III.3.,* the mucosa harvested from two different regions – anterior and posterior – shown in *Figure III.1.6.*, was named as "nasal mucosa", instead of "sinonasal mucosa".

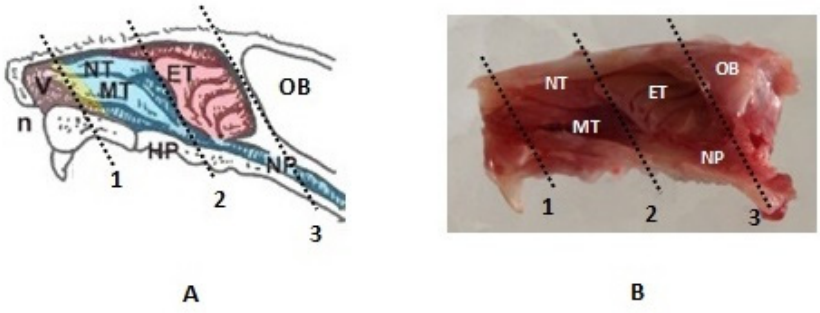

Figure III.1.6. Schematic representation of the right nasal passage of a rodent (A) and the corresponding image obtained during the dissection procedure of a Wistar rat nose in the *in vivo* pharmacokinetic studies (B). Pink, yellow, blue, and red represent the squamous, transitional, respiratory and olfactory epithelium, respectively. 1–2, anterior nasal region; 2–3, posterior nasal region. ET, ethmoid turbinates; HP, hard palate; MT, maxilloturbinate; n, nostril; NP, nasopharynx; NT, nasoturbinate; OB, olfactory bulb; V, nasal vestibule (A: adapted from Harkema, 2015).

III.2. INTRANASAL DELIVERY OF CIPROFLOXACIN TO RATS: A TOPICAL APPROACH USING A THERMOREVERSIBLE *IN SITU* GEL

III.2.1. INTRODUCTION

Ciprofloxacin, a second-generation FQ, is a synthetic antibiotic used in the treatment of a variety of bacterial infections particularly those of the respiratory, genitourinary and gastrointestinal tracts (Emmerson and Jones, 2003; Oliphant and Green, 2002; Sousa et al., 2011). Amongst respiratory diseases, CRS continues to be a significant medical problem without any specific medication globally approved. Several clinical studies have been conducted using FQs including CIP to evaluate medical responses (Comstock et al., 2010; Vaughan, 2004).

Chronic rhinosinusitis is a persistent symptomatic inflammation of the mucosa of the nasal cavity and paranasal sinuses, affecting ca. 10% of adult European population. Not only it represents a considerable economical burden in society due to direct costs in health service and indirect costs in productivity, but it also has a significant impact in patients' quality of life (Ramakrishnan et al., 2015; Suh and Kennedy, 2011). Although the exact etiology and pathophysiology of CRS are still unclear, the theory of bacterial biofilms and its contribution to the recalcitrant and persistent nature of CRS are gaining prominence. Several studies have confirmed the presence of bacterial biofilms in the sinonasal mucosa of patients with CRS and some of the bacteria identified include *S. pneumonia*, *H. influenza*, *S. aureus* and *P. aeruginosa*, the latter two being the most prevalent in refractory CRS patients (Al-Mutairi and Kilty, 2011; Bendouah et al., 2006; Kilty and Desrosiers, 2008; Psaltis et al., 2008; Schwartz et al., 2016; Serralheiro et al., 2013; Suh and Kennedy, 2011). Current American and European guidelines for the management of CRS recommend the use of topical corticosteroids and broad-spectrum or culture-directed oral antibiotics. Only patients who do not respond to these pharmacological therapies should be submitted to endoscopic sinus surgery (Fokkens et al., 2012; Rosenfeld et al., 2015; Schwartz et al., 2016). Studies have shown that bacterial biofilms are as high as 1000 times less susceptible to antibiotics than their corresponding planktonic form, which explains the lack of effectiveness of oral antibiotics in this clinical condition (Comstock et al., 2010;

Desrosiers et al., 2007; Ramakrishnan et al., 2015). Increasing the concentration of antibiotics, especially those with concentration-dependent antibacterial activity such as FQs, would overcome this drawback. However, high concentrations at the infected site are difficult to attain by traditional routes of administration (e.g. oral) without significant risks of systemic toxicity. Topical administration appears as an alternative approach to deliver high concentrations of antibiotics directly to the site of infection, using lower effective doses and thus minimizing systemic absorption and reducing the potential for systemic adverse effects (Chiu et al., 2007; Chono et al., 2007; Comstock et al., 2010; Desrosiers et al., 2007; Serralheiro et al., 2013). Presently, the information about topical IN administration of antibiotics in the management of CRS is scarce and often inconclusive (Lim et al., 2008; Wei et al., 2013). Evidence for clear benefits of topical antibiotics in patients with CRS is insufficient and therefore not recommended as first-line therapy; nonetheless, literature suggests that it is a reasonable treatment option for patients that are refractory to traditional oral antibiotics and surgical therapies (Lee and Chiu, 2014; Lim et al., 2008; Suh and Kennedy, 2011). Scheinberg and Otsuji (2002) conducted a clinical study where several antibiotics, including CIP and LEV, were administered via nebulization to individuals with recalcitrant CRS. Based on a comparison of symptoms before and after treatment, they found that the use of nebulized antibiotics was effective and safe (Scheinberg and Otsuji, 2002). Furthermore, symptomatic and endoscopic data before and after nebulization with FQs (e.g. CIP, LEV and ofloxacin) to postsurgical CRS patients revealed a significantly longer average infection-free period (Vaughan and Carvalho, 2002).

Ciprofloxacin is the most potent of the currently marketed FQs against *P. aeruginosa* which is known to be associated with recalcitrant CRS (Bendouah et al., 2006; Emmerson and Jones, 2003; Oliphant and Green, 2002; Zhanel et al., 2002). Therefore, IN administration of CIP is expected to be well succeeded as a culture-directed therapy for refractory CRS patients, since it probably allows a topical antibiotic effect with lower potential for systemic toxicity. Presently, topical formulations of CIP are commercially available as ophthalmic and otic solutions/suspensions (Cilodex® SPC; Ciloxan® SPC; Ciprodex® Prescribing Information; Cetraxal® Prescribing Information). An otic suspension was tested by Sahin-Yilmaz et al. (2008) to deliver the antibiotic into sinonasal mucosa and no significant improvement on the bacterial infection was found probably because of the rapid nasal clearance of the drug (Sahin-Yilmaz et al., 2008). *In situ* gels have recently

attracted a lot of attention as favorable delivery systems used to increase drug residence time, due to their viscosity and ability to undergo transition into a gel at the infection site. Indeed, several ophthalmic *in situ* gel forming systems for CIP have been designed with sol-gel transitions induced by a shift in pH, temperature, or ionic strength (Abdelkader and Mansour, 2015; Ahmed et al., 2014; Al-Kassas and El-Khatib, 2009; Makwana et al., 2016; Mansour et al., 2008; Varshosaz et al., 2008).

Following this line of research, a thermoreversible *in situ* gel containing CIP was used in the present work for IN administration to rats. CIP concentrations in plasma, olfactory bulb and nasal mucosa were determined and the pharmacokinetic parameters were assessed and compared, following IN and IV administrations to rats. To the best of our knowledge, only one *in vivo* study has been performed with IN gel formulations of CIP; however only data about plasma pharmacokinetic properties of CIP were given (Ózsoy et al., 2000). Thus, this is the first study that simultaneously characterizes the pharmacokinetic behavior of CIP delivered by IN route in nasal mucosa, plasma and olfactory bulb, giving key information about drug exposure at the biophase and also assessing the potential for systemic and brain drug exposure.

III.2.2. MATERIAL AND METHODS

III.2.2.1. Chemicals, Materials and Reagents

Ciprofloxacin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and gatifloxacin, used as IS, was obtained from Biokemix (New Mills, Derbyshire, UK). Methanol and acetonitrile (both HPLC gradient grade) were purchased from Fisher Scientific (Leicestershire, UK). Ultrapure water (HPLC grade, 18.2 MΩ.cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Reagents such as fuming hydrochloric acid (37%), formic acid (98–100%) and TEA were acquired from Merck KGaA (Darmstadt, Germany), and sodium hydroxide and trichloroacetic acid were obtained in solid state from Sigma-Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate and ortho-phosphoric acid (85%), used to prepare 25mM phosphate buffer pH 3.0 solution, were obtained from Merck KGaA (Darmstadt, Germany) and Panreac (Barcelona, Spain), respectively. PF-127 and propylene glycol were supplied by Sigma-Aldrich (St. Louis,

MO, USA). Injectable solution of pentobarbital sodium was commercially available under the brand Eutasil® (200 mg/mL; Ceva Saúde Animal). Sodium chloride 0.9% solution was acquired from B.Braun Medical (Queluz de Baixo, Portugal).

III.2.2.2. Animals

Healthy adult male Wistar rats (RccHan:WIST; *n* = 48) weighing 290–340 g were used in this study and purchased from a professional stockbreeder (Harlan®, Barcelona, Spain). The animals were housed under controlled environmental conditions (12 h light/dark cycle; temperature 20 ± 2 °C; relative humidity 55 ± 5%) for a minimum of five days before the experiments. During this period, animals had free access to a standard rodent diet (4RF21, Mucedola®, Italy) and tap water. Rats were fasted overnight (12 h) prior to drug administration and kept on fast during the first 4 h of the study. All experimental and care procedures were conducted in accordance with the European Directive (2010/63/EU) regarding the protection of laboratory animals used for scientific purposes (European Parliament and Council of the European Union, 2010) and with the Portuguese law on animal welfare (Decreto-Lei 113/2013, 2013). The experimental procedures were reviewed and approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária).

III.2.2.3. Preparation and Optimization of Ciprofloxacin Formulations

For IN and IV administration, CIP was first dissolved at the concentration of 30 mg/mL in an aqueous acidic solution of 1% (v/v) of fuming hydrochloric acid (37%).

Pluronic F-127 was used to prepare the thermoreversible gel for IN administration by the cold method described by Schmolka (1972). A weighed amount of 9.6 g of PF-127 was slowly added to 40 mL of ultrapure cold water while providing gentle mixing and subsequent magnetic stirring to promote hydration of the flakes. The mixture was stored at 4 °C over night to achieve complete dissolution of PF-127 at 24% (w/v). A volume of 200 µL of the acidic CIP solution (30 mg/mL) previously prepared was then incorporated in 1800 µL of the thermoreversible gel (24% PF-127, w/v), leading to a final thermosensitive formulation with 3 mg/mL CIP and 21.6% (w/v) PF-127. Finally, the pH of this IN

formulation was adjusted to 5.3-5.5 with minor amounts of 0.5 M sodium hydroxide in order to avoid nasal irritation.

To ensure gelation of the thermoreversible gel at nasal physiological temperature, the percentage of PF-127 was optimized and three different concentrations of drug-free PF-127 gel were tested. Gelation temperatures were determined by the test tube inverting method described by Gilbert et al. (1987) and Mansour et al. (2008). A falcon tube containing 2 mL of the thermoreversible gel, sealed with parafilm, was immersed in a water bath. The temperature of the bath was increased by 2 °C from room temperature and left to equilibrate for 10 min at each new temperature. The sample was then examined for gelation, which is considered to have occurred if the meniscus no longer moves when tilted more than 90°. Tests were performed in triplicate (n = 3) for each drug-free PF-127 gel. As a result of this optimization procedure, a starting 24% (w/v) PF-127 gel was selected to fulfill the above gelation criterion, that was used to incorporate CIP and thus prepare the IN formulation.

For IV administration, the acidic CIP solution (30 mg/mL) was diluted 12-fold in a vehicle composed of propylene glycol–sodium chloride 0.9% (50:50, v/v) to attain a concentration of 2.5 mg/mL of CIP.

III.2.2.4. *In Vivo* Pharmacokinetic Studies

Rats were randomly assigned to either an IN administration group (n = 24) or an IV administration group (n = 24) of CIP. Prior to the experiments, they were anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). Under anesthesia, CIP was administered intravenously to one of the groups by a 1-min injection of the formulation into the lateral tail vein at a dose of 10 mg/kg, with a total volume of administration of 4 mL/kg. In the other group, IN administration of CIP was performed at a dose of 0.24 mg/kg with rats placed in the lateral recumbent position. One half of a total volume in the range of 23-27 µL of the thermoreversible *in situ* gel was delivered through the right and left nostrils, according to the weight of the animal (80 µL/kg). IN administration was made via a polyurethane catheter (Introcan Safety®; 24G; 0.7 x 19 mm) inserted 1 cm deep into nasal cavity and attached to a 50 µL microliter syringe (Hamilton®, USA). Animals were kept at the above mentioned position and, at predetermined time

points (10, 30, 60, 90, 120, 240 min) after IN and IV administration; four rats per time point (n = 4) were sacrificed by decapitation followed by exsanguination. Blood samples were immediately collected into heparinized tubes and plasma samples were obtained after centrifugation at 1514 *g* for 10 min at 4 °C. In the IN group, the nose of the rats was flushed with 20 mL of an ice-cold 0.9% sodium chloride solution, after decapitation and exsanguination, in order to remove the thermoreversible gel formulation. This nasal lavage procedure was completed within 2 min and performed by insertion into each nostril of 1 cm of another catheter (Introcan® Certo; 22G; 0.9 x 25 mm) attached to an appropriate syringe.

In both groups, the brain was removed from the skull to expose the olfactory bulb that was further collected and weighed. A median-sagittal cut starting at the remaining frontal bone was made to bisect the nose and expose the nasal cavity (*Figure III.1.6.*). The nasal septum was removed to enable access to the right and left mucosal surface of the nasal lateral wall and turbinates. The nasal mucosa of two different regions was harvested with the help of a scalpel and forceps (Harkema et al., 2006). The anterior nasal mucosa (ANM) was collected from the naso- and maxilloturbinates and contiguous lining lateral wall epithelium, while the posterior nasal mucosa (PNM) was isolated from the ethmoid turbinates. ANM (right and left) and PNM (right and left) tissue samples, after being separated from any bony remnants, were gently blotted with sterile gauze to remove adherent surface blood and subsequently carefully weighed. All plasma and tissues samples were stored frozen at -24 °C until drug analysis.

III.2.2.5. Ciprofloxacin Bioanalysis

Quantitative determination of CIP in plasma and tissue samples obtained from the *in vivo* pharmacokinetic studies was performed by HPLC analysis. The HPLC method was developed and partially validated in each biological matrix as described in *Chapter II, section II.4.*. This HPLC method fulfilled the requirements established by the international guidelines (European Medicines Agency, 2011; US Food and Drug Administration, 2001) and the main parameters of the partial validation are summarized in *Table III.2.1.*.

Table III.2.1. Main parameters of the partial HPLC method validation (*n* = 3) used for the quantification of ciprofloxacin in plasma, olfactory bulb and nasal mucosa matrices.

Validation Parameters	*Plasma*	*Olfactory Bulb*	*Nasal Mucosa*
Calibration range	0.03 – 5 µg/mL	12.5 – 500 ng	12.5 – 4000 ng
r^2	0.998	0.996	0.996
LLOQ	0.03 µg/mL	12.5 ng	12.5 ng
Precision (%CV)[a]	≤ 16.6% (QC_{LLOQ}) ≤ 6.5% ($QC_{L,M,H}$)	≤ 7.7% (QC_{LLOQ}) ≤ 8.2% ($QC_{L,M,H}$)	≤ 10.8% (QC_{LLOQ}) ≤ 9.7% ($QC_{L,M,H}$)
Accuracy (%*bias*)[a]	-2.8 – 3.6%	-5.1 – 5.4%	-12.2 – 11.3%

[a] Inter-day and intra-day values (*n* =3).
LLOQ, Lower limit of quantification; CV, Coefficient of variation; *bias*, Deviation from nominal value; QC, Quality control samples at the lower limit of quantification (QC_{LLOQ}) and at low (QC_L), medium (QC_M) and high (QC_H) concentrations of the calibration range.

Briefly, each aliquot of plasma (100 µL) was spiked with 30 µL of IS working solution (gatifloxacin: 16 µg/mL) and mixed with 30 µL of 20% (w/v) trichloroacetic acid. The final plasma sample was vortex-mixed for 30 s and centrifuged at 12100 *g* for 5 min at room temperature to precipitate plasma proteins and obtain a clear supernatant that was directly injected (20 µL) into HPLC system for drug analysis. Samples of olfactory bulb (53.7 ± 7.5 mg), ANM (25.5 ± 3.1 mg) and PNM (35.3 ± 5.9 mg) were extracted and homogenized with 1 mL of 0.025 M phosphate buffer solution pH 3.0 in an Ultra-Turrax device (Ystral® GmbH, Germany) for 2-5 min at room temperature, after addition of 10 µL of IS working solution (gatifloxacin: 16 µg/mL for olfactory bulb and 48 µg/mL for nasal mucosa). The homogenate was centrifuged at 17530 *g* for 10 min at 4 °C and 400 µL of the resultant supernatant were mixed with 120 µL of 20% (w/v) trichloroacetic acid. The following vortex-mixing and centrifugation steps were the same as for plasma samples, leading to cleaner and transparent samples, ready to be directly injected (40 µL) into HPLC system for drug quantification.

The HPLC analysis was carried out on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a solvent delivery model (LC-20A), a degasser (DGU-20A5), an autosampler (SIL-20AHT), a column oven (CTO-10ASVP) and fluorescence detector (RF-20AXS). The HPLC apparatus and data acquisition were controlled by LCsolution software (Shimadzu Corporation, Kyoto, Japan). CIP and IS, present in the pre-treated samples, were separated on a reversed-phase LiChroCART® Purospher Star C_{18}

column (55 mm × 4 mm, 3 µm particle size; Merck KGaA, Darmstadt, Germany) at 25 °C by isocratic elution of a mobile phase composed of 0.1% aqueous formic acid (pH 3.0, adjusted with TEA)–acetonitrile–methanol (84:1:15, v/v/v) pumped at a flow rate of 1.0 mL/min. The excitation and emission wavelengths selected for FD were 278 and 470 nm, respectively.

III.2.2.6. Pharmacokinetic Analysis

The maximum concentration (C_{max}) of CIP and the corresponding time to reach C_{max} (t_{max}) in plasma after IN delivery and in tissues after IN and IV administration were directly obtained from the experimental data by graphic observation of the concentration-time profiles. The remaining pharmacokinetic parameters were estimated from the mean concentration values (n = 4) determined at each time point by non-compartmental analysis using the WinNonlin® version 5.2 software. The initial concentration (C_0) of CIP in plasma after IV administration was estimated by back-extrapolation to time zero (t_0). For all matrices in both administration groups, the area under drug concentration-time curve (AUC) from time zero to the time of the last measurable drug concentration (AUC_{0-t}) was calculated by trapezoidal rule; the AUC from time zero to infinity (AUC_{0-inf}) was determined by addition of AUC_{0-t} and the AUC extrapolation to infinity (AUC_{t-inf}) according to: $AUC_{0-t} + AUC_{t-inf} = AUC_{0-t} + C_{last}/k_e$, where C_{last} is the last observed concentration and k_e the apparent elimination rate constant. The percentage of the extrapolated AUC_{t-inf} indicated as AUC_{extrap} (%) and k_e which was estimated by semi-logarithmic linear regression of the terminal segment of the concentration-time profiles were evaluated, as well as the elimination half-life ($t_{1/2}$) and the mean residence time (MRT). Plasma and tissues concentrations below the LLOQ of the analytical method were considered as zero for the pharmacokinetic data analysis.

III.2.3. RESULTS

III.2.3.1. Thermoreversible *In Situ* Gel

Gelation temperature was measured by visual inspection according to the test tube inverting method described in *section III.2.2.3.* for three different concentrations of drug-

free PF-127 gel. To mimic the incorporation of the drug, the same volume of the plain solvent [1% (v/v) fuming hydrochloric acid] was added to the prepared 20%, 22% and 24% (w/v) PF-127 gels under investigation, leading to PF-127 final concentrations of 18%, 19.8% and 21.6% (w/v; prior to adjustment of pH), respectively. As expected, it was found that gelation temperature decreased with increasing concentration of plain PF-127. The first drug-free PF-127 formulation tested (C_{final} = 18%, w/v) did not show gelling properties at any temperature (in the range of 25 °C to 39 °C). The drug-free PF-127 gel of intermediate concentration (C_{final} = 19.8%, w/v) exhibited a gelation temperature at 35-37 °C and the thermoreversible gel with the highest concentration of drug-free PF-127 (C_{final} = 21.6%, w/v) underwent transition into a gel at 29-31 °C. Gelation temperatures higher than 25 °C and lower than 34 °C have been considered suitable. For gelation temperatures lower than 25 °C, a gel might easily be formed at room temperature hindering the preparation, handling and administration of the formulation. On the other hand, at gelation temperatures higher than 34 °C, a liquid form may still be stable at the temperature of nasal cavity (34 °C). In order to ensure gelation of the thermoreversible gel at nasal physiological temperature, a gelation temperature lower than 34 °C was chosen. Therefore, a starting 24% (w/v) PF-127 gel was selected and used to incorporate CIP and prepare the IN formulation with the final PF-127 concentration of 21.6% (w/v). This thermoreversible *in situ* gel also allows for the effect of the watery medium of the nasal cavity that could slightly increase the measured gelation temperature.

III.2.3.2. Pharmacokinetics of Ciprofloxacin after Intranasal and Intravenous Administration

The mean concentration-time profiles (*n* = 4) of CIP in plasma, olfactory bulb and nasal mucosa obtained in rats after administration of a single dose of the prepared IN thermoreversible *in situ* gel (0.24 mg/kg) and the above mentioned IV solution (10 mg/kg) are shown in *Figure III.2.1.(A-C).* In the case of nasal mucosa, CIP concentration was normalized for the amount of drug (in μg) administered to rats by the corresponding administration route, according to the IN or IV dose defined; these dose-normalized concentration-time profiles are also depicted in *Figure III.2.1.(D)*.

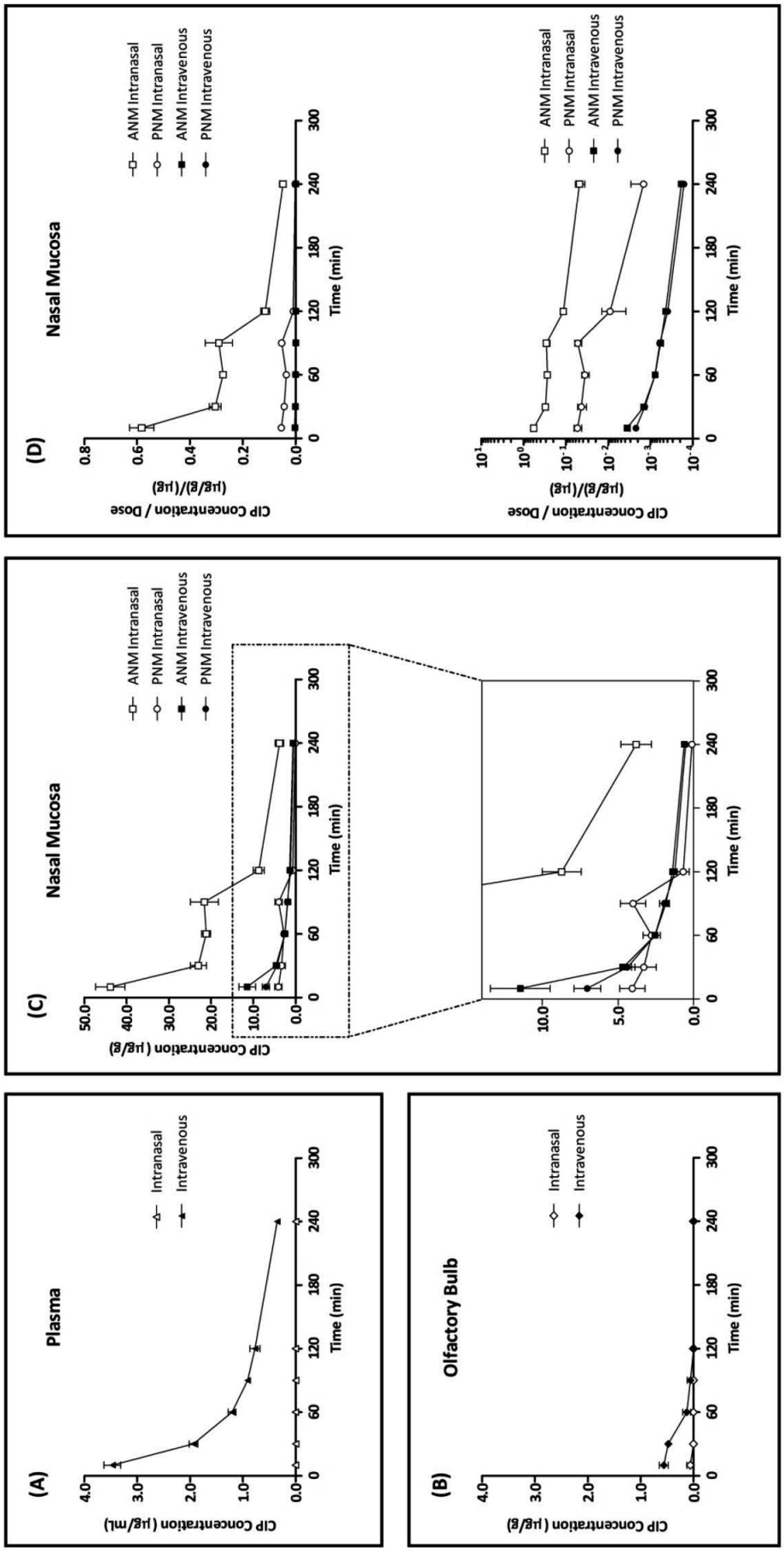

Figure III.2.1. Concentration-time profiles of ciprofloxacin (CIP) in plasma (A), olfactory bulb (B) and nasal mucosa (C) following intranasal (IN, 0.24 mg/kg) and intravenous (IV, 10 mg/kg) administration to rats. Dose-normalized concentration-time profiles of CIP in nasal mucosa (D – linear and semi-logarithmic graphs). Symbols represent the mean values ± standard error of the mean of four determinations per time point (n = 4).

The mean pharmacokinetic parameters of CIP in plasma, olfactory bulb and nasal mucosa estimated by non-compartmental analysis are summarized in *Table III.2.2.* and only the main exposure pharmacokinetic parameters were dose-normalized and presented in *Table III.2.3.*. It should be noted that these normalized values have a limited pharmacokinetic value and were used to enable some degree of comparability between the two administration routes.

Regarding the plasma samples, CIP concentration achieved after IN administration was very low and for the majority of samples was below the LLOQ of the analytical method. Vestigial concentrations were found at three different time points spread over the entire study and the mean C_{max} of CIP in plasma (0.009 µg/mL) after IN delivery was attained at 60 min post-dosing. Thus, due to the lack of concentration-time data, it was not possible to estimate other plasma pharmacokinetic parameters (*Table III.2.2.*). In contrast to the IN administration, CIP concentration in plasma was always above the LLOQ after IV administration and a more complete concentration-time profile of CIP in plasma was achieved over the 4 h of the study (*Figure III.2.1.(A)*), with the corresponding plasma pharmacokinetic parameters being depicted in *Table III.2.2.*. The theoretical C_0 parameter of CIP after IV administration was estimated to be 4.652 µg/mL, while the maximum experimental plasma concentration was immediately achieved at the first post-dosing time point (10 min) after IV administration and was found to be 3.473 µg/mL. From the results obtained for an IN dose 41.7-fold lower than IV dose (0.24 mg/kg and 10 mg/kg, respectively), it is evident that there is a significantly lower plasma exposure of CIP after IN delivery. Comparison of the dose-normalized C_{max} and C_0 parameters in plasma following IN and IV administration shows that C_{max}/dose obtained after IN delivery is 12.3-fold lower than C_0/dose of the IV route which confirms the advantage of a lower systemic exposure for IN delivery (*Table III.2.3.*).

The olfactory bulb was also analyzed in this study and, as for plasma samples, the only pharmacokinetic parameters obtained after IN administration were those taken from the experimental data, namely the C_{max} and t_{max}, whereas in the case of IV administration all the other pharmacokinetic parameters estimated from the non-compartmental analysis were also calculated (*Table III.2.2.*). When CIP was delivered by IN route, only one of the olfactory bulb samples was quantified above the LLOQ, leading to the only non-zero mean value in this matrix which corresponds to the mean C_{max} achieved at 10 min post-dosing

(t_{max}). These results are reflected in the concentration-time profiles depicted in *Figure III.2.1.(B)*. Taking into account the different doses administered by IN and IV route, the dose-normalized parameter (C_{max}/dose) becomes 4.6-fold higher after IN delivery in the olfactory bulb (*Table III.2.3.*). However, it should be noted that the mean value (C_{max}) obtained after the applied IN dose (0.24 mg/kg) is significantly lower than the C_{max} after IV administration (10mg/kg), namely 0.062 *versus* 0.562 µg/mL (*Table III.2.2.*).

In nasal mucosa, two concentration-time profiles of CIP were obtained for each administration route; one corresponding to the ANM and the other to the PNM. From *Figure III.2.1.* and *Table III.2.2.*, it is evident that C_{max} in both nasal mucosa (ANM and PNM) was always achieved at 10 min post-dosing, independently of the administration route. After t_{max}, CIP concentrations following IV administration were quite similar in both ANM and PNM. In the case of topical administration, CIP concentration in ANM did not change significantly in the interval 30 – 90 min after dosing, leading to a plateau also observed in PNM, although less evident probably due to the lower concentration levels. The concentration-time profile of CIP in ANM following IN administration (0.24 mg/kg) was markedly higher than that following IV administration (10 mg/kg), while in the case of PNM the concentration-time profiles for the two routes were close in magnitude to each other, as can be observed in *Figure III.2.1.(C)*. The separation between dose-normalized concentration-time profiles of CIP in ANM after each route increased, as shown in *Figure III.2.1.(D).* (linear scale), while the dose-normalized concentration-time profile in PNM after IN administration became higher than the one obtained after IV administration, as is better visualized in the semi-logarithmic scale graph. The C_{max}/dose, AUC_{0-t}/dose and AUC_{0-inf}/dose of CIP in ANM were 160.9-fold, 254.1-fold and 243.6-fold greater, respectively, after IN than after IV delivery; the same dose-normalized parameters in PNM became 24.1-fold, 35.9-fold and 32.3-fold higher for IN route *versus* IV route (*Table III.2.3.*). Regarding the estimated pharmacokinetic parameters of elimination, the $t_{1/2}$ of CIP was lower in both nasal mucosa specimens after IN delivery, differing from IV administration in approximately 29 and 38 min in ANM and PNM, respectively.

Table III.2.2. Mean pharmacokinetic parameters of ciprofloxacin following intranasal (IN) and intravenous (IV) administration to rats of 0.24 mg/kg and 10 mg/kg doses, respectively.

Pharmacokinetic Parameters[a]	*Plasma*		*Olfactory Bulb*		*Anterior Nasal Mucosa*		*Posterior Nasal Mucosa*	
	IN	*IV*	*IN*	*IV*	*IN*	*IV*	*IN*	*IV*
C_0 (µg/mL or µg/g)	-	4.652	-	-	-	-	-	-
t_{max} (min)	60.0	-	10.0	10.0	10.0	10.0	10.0	10.0
C_{max} (µg/mL or µg/g)	0.009	-	0.062	0.562	43.830	11.464	4.059	7.035
AUC_{0-t} (µg.min/mL or µg.min/g)	ND	266.193	ND	25.145	3398.655	563.265	406.045	474.700
AUC_{0-inf} (µg.min/mL or µg.min/g)	ND	321.308	ND	26.878	3777.220	652.002	413.008	537.044
AUC_{extrap} (%)	ND	17.2	ND	6.4	10.0	13.6	1.7	11.6
k_e (min^{-1})	ND	0.006	ND	0.035	0.010	0.007	0.016	0.009
$t_{½}$ (min)	ND	108.5	ND	20.0	69.0	98.1	42.7	80.9
MRT (min)	ND	125.9	ND	35.8	101.2	107.8	70.0	103

[a] Parameters were estimated using the mean concentration-time profiles obtained from four different animals per time point (n = 4).

AUC_{0-inf}, Area under the concentration time-curve from time zero to infinite; AUC_{0-t}, Area under the concentration time-curve from time zero to the last measurable drug concentration; C_0, Plasma concentration back-extrapolated to time zero; C_{max}, Maximum concentration; k_e, Apparent elimination rate constant; MRT, Mean residence time; ND, Not determined; $t_{1/2}$, Elimination half-life; t_{max}, Time to achieve the maximum concentration.

Table III.2.3. Dose-normalized pharmacokinetic parameters of ciprofloxacin following intranasal (IN) and intravenous (IV) administration to rats.

Dose-Normalized Pharmacokinetic Parameters[a]	*Plasma*		*Olfactory Bulb*		*Anterior Nasal Mucosa*		*Posterior Nasal Mucosa*	
	IN	*IV*	*IN*	*IV*	*IN*	*IV*	*IN*	*IV*
C_0 / Dose (µg/mL or µg/g)/(µg)	-	1.455 $x10^{-3}$	-	-	-	-	-	-
C_{max} / Dose (µg/mL or µg/g)/(µg)	0.118 $x10^{-3}$	-	0.816 $x10^{-3}$	0.176 $x10^{-3}$	0.577	3.586 $x10^{-3}$	0.053	2.201 $x10^{-3}$
AUC_{0-t} / Dose (µg.min/mL or µg.min/g)/(µg)	ND	0.083	ND	7.865 $x10^{-3}$	44.719	0.176	5.343	0.149
AUC_{0-inf} / Dose (µg.min/mL or µg.min/g)/(µg)	ND	0.101	ND	8.407 $x10^{-3}$	49.700	0.204	5.434	0.168

[a] IN and IV pharmacokinetics parameters divided by the average amount of CIP administered by IN (76.0 µg) and IV (3197 µg) routes, respectively.
AUC_{0-inf}, Area under the concentration time-curve from time zero to infinite; AUC_{0-t}, Area under the concentration time-curve from time zero to the last measurable drug concentration; C_0, Plasma concentration back-extrapolated to time zero; C_{max}, Maximum concentration; ND, Not determined.

III.2.4. DISCUSSION AND CONCLUSION

In the present study, pharmacokinetics of CIP following IN and IV administration to rats was investigated and compared. Although, in clinical practice, IV administration of CIP is not recommended for routine use in the management of CRS, this route was herein chosen because oral bioavailability of CIP is only around 20% in rats (Hwang et al., 2012), a value much lower than that assessed for humans (approximately 70%) (Ravi et al., 2012; Vance-Bryan et al., 1990). On the other hand, when testing a new route of administration, it is recommended to compare it with IV administration where drug bioavailability is 100%. For these reasons, CIP was intravenously administered, thus avoiding the intestinal absorption by delivering directly the drug into the systemic circulation, being an appropriate route to compare with IN administration. The IV dose used in the study was determined by the requirement of a clear solution adequate for IV injection; this is governed by the solubility of CIP which is limited by the pH of the formulation. The dose of 10 mg/kg is in the range of doses mentioned in literature (lower limit) (Hwang et al., 2012) and fulfills the requirements above. For IN administration, the dose selected was in

agreement with a study of intrapulmonary delivery of CIP to rats which, to our knowledge, used one of the lowest doses cited in literature (Chono et al., 2007).

In the case of IV administration (10 mg/kg), the result obtained for plasma $AUC_{0\text{-}inf}$ (321.3 µg.min/mL, i.e. 5.4 µg.h/mL) was comparable to that reported by Hwang et al., (2012) in Sprague-Dawley rats (4.6 µg.h/mL) and approximately 5-fold lower than plasma $AUC_{0\text{-}inf}$ estimated as 1565.8 µg.min/mL for an IV dose 5 times higher (50 mg/kg) administered to Sprague-Dawley rats in the study conducted by (Nouaille-Degorces et al., 1999). The plasma $t_{1/2}$ obtained herein (108.5 min) is in accordance with the $t_{1/2}$ of 102.3 min reported by (Nouaille-Degorces et al., 1999). Both values differ from plasma $t_{1/2}$ of CIP in humans which is 3-5 h (Bolon, 2009) demonstrating that CIP is eliminated faster in rodents than in humans, as reported also for other FQs, namely gatifloxacin (Tasso et al., 2008). CIP, as compared to other antibacterials of its class, has a good tissue penetration and is rapidly distributed to many different body tissues. However, the penetration into cerebrospinal fluid and brain is low, indicating that CIP distribution into this organ is hindered by the complex blood-brain barrier (Bergan, 1990; De Lange et al., 2000; Emmerson and Jones, 2003; Siefert et al., 1986). In fact, in our study, low levels of CIP in olfactory bulb were found after IV administration (10 mg/kg) and CIP concentration in nasal mucosa specimens (ANM and PNM) was consistently higher than in olfactory bulb with tissue-to-plasma ratios of 2.03 ($AUC_{0\text{-}inf(ANM)}/AUC_{0\text{-}inf(plasma)}$), 1.67 ($AUC_{0\text{-}inf(PNM)}/AUC_{0\text{-}inf(plasma)}$) and 0.08 ($AUC_{0\text{-}inf(olfactory\ bulb)}/AUC_{0\text{-}inf(plasma)}$), respectively. Moreover, in nasal mucosa CIP was homogenously distributed between ANM and PNM after this parenteral route with similar elimination rates (*Table III.2.2.*).

In the case of IN administration, ANM was more exposed to CIP than PNM, suggesting a heterogeneous deposition pattern of CIP in rat nasal cavity (*Figure III.2.1.(C and D)*). The present data suggests that application of the thermoreversible gel was mainly in the ANM region and that part of its volume also reached the PNM region. Although both studied nasal regions form a unique nasal passage without any physical barrier separating them, CIP deposited and released from the formulation in ANM region appears to have had no influence in diffusion and transport of CIP to PNM, probably due to nasal MCC processes. In fact, we can observe from the concentration-time profiles of CIP in both regions (*Figure III.2.1.(C and D)*) that there was no increase of CIP concentration in PNM

with decreasing CIP concentration in ANM. This is in agreement with the mucus flow pattern reported by (Morgan et al., 1984) in rats; they found that different regions of the rat nose have characteristic mucus flow rate and flow pattern and that in the case of the corresponding region of our ANM, the direction of mucus flow was ultimately towards the nasopharynx without passing through the region of the ethmoid turbinates (our PNM region) (Morgan et al., 1984). This mucus flow of ANM region is a consequence of a vital key defense mechanism, MCC, which is one of the major drawbacks of IN drug delivery by limiting the contact time of the drug with nasal mucosa (Arora et al., 2002). However, in this work, the *in situ* thermoreversible gel of PF-127 was used specifically to avoid the rapid nasal MCC of CIP. Due to its temperature-responsive properties, this type of formulation has the advantage of being easily administered into the nostrils as a liquid form and undergo transition to a viscous gel at physiological temperature within the nose, lowering the effect of mucus flow and thus increasing the residence time of CIP in nasal cavity (Ma et al., 2008; Serralheiro et al., 2015, 2014). Another important feature reported for this type of *in situ* gel forming polymeric systems is its ability to release the drug in a controlled/sustained manner (Aliabadi et al., 2008; Swamy and Abbas, 2012; Taha et al., 2014; Yang and Alexandridis, 2000). Taha et al. (2014) showed that PF-127 micellar systems could enhance CIP solubility by the presence of the hydrophobic chain of the polymer, suggesting that CIP would be located mainly in the hydrophobic interior of micelles or in both micelles' core and hydrophilic micelles' shell, thus affecting and slowing down the release rate of CIP, which is dependent on the polymer concentration. Some investigations performed with CIP ophthalmic *in situ* gels have demonstrated that the release of CIP decreases as the concentration of PF-127 increases, being much lower than CIP solution (Abdelkader and Mansour, 2015; Mansour et al., 2008). From our study, a plateau was observed in the concentration-time profile of CIP in ANM and PNM (*Figure III.2.1.(C and D)*) after IN administration of the thermoreversible *in situ* gel; this was not observed after IV administration and may be explained by the residence time of the in situ gel in nasal cavity and by the release properties of the nasal formulation, which seem to contribute to the sustained concentration pattern observed up to 90 min post-dose. The dose-normalized results obtained for this matrix demonstrate that the topical IN administration of CIP gel offers, by itself, higher concentration values and pharmacokinetic

exposure parameters in both nasal mucosa specimens compared to IV delivery. These differences are expected to be even more evident if compared to oral administration.

With respect to systemic exposure, IN administration of CIP thermoreversible gel (0.24 mg/kg) resulted in vestigial concentrations at three different time points with a later t_{max} at 60 min post-dose, resulting in very little systemic absorption from nasal cavity. The residual or zero CIP concentration mean values obtained in plasma after this IN administration and the lower C_{max}/dose parameter compared to that of IV administration demonstrate that IN delivery has the important advantage of avoiding or minimizing CIP distribution to other organs and thus the development of adverse effects. This is particularly relevant for patients with CRS because FQs are associated with increased risk for tendinitis and tendon rupture which is more pronounced with the concurrent use of corticosteroids, making these patients a more susceptible population to this musculoskeletal disorder, due to the simultaneous implementation of the two types of drug in CRS therapy (Sousa et al., 2014; Stahlmann and Lode, 2013).

The unique anatomical connection between nose and brain has drawn our attention to assess CIP in olfactory bulb for the evaluation of its potential to develop CNS adverse effects, which are the second most commonly reported for FQs (Sousa et al., 2014). The higher IN dose-normalized value found for mean C_{max} compared to the corresponding IV value may suggest a direct transport of the drug from nasal cavity. However, this is not a real drawback because, in practice, the dose administered by IN route is usually small due to the restrictions imposed by the anatomical and physiological characteristics of nasal cavity, as well as the physicochemical properties of the drug. In the present work, for an IN dose 41.7-fold lower than IV dose (0.24 mg/kg and 10 mg/kg, respectively) the only mean concentration of CIP in olfactory bulb after IN delivery found at the first sampling time (t_{max} = 10 min) was markedly lower (one order of magnitude lower) than that obtained after IV delivery (*Table III.2.2.*), which proves that in these conditions the olfactory bulb exposure is negligible. Although CIP total brain exposure was not herein considered, the results obtained from olfactory bulb and plasma with the administered IN dose of 0.24 mg/kg will clearly favor a safe profile for adverse effects in the CNS.

In conclusion, we have demonstrated that topical IN administration is advantageous for delivering CIP to biophase, leading to negligible systemic and brain

exposure with a IN dose of 0.24 mg/kg, 41.7-fold lower than IV dose. Moreover, the thermoreversible *in situ* gel used for topical administration is an interesting option for a local delivery of CIP. The topical IN delivery of CIP proposed in this study is expected to be an alternative and attractive approach in culture-directed therapy for refractory CRS patients, reducing the possible adverse effects inherent to systemic antibiotherapy, when trying to eradicate bacterial biofilms.

III.3. INTRANASAL DELIVERY OF TOPICALLY-ACTING LEVOFLOXACIN TO RATS: A PROOF-OF-CONCEPT PHARMACOKINETIC STUDY

III.3.1. INTRODUCTION

Levofloxacin, the optical S-(−)-isomer of the racemic ofloxacin, is a third-generation FQ antibacterial agent. This and the subsequent fourth-generation have emerged from an attempt to expand antibacterial activity spectrum and also improve pharmacokinetic properties. LEV is one of the outstanding representatives of the third-generation of the quinolone class, evidencing a broad spectrum of antibacterial activity against most aerobic gram-negative and gram-positive bacteria and demonstrating a moderate activity against anaerobes (Anderson and Perry, 2008; Ball, 2003; Davis and Bryson, 1994; Sousa et al., 2014; Zhanel et al., 2002).

Chronic rhinosinusitis is one of the most common chronic diseases with continued increasing prevalence despite the evolution of conventional antibiotic therapy, which is one of the most prescribed medication by physicians for the treatment of this medical condition. CRS is characterized by inflammation of the nose and mucosa of paranasal sinuses with symptoms and signs persisting for more than 12 consecutive weeks. The optimal clinical management of CRS is difficult to define due to the heterogeneity of this disease whose etiology and pathology are still not clearly established. However, the role of bacterial biofilms is increasingly recognized in CRS patients and may explain the recurrent and resistant nature of the disease (Cain and Lal, 2013; Elliott and Stringer, 2006; Ezzat et al., 2015). The mainstay of treatment is the use of topical corticosteroids and oral antibiotics, endoscopic sinus surgery being reserved only for patients who are refractory to this drug therapy (Fokkens et al., 2012; Rosenfeld et al., 2015; Schwartz et al., 2016). Notwithstanding, in clinical practice a significant subpopulation of CRS patients remains resistant to cure even after multiple pharmacological and surgical therapies, making CRS a challenging and sometimes frustrating disease to treat.

Bacterial biofilms present in sinonasal mucosa consist of an organized community of bacteria anchored to its surface and embedded in an exopolysaccharide matrix. Once fully formed, they represent a polymicrobial community highly resistant to host defenses

and antibiotics (Bendouah et al., 2006; Cain and Lal, 2013; Cohen et al., 2009). This increased resistance calls for alternative therapeutic approaches to effectively eradicate biofilms. In this context, topical IN administration of antibiotics has emerged with the benefit of delivering high concentrations directly to the site of infection without attaining high drug concentrations in serum as it would be the case with traditional administration routes, thus reducing the risk of systemic adverse effects (Elliott and Stringer, 2006; Suh and Kennedy, 2011). There is insufficient and often inconclusive evidence to support a clear benefit of topical antibiotics in CRS patients and larger and better-designed clinical trials are required to thoroughly evaluate this alternative strategy of treatment. However, literature suggests that it is a viable alternative option for patients refractory to oral antibiotics and surgical therapies (Lim et al., 2008; Wei et al., 2015). Several studies have been performed where antibiotics such as CIP, LEV and ofloxacin were administered via IN route to individuals with refractory CRS; they found that the applied topical antibiotic therapies were effective and safe not only by reduction of symptoms and infrequent side effects, but also by complete disappearance of biofilms and longer infection-free periods (Cain and Lal, 2013; Ezzat et al., 2015; Scheinberg and Otsuji, 2002; Vaughan and Carvalho, 2002).

The most common bacteria isolated from biofilms of refractory CRS patients are *S. aureus* and *P. aeruginosa* (Bendouah et al., 2006; Desrosiers and Kilty, 2008; Elliott and Stringer, 2006; Ezzat et al., 2015; Prince et al., 2008; Schwartz et al., 2016). LEV is active against these target microorganisms and was selected for the present study to investigate the advantages of its topical therapy that will favor its use in clinical practice for refractory cases of post-surgical CRS patients (Andersson and MacGowan, 2003; Cohen et al., 2009). The residence time of the drug in nasal cavity is of great importance for IN drug delivery. Bearing this in mind, a thermoreversible *in situ* gel was used in this study for IN administration of LEV to rats. To the best of our knowledge, this is the first study that quantifies LEV in nasal mucosa, plasma and olfactory bulb after topical delivery, giving key information about drug levels at biophase as well as systemic and brain exposure. The pharmacokinetic parameters were also assessed and compared with those obtained after IV administration of LEV to rats, in order to establish the potential of this topical administration route.

III.3.2. MATERIAL AND METHODS

III.3.2.1. Chemicals, Materials and Reagents

Levofloxacin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and gatifloxacin, used as IS, was obtained from Biokemix (New Mills, Derbyshire, UK). Methanol and acetonitrile (both HPLC gradient grade) were acquired from Fisher Scientific (Leicestershire, UK). Ultrapure water (HPLC grade, 18.2 MΩ.cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Reagents such as fuming hydrochloric acid (37%), formic acid (98–100%) and TEA were obtained from Merck KGaA (Darmstadt, Germany), and PF-127, sodium hydroxide and trichloroacetic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA). Potassium dihydrogen phosphate and ortho-phosphoric acid (85%), used to prepare 25 mM phosphate buffer pH 3.0 solution, were acquired from Merck KGaA (Darmstadt, Germany) and Panreac (Barcelona, Spain), respectively. Injectable solution of pentobarbital sodium was commercially available under the brand Eutasil® (200 mg/mL; Ceva Saúde Animal) and sodium chloride 0.9% solution was purchased from B.Braun Medical (Queluz de Baixo, Portugal).

III.3.2.2. Animals

A total of 48 healthy adult male Wistar rats (RccHan:WIST, Harlan®, Barcelona, Spain) weighing 280–335 g were used in this study. All animals were maintained in steady environmental conditions at 20 ± 2 °C with a 12-h light/dark cycle and a relative humidity of 55 ± 5% for a minimum of five days before the experiments. A standard rodent diet (4RF21, Mucedola®, Italy) was provided with water available *ad libitum*. Twelve-hours before drug administration rats were fasted and kept on fast during the 4 h of the study with free access to tap water. All experimental and care procedures were conducted in accordance with the European Directive (2010/63/EU) regarding the protection of laboratory animals used for scientific purposes (European Parliament and Council of the European Union, 2010), the Portuguese law on animal welfare (Decreto-Lei 113/2013, 2013) and the experimental procedures were reviewed and approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária).

III.3.2.3. Preparation of Levofloxacin Formulations

For IV injection, a solution of LEV in sterile sodium chloride 0.9% with a concentration of 2.5 mg/mL was prepared.

For IN administration, LEV was first dissolved in an aqueous acidic solvent of 1% (v/v) of fuming hydrochloric acid (37%) to prepare a concentrated solution at 30 mg/mL. This solution was incorporated in the thermoreversible gel composed of PF-127 that was prepared by the cold method described by (Schmolka, 1972) and used as the IN formulation vehicle.

The percentage of PF-127 was optimized to ensure gelation of the thermoreversible gel at nasal physiological temperature and a starting 24% (w/v) PF-127 gel was selected to fulfill this requirement. A weighed amount of 9.6 g of PF-127 was slowly added to 40 mL of ultrapure cold water while providing gentle mixing followed by magnetic stirring to promote hydration of the flakes. The mixture was stored at 4 °C over night to achieve complete dissolution of PF-127 at 24% (w/v). Subsequently, a volume of 200 µL of the acidic LEV solution (30 mg/mL) previously prepared was added to 1800 µL of the thermoreversible gel (24% PF-127, w/v), leading to a final thermoreversible gel formulation with 3 mg/mL LEV and 21.6% (w/v) PF-127. Finally, the pH of this IN formulation was adjusted to 5.3-5.5 with minor amounts of 0.5 M sodium hydroxide in order to avoid nasal irritation. The drug-free final IN gel formulation exhibited a gelation temperature lower than 34 °C as reported in *section III.2.2.3.*.

III.3.2.4. *In Vivo* Pharmacokinetic Studies

Rats were randomly divided into an IV administration group (n = 24) and an IN administration group (n = 24). Before LEV dosing, they were all anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). In both groups, LEV was administered to four rats per each of the six time points predetermined for this experiment (n = 4). IV administration was performed by a 1-min injection into the lateral tail vein at a dose of 10 mg/kg, with a total volume of administration of 4 mL/kg. For IN administration, rats were placed in the lateral recumbent position and a polyurethane catheter (Introcan Safety®; 24G; 0.7 x 19 mm) was inserted 1 cm deep into nasal cavity and

attached to a 50 µL microliter syringe (Hamilton®, USA). A dose of 0.24 mg/kg (80 µL/kg) was delivered in a total volume of 22-27 µL in accordance with the weight of the animals, one half of which was administered to each nostril. Animals from both groups were kept at the above mentioned position and were sacrificed by decapitation followed by exsanguination at 10, 30, 60, 90, 120, 240 min after LEV administration. Blood samples were immediately collected into heparinized tubes and plasma samples were obtained after centrifugation at 1514 *g* for 10 min at 4 °C and stored at -24 °C until drug analysis. To remove the thermoreversible gel formulation, a nasal lavage was performed in the IN group after decapitation and exsanguination; the nose of the rats was flushed with 20 mL of an ice-cold 0.9% sodium chloride solution by insertion into each nostril of 1 cm of another catheter (Introcan® Certo; 22G; 0.9 x 25 mm) attached to an appropriate syringe. This lavage procedure was completed within two minutes injecting 10 mL in each nostril.

In both groups, their skin and soft tissue of the head was excised and the brain was removed from the skull. Then, a median-sagittal cut starting at the remaining frontal bone was made to expose the olfactory bulb and bisect the nose, exposing the nasal cavity. The nasal septum was removed to enable access to the right and left mucosal surface of the nasal lateral wall and turbinates. The nasal mucosa of two different regions was harvested with the help of a scalpel and forceps (*Figure III.1.6.*) (Harkema et al., 2006). The ANM was collected from the naso- and maxilloturbinates and contiguous lining lateral wall epithelium while the PNM was isolated from the ethmoid turbinates. After separating any bony remnants, ANM (right and left) and PNM (right and left) tissue samples were gently blotted with sterile gauze to remove adherent surface blood. After collection of olfactory bulb and nasal mucosa samples, they were carefully weighed and stored frozen at -24 °C until drug analysis.

III.3.2.5. Levofloxacin Bioanalysis

Plasma and tissue samples obtained from the *in vivo* pharmacokinetic studies were analyzed by HPLC to quantify LEV. The HPLC method was developed and partially validated in each biological matrix as described in *Chapter II, section II.4.*. Each aliquot of plasma (100 µL) was spiked with 30 µL of IS working solution (gatifloxacin: 16 µg/mL) and mixed with 30 µL of 20% (w/v) trichloroacetic acid. The final sample was vortex-mixed for 30 s

and centrifuged at 12100 *g* for 5 min at room temperature to precipitate plasma proteins. The clear supernatant obtained was directly injected (20 µL) into HPLC system for drug analysis. After addition of 10 µL of IS working solution (gatifloxacin: 16 µg/mL for olfactory bulb and 48 µg/mL for nasal mucosa), olfactory bulb (54.7 ± 5.5 mg), ANM (25.0 ± 3.1 mg) and PNM (33.8 ± 4.9 mg) samples were homogenized with 1 mL of 0.025 M phosphate buffer solution pH 3.0 in an Ultra-Turrax device (Ystral® GmbH, Germany) for 2-5 min at room temperature. The homogenate was centrifuged at 17530 *g* for 10 min at 4 °C and 400 µL of the resultant supernatant were mixed with 120 µL of 20% (w/v) trichloroacetic acid. This was followed by vortex-mixing and centrifugation steps as described for plasma samples. Then, the samples obtained were directly injected (40 µL) into HPLC system for drug quantification.

The HPLC analysis was carried out on a Shimadzu® HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a solvent delivery model (LC-20A), a degasser (DGU-20A5), an autosampler (SIL-20AHT), a column oven (CTO-10ASVP) and fluorescence detector (RF-20AXS). The HPLC apparatus and data acquisition were controlled by LCsolution software (Shimadzu Corporation, Kyoto, Japan). LEV and IS, present in the pre-treated samples, were separated on a reversed-phase LiChroCART® Purospher Star C_{18} column (55 mm × 4 mm, 3 µm particle size; Merck KGaA, Darmstadt, Germany), thermostated at 25 °C, by isocratic elution of a mobile phase composed of 0.1% aqueous formic acid (pH 3.0, adjusted with TEA)–acetonitrile–methanol (84:1:15, v/v/v) pumped at a flow rate of 1.0 mL/min. Excitation and emission wavelengths of 278 and 470 nm, respectively, were selected for FD. The described HPLC method fulfilled the requirements established by the international guidelines (European Medicines Agency, 2011; US Food and Drug Administration, 2001) and the main parameters of the partial validation are summarized in *Table III.3.1.*.

Table III.3.1. Main parameters of the partial HPLC method validation (*n* = 3) used for the quantification of levofloxacin in plasma, olfactory bulb and nasal mucosa matrices.

Validation Parameters	*Plasma*	*Olfactory Bulb*	*Nasal Mucosa*
Calibration range	0.04 – 5 µg/mL[b]	18 – 500 ng	18 – 4000 ng
r^2	0.997	0.992	0.997
LLOQ	0.04 µg/mL	18 ng	18 ng
Precision (%CV)[a]	≤ 15.5% (QC_{LLOQ})	≤ 8.6% (QC_{LLOQ})	≤ 8.4% (QC_{LLOQ})
	≤ 7.1% ($QC_{L,M,H}$)	≤ 8.6% ($QC_{L,M,H}$)	≤ 8.8% ($QC_{L,M,H}$)
	≤ 3.2% (QC_{Dil})	-	-
Accuracy (%*bias*)[a]	-13.0 –1.5%	-4.0 – 7.0%	-7.0 – 11.6%

[a] Inter-day and intra-day values (*n* =3). [b] 5-fold dilution effect evaluated in plasma using a quality control sample at the concentration of 10 µg/mL.
LLOQ, Lower limit of quantification; CV, Coefficient of variation; *bias*, Deviation from nominal value; QC, Quality control samples at the lower limit of quantification (QC_{LLOQ}) and at low (QC_L), medium (QC_M) and high (QC_H) concentrations of the calibration range and following a sample dilution by a 5-fold factor (QC_{Dil}).

III.3.2.6. Pharmacokinetic Analysis

The maximum concentration (C_{max}) of LEV and the time to attain it (t_{max}) in plasma after IN delivery and in tissues after IN and IV administration were directly obtained from the experimental data by graphic observation of the concentration-time profiles. The remaining pharmacokinetic parameters were estimated from the mean concentration values (*n* = 4) determined at each time point by non-compartmental analysis using the WinNonlin® version 5.2 software. The initial concentration (C_0) of LEV in plasma after IV administration was estimated by back-extrapolation to time zero (t_0). For all matrices, in both administration groups, the AUC_{0-t} and AUC from time zero to the last time point of the study (AUC_{all}) were calculated by trapezoidal rule; the AUC_{0-inf} was determined according to: $AUC_{0-t} + AUC_{t-inf} = AUC_{0-t} + C_{last}/k_e$, where AUC_{t-inf} represents the AUC extrapolation to infinity, C_{last} is the last observed concentration and k_e the apparent elimination rate constant. The percentage of the extrapolated AUC_{t-inf} indicated as AUC_{extrap} (%) and k_e estimated by semi-logarithmic linear regression of the terminal segment of the concentration-time profiles were evaluated, as well as $t_{1/2}$ and the MRT. Plasma and tissues concentrations below the LLOQ of the analytical method were considered as zero for the pharmacokinetic data analysis.

III.3.3. RESULTS

The mean plasma, olfactory bulb and nasal mucosa concentration-time profiles (*n* = 4) obtained in rats after administration of a single dose of the IN thermoreversible *in situ* gel (0.24 mg/kg) and of the IV formulation (10 mg/kg) are displayed in *Figure III.3.1.(A-C)*. LEV concentration in nasal mucosa was also normalized for the amount of drug (in µg) administered to rats by IN or IV route, according to the dose defined for the corresponding administration route (*Figure III.3.1.(D)*). The mean pharmacokinetic parameters of LEV in plasma, olfactory bulb and nasal mucosa obtained by non-compartmental analysis are summarized in *Table III.3.2.* and the main exposure pharmacokinetic parameters were dose-normalized and shown in *Table III.3.3.*

LEV mean concentrations achieved in plasma after IN administration were low at the first three time points of the study (10, 30 and 60 min), although the values of the individual samples were consistently above the LLOQ of the analytical method. For the remaining time points, plasma samples were either below the LLOQ or undetected. This limited data requires careful interpretation of some of the pharmacokinetic parameters obtained (*Table III.3.2.*). After IV administration, LEV concentrations in plasma were always above the LLOQ, leading to a more complete concentration time profile over the 4 h of the study (*Figure III.3.1.(A)*), which enabled a more reliable set of pharmacokinetic parameters to be calculated for LEV after this administration route (*Table III.3.2.*). The mean plasma C_{max} of LEV after IN delivery was attained at 10 min post-dosing and was found to be 0.085 µg/mL, a much lower value than the theoretical C_0 parameter and the experimental C_{max} (t_{max} = 10 min) of LEV after IV administration, 7.131 µg/mL and 5.203 µg/mL, respectively. The above results were obtained after administration of an IN dose 41.7-fold lower than the IV dose. Taking into account these different doses, comparison of dose-normalized C_{max} and C_0 parameters in plasma following IN and IV administration shows that C_{max}/dose obtained after IN delivery is 2.1-fold lower than C_0/dose of the IV route. A similar relationship (2.3-fold lower in magnitude) occurs with dose-normalized AUC_{all} values which, for the present data, are more appropriate for comparison between the two routes than $AUC_{0\text{-}inf}$ (*Table III.3.3.*).

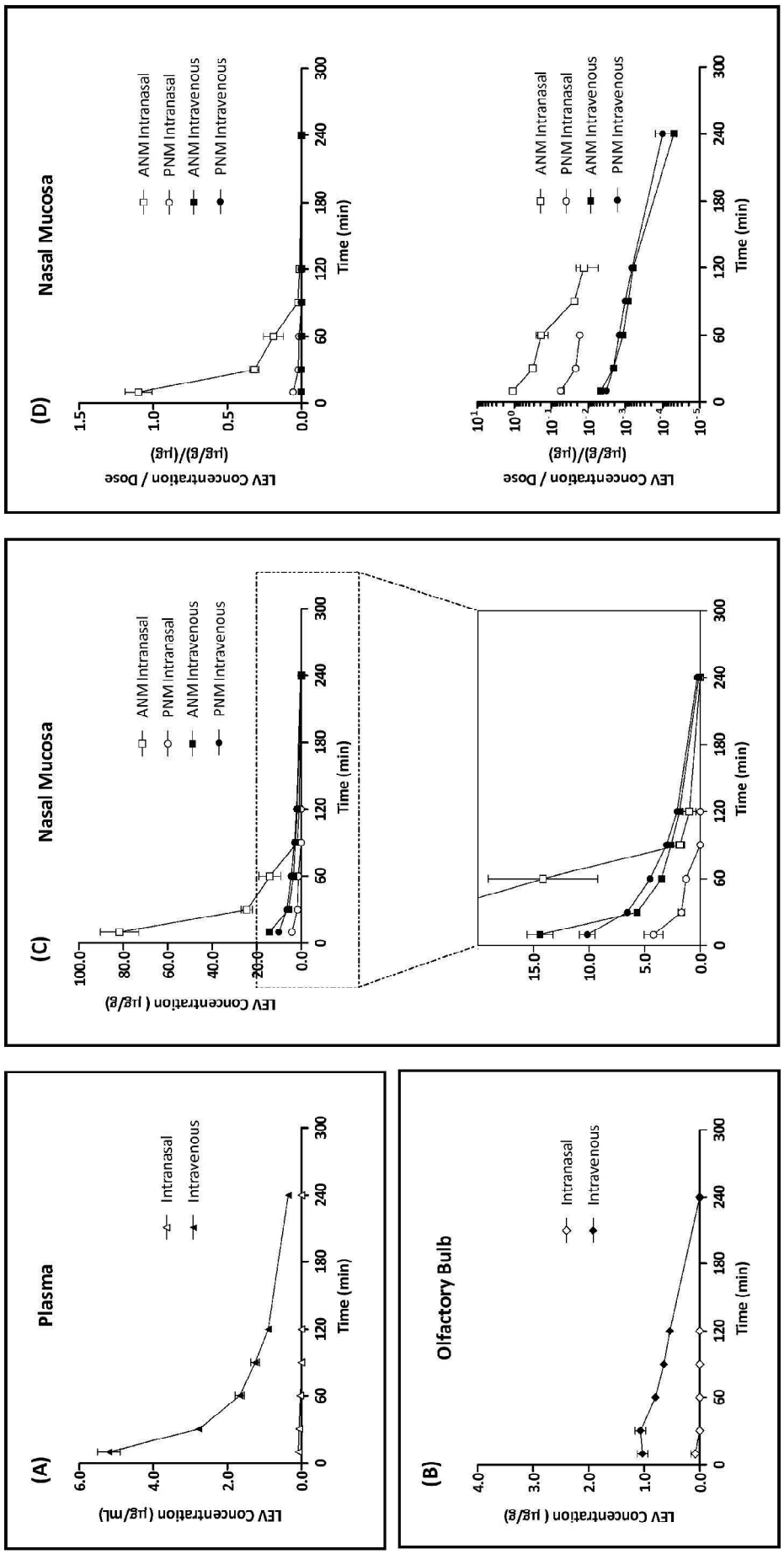

Figure III.3.1. Concentration-time profiles of levofloxacin (LEV) in plasma (A), olfactory bulb (B) and nasal mucosa (C) following intranasal (IN, 0.24 mg/kg) and intravenous (IV, 10 mg/kg) administration to rats. Dose-normalized concentration-time profiles of LEV in nasal mucosa (D – linear and semi-logarithmic graphs). Symbols represent the mean values ± standard error of the mean of four determinations per time point (n = 4).

Table III.3.2. Mean pharmacokinetic parameters of levofloxacin following intranasal (IN) and intravenous (IV) administration to rats of 0.24 mg/kg and 10 mg/kg doses, respectively.

Pharmacokinetic Parameters[a]	*Plasma*		*Olfactory Bulb*		*Anterior Nasal Mucosa*		*Posterior Nasal Mucosa*	
	IN	*IV*	*IN*	*IV*	*IN*	*IV*	*IN*	*IV*
C_0 (μg/mL or μg/g)	-	7.131	-	-	-	-	-	-
t_{max} (min)	10.0	-	10.0	30.0	10.0	10.0	10.0	10.0
C_{max} (μg/mL or μg/g)	0.085	-	0.083	1.073	81.751	14.402	4.211	10.164
$AUC_{0\text{-}t}$ (μg.min/mL or μg.min/g)	3.385	359.439	ND	94.025	2333.415	692.890	125.370	720.065
AUC_{all} (μg.min/mL or μg.min/g)	3.895	359.439	ND	126.485	2392.935	692.890	144.825	720.065
$AUC_{0\text{-}inf}$ (μg.min/mL or μg.min/g)	5.219	402.069	ND	176.216	2357.812	700.931	183.218	738.990
AUC_{extrap} (%)	35.1	10.4	ND	46.6	1.0	1.1	31.6	2.6
k_e (min^{-1})	0.019	0.008	ND	0.007	0.041	0.019	0.022	0.016
$t_{½}$ (min)	37.4	82.2	ND	105.3	17.0	35.7	30.9	44.0
MRT (min)	57.5	93.9	ND	156.0	28.1	59.0	49.8	70.1

[a] Parameters were estimated using the mean concentration-time profiles obtained from four different animals per time point (n = 4).

AUC_{all} Area under the concentration time-curve from time zero to the last time point; $AUC_{0\text{-}inf}$, Area under the concentration time-curve from time zero to infinite; $AUC_{0\text{-}t}$, Area under the concentration time-curve from time zero to the last measurable drug concentration; C_0, Plasma concentration back-extrapolated to time zero; C_{max}, Maximum concentration; k_e, Apparent elimination rate constant; MRT, Mean residence time; ND, Not determined; $t_{1/2}$, Elimination half-life; t_{max}, Time to achieve the maximum concentration.

Table III.3.3. Dose-normalized pharmacokinetic parameters of levofloxacin following intranasal (IN) and intravenous (IV) administration to rats.

Dose-Normalized Pharmacokinetic Parameters[a]	*Plasma*		*Olfactory Bulb*		*Anterior Nasal Mucosa*		*Posterior Nasal Mucosa*	
	IN	*IV*	*IN*	*IV*	*IN*	*IV*	*IN*	*IV*
C_0 / Dose (µg/mL or µg/g)/(µg)	-	2.317 x10^{-3}	-	-	-	-	-	-
C_{max} / Dose (µg/mL or µg/g)/(µg)	1.124 x10^{-3}	-	1.098 x10^{-3}	0.349 x10^{-3}	1.081	4.679 x10^{-3}	0.056	3.302 x10^{-3}
AUC_{0-t} / Dose (µg.min/mL or µg.min/g)/(µg)	0.045	0.117	ND	0.031	30.865	0.225	1.658	0.234
AUC_{all} / Dose (µg.min/mL or µg.min/g)/(µg)	0.052	0.117	ND	0.041	31.653	0.225	1.916	0.234
AUC_{0-inf} / Dose (µg.min/mL or µg.min/g)/(µg)	0.069	0.131	ND	0.057	31.188	0.228	2.424	0.240

[a] IN and IV pharmacokinetics parameters divided by the average amount of levofloxacin administered by IN (75.6 µg) and IV (3078 µg) routes, respectively.
AUC_{all}, Area under the concentration time-curve from time zero to the last time point; AUC_{0-inf}, Area under the concentration time-curve from time zero to infinite; AUC_{0-t}, Area under the concentration time-curve from time zero to the last measurable drug concentration; C_0, Plasma concentration back-extrapolated to time zero; C_{max}, Maximum concentration; ND, Not determined.

With respect to olfactory bulb, only one of the samples obtained after IN administration was quantified above the LLOQ, yielding the only non-zero mean value in this matrix which corresponds to the mean C_{max} achieved at 10 min post-dosing (t_{max}). Therefore, these are the only pharmacokinetic parameters shown in *Table III.3.2.* following this topical administration route. In the case of IV administration, C_{max} was attained in olfactory bulb at 30 min post-dose (t_{max}) and samples were quantified at all time points except the last one (240 min), as depicted in *Figure III.3.1.(B)*. All the pharmacokinetic parameters of LEV in olfactory bulb following IV injection were estimated and listed in *Table III.3.2.*. The dose-normalized C_{max} parameter (C_{max}/dose) obtained for IN delivery is 3.1-fold higher than for IV administration, as shown in *Table III.3.3.*. However, the dose administered by IN route (0.24 mg/kg) was lower than the IV dose (10 mg/kg), resulting in a mean C_{max} of 0.083 µg/g for the IN group which is considerably lower than the value of 1.073 µg/g (C_{max}) obtained after IV administration (*Table III.3.2.*).

Nasal mucosa of two different nasal regions was analysed in this study and concentration-time profiles of LEV in ANM and PNM were obtained for each administration route (*Figure III.3.1.(C)*). In both ANM and PNM, C_{max} was attained at 10 min post-dose via IN and IV delivery (*Table III.3.2.*). After IV administration, the concentration-time profiles of LEV in ANM and PNM were comparable. The same was not observed in the case of IN administration where the concentrations attained in ANM were clearly higher than those in PNM during the first 60 min of the study. A rapid and continuous decay of the concentration values was observed in ANM after this topical administration that resulted in much lower concentrations at the following 90 min and 120 min post-dose and in samples either below the LLOQ or undetected at the last time point (240 min post-dose). In PNM, at the three last time points of the study, samples were always below the LLOQ or undetected, which constraints the information about the pharmacokinetic behavior of LEV in this nasal mucosa region. Taking in consideration these nasal mucosa profiles (*Figure III.3.1.(C)*), it is evident that the concentration-time profile in ANM during the first 60 min post-dose reveals markedly higher concentrations after IN administration than the corresponding ones obtained after IV administration, whereas in PNM the only three quantified mean concentrations were lower following IN delivery. As for the last three time points, the concentration-time profile in ANM for the IN group followed closely that of the IV group with lower values. Having in mind that the dose administered by IN route (0.24 mg/kg) was approximately 41.7-fold inferior to the IV dose (10 mg/kg), dose-normalized concentrations in PNM become higher than the corresponding values for IV administration (at 10, 30 and 60 min post-dose), while in ANM dose-normalized concentrations after IN administration are always above those found for IV route, not only at the first three time points but even up to 120 min (*Figure III.3.1.(D)*).

Dose-normalized pharmacokinetic parameters given in *Table III.3.3.* reflect these results. The C_{max}/dose and $AUC_{0\text{-}inf}$/dose of LEV in ANM are 231.0-fold and 136.8-fold greater, respectively, after IN compared with their equivalents after IV delivery. In PNM, C_{max}/dose for IN route is only 17.0-fold higher than that after IV administration and AUC_{all}/dose, more appropriate for comparison, is 8.2-fold higher following IN administration.

III.3.4. DISCUSSION AND CONCLUSION

The aim of the present study was to investigate and compare the pharmacokinetic behavior of LEV in plasma, olfactory bulb and nasal mucosa after drug IN and IV administration to rats. Antibiotics are commonly administered orally in CRS and there is no clear benefit supporting the use of IV antibiotics (Craig and Goyal, 2015). However, IV route was selected for this study because in rats the absolute oral bioavailability of LEV is just 59% (Fujieda et al., 1996) unlike in humans where it approaches 100% (Davis and Bryson, 1994) and when testing the potential of a new route of administration it is recommended to compare it with IV administration where drug bioavailability is 100% avoiding the limiting factor of intestinal absorption. For IN delivery, a thermoreversible *in situ* gel was used with a final percentage of PF-127 that is in the range of commonly reported ophthalmic and nasal gel formulations. PF-127 is generally considered as non-toxic and non-irritant and proved by some authors to be safe with respect to nasal mucosa integrity even in higher percentages (Abdelkader and Mansour, 2015; Bankhele et al., 2012; Bhandwalkar and Avachat, 2013; Jagdale et al., 2016; Majithiya et al., 2006; Pathak et al., 2014; Perez et al., 2012; Rao et al., 2017). Moreover, during the *in vivo* experiments and at the moment of rat nose dissection, no signs of bleeding or nasal mucosa shrinkage were observed.

In plasma, dose-normalized exposure pharmacokinetic parameters C_{max}/dose and AUC_{all}/dose of LEV following IN administration were inferior to C_0/dose and AUC_{all}/dose obtained after IV delivery (*Table III.3.3.*). The systemic absolute bioavailability of LEV following IN administration of the thermoreversible *in situ* gel (estimated as the ratio of the dose normalized $AUC_{0\text{-}inf}$ parameters) was approximately 53%. These results demonstrate that IN route leads to a moderate systemic exposure to LEV when using the prepared thermoreversible *in situ* gel for IN delivery. As mentioned before, taking into account the percentage of extrapolation in the calculation of $AUC_{0\text{-}inf}$, as well as the number of non-zero samples and magnitude of the drug elimination half-life, the AUC_{all} is more appropriate than $AUC_{0\text{-}inf}$ for comparison between the two routes in this matrix and the latter obtained for IN route should be carefully interpreted. The above conclusion of an IN lower systemic exposure than IV delivery, drawn from the absolute (dose independent) pharmacokinetic parameters, is even more evident for the real doses administered by IN

and IV routes (0.24 mg/kg and 10 mg/kg, respectively). The three mean plasma concentration values attained with the lower topical dose were much lower than those obtained after IV delivery, confirming that systemic exposure of LEV following the IN dose of 0.24 mg/kg is minimal when compared to the IV administered dose. Therefore, IN delivery will guarantee a reduced LEV distribution to other organs, having the important advantage of minimizing or even avoiding the development of adverse effects; this is an important issue for CRS patients that often require multiple courses of antibiotics and that concomitantly use corticosteroids, increasing the risk of development of tendinitis and tendon rupture (Craig and Goyal, 2015; Sousa et al., 2014).

LEV is widely distributed throughout the body and penetrates well into most body tissues and fluids with exception of those of the CNS (Fish and Chow, 1997; Sharma et al., 2009). In fact, in the present study, mean LEV concentrations measured in olfactory bulb of the rats were lower than those in nasal mucosa tissue after IV injection. The fact that more quantifiable sampling points would be required for a more reliable determination of $AUC_{0\text{-}inf}$ in this matrix suggests that the significance of $AUC_{0\text{-}inf(olfactory\ bulb)}/AUC_{0\text{-}inf(plasma)}$ value of 0.44 should be assessed in the light of this limitation. However, this tissue-to-plasma ratio may still be compared with its analogue for nasal mucosa (given below) and interpreted as a sign of the limited distribution of LEV into brain which is probably hindered by the complex blood-brain barrier. In spite of this limited access to brain by traditional systemic administration routes, the potential of IN route to deliver drugs directly to brain by circumventing the blood-brain barrier, has drawn our attention to assess LEV in olfactory bulb after this topical nasal route, due to its special connection with nasal cavity. The higher IN dose-normalized value found for mean C_{max} compared to the corresponding IV value supports this concept of a direct transport to brain (*Table III.3.3.*). However, this is not a real drawback because, in practice, the dose administered by IN route is usually small due to the restrictions imposed by the anatomical and physiological characteristics of nasal cavity, as well as the physicochemical properties of the drug in the preparation of nasal formulations. In the present work, for an IN dose 41.7-fold lower than IV dose (0.24 mg/kg and 10 mg/kg, respectively) the only mean concentration value of LEV in olfactory bulb after IN delivery found at the first sampling time (t_{max} = 10 min) was markedly lower than that obtained after IV delivery at t_{max} = 30 min (*Table III.3.2.*), which proves that in these conditions the olfactory bulb exposure is negligible. Although total brain exposure was not

herein assessed, the results from olfactory bulb will guarantee in combination with plasma results, a safe profile for CNS adverse effects with the administered dose of 0.24 mg/kg.

LEV mean concentrations were comparable in ANM and PNM after IV administration, leading to the conclusion that drug was homogenously distributed between both nasal regions after this parenteral route. This is supported by ANM and PNM tissue-to-plasma ratios which were quite similar to each other, namely 1.74 ($AUC_{0\text{-}inf(ANM)}/AUC_{0\text{-}inf(plasma)}$) and 1.84 ($AUC_{0\text{-}inf(PNM)}/AUC_{0\text{-}inf(plasma)}$), respectively. Moreover, these ratios demonstrate an excellent penetration of LEV into nasal mucosa tissue, contrasting with the poor concentration levels and tissue-to-plasma ratio found for olfactory bulb following IV delivery. In the case of IN administration, the nasal mucosa exposure to LEV (assessed by C_{max} and AUC parameters) was higher in ANM than in PNM. This suggests a heterogeneous deposition pattern of LEV formulation in nasal cavity, which is consistent with the fact that the thermoreversible *in situ* gel was applied in the ANM region and that a fraction of its volume also reached PNM. In addition, the IN concentration-time profiles of LEV in both nasal regions show that there was no increase of LEV concentration in PNM with decreasing LEV concentration in ANM. This is in agreement with the mucus flow pattern reported by (Morgan et al., 1984) in rats where mucus flow of the nasal region corresponding to our ANM region was ultimately towards the nasopharynx without passing through our PNM region. This mucus flow is a consequence of nasal MCC that is responsible for the reduced residence time of the drug in nasal cavity. To overcome this problem a gel of PF-127 was prepared in order to ensure *in situ* gelation and prevent the drug from being easily removed from the nasal cavity by MCC. From the concentration-time profile of LEV in ANM (*Figure III.3.1.(C and D)*) following IN administration a rapid decrease of its concentration was observed. This result contrasts with results obtained for CIP and with the ability of *in situ* gelling polymeric systems of PF-127 to control drug release by self-assembling into a compact aggregate of micelles that incorporate the drug, slowing its release at the local delivery site over a period of time. However, drug release from these *in situ* gelling polymeric systems is a complex process which depends on the concentration of the polymer, drug solubility in the gel, initial state of the drug-loaded polymer, polymer swelling ability, diffusion rate of the drug within the gel and dissolution pattern of the gel (Aliabadi et al., 2008; Anand et al., 2012; Anderson et al., 2001; Taha et al., 2014; Yang and Alexandridis, 2000). This calls for an effort to improve the composition

of the gel possibly by combining PF-127 with other mucoadhesive polymers that would simultaneously control and prolong LEV delivery of formulation in nasal cavity, providing sustained higher concentrations in the nasal mucosa over a higher residence time (Anand et al., 2012; Gonda and Gipps, 1990; Swamy and Abbas, 2012). Notwithstanding, the rapid decay of LEV concentration observed in ANM (*Figure III.3.1.(C)*) using the prepared thermoreversible *in situ* gel of PF-127, suggests that more frequent administrations of this IN formulation need to be given to attain high concentrations of LEV in nasal mucosa for a pre-determined time of treatment. In PNM, the only three measured concentrations of LEV after IN administration of 0.24 mg/kg were low and have limited a reliable estimation of some of the pharmacokinetic parameters (namely $AUC_{0\text{-}inf}$) and the interpretation of the results. Despite this handicap, the dose-normalized results obtained for ANM and PNM demonstrate that the topical IN administration of LEV PF-127 gel offers, by itself, higher pharmacokinetic exposure parameters (C_{max}/dose, $AUC_{0\text{-}inf}$/dose or AUC_{all}/dose) compared to IV delivery (*Table III.3.3.*); moreover, no further conclusions about dose-normalized concentrations can be drawn after 120 min post-dose in ANM and after 60 min post-dose in PNM (*Figure III.3.1.(D)*). The advantages of IN over oral administration, as far as nasal mucosa exposure is concerned, are expected to be even more evident than those of IN over IV administration, since absolute oral bioavailability in rats is only 59% (Fujieda et al., 1996).

The present study should be understood as a proof-of-concept study which demonstrates the potential of delivering LEV to biophase by IN route in comparison to IV administration. A further positive development would be the optimization of the thermoreversible *in situ* gel to sustain the delivery of LEV and enable extension of reliable conclusions for the total time of the study. This study has proved that – using an IN dose of 0.24 mg/kg, much lower than IV dose – minimal or negligible systemic and brain exposure occurs. Therefore, it is expected that topical IN delivery of LEV will be an alternative and safe approach in the management of refractory CRS.

CHAPTER IV

GENERAL DISCUSSION / CONCLUSION

The present thesis relies on *in vivo* studies performed to investigate the pharmacokinetic behavior of two FQs, CIP and LEV, after IN administration, as a promising strategy to achieve higher concentrations locally that could not be attained by systemic administration, sparing the systemic adverse effects of oral and intravenous antibiotics. These are important issues for the treatment of a resistant bacteria infection present in CRS.

- The IN administration can be evaluated by several *in vitro*, *ex vivo* and *in vivo* techniques. *In vitro* and *ex vivo* experiments have the ability to control a lot of variables, enabling the investigation of molecular mechanisms involved in nasal drug permeation and metabolism and the evaluation of local toxicity of drugs and excipients. These techniques also allow high throughput screening of a large number of drug candidates, reducing the number of animals to be used at later stages. However, the simplified environmental conditions under which these experiments are performed do not fully mimic the complexity of nasal mucosa physiology (including MCC, nasal cycle and breathing), the role of blood flow and the different possible pathways that can lead to local, systemic and CNS drug disposition. Therefore, the relevance of *in vitro* and *ex vivo* studies is limited and *in vivo* experiments are essential. In fact, only *in vivo* techniques, which use the whole animal, are able to follow the evolution of drug concentrations in body compartments over time and thus fully assess drug pharmacokinetics.

A rabbit model to study rhinosinusitis was developed in the early 20th century and became the main tool in animal experiments in this field (Hilding, 1941). The relative lack of experimental reagents and the inability to genetically manipulate these animals have favored the transition to the use of rodents, which do not present these disadvantages, despite their anatomical lack of true paranasal sinuses (Jacob et al., 2001; Jeon et al., 2005). This eventual drawback is overweighed by the advantages of low cost, availability and easy maintenance and handling. In fact, rodents are common and reliable animals used in *in vivo* IN delivery studies, particularly in pharmacokinetic research, that is the main purpose of this work, and can be complemented with pharmacodynamic studies. These considerations justified the use of male Wistar rats – rodents of larger size than mice – in the *in vivo* pharmacokinetic studies previously described in this thesis.

- One of the first aims of the work presented in this thesis was the development and validation of a bioanalytical method to quantify FQs in different biological matrices, to support subsequent *in vivo* pharmacokinetic studies. This goal was achieved in several steps, starting with the choice of RP-HPLC – the preferred quantification method used in bioanalytical laboratories for the determination of FQs – and with the acquisition of know-how in the bioanalytical field and optimization of analytical conditions using human plasma, to be subsequently transposed to bioanalysis of FQs in rat matrices. The selection of human plasma for this purpose was dictated by the fact that this matrix is easily accessible, available in large quantities and ethically more acceptable, reducing the number of rats to be sacrificed in further steps.

Following this line of work, two RP-HPLC methods coupled with FD were developed and fully validated in human plasma, one for the quantification of second-generation (NOR, CIP and LOM) and another for the quantification of third- and fourth-generation (LEV, PAZ, GAT, MOX and TRO) FQs. Both methods demonstrated to fulfill the validation international requirements for the reliable quantification of above mentioned FQs in a wide plasma concentration range, with good selectivity and sensitivity and sample dilution feasibility. Therefore, they can be applied to monitor plasma drug concentrations in clinical practice, consequently improving antibiotherapy efficacy and preventing the development of bacterial resistance, since it is not possible to routinely measure tissue drug concentration at the site of action. In addition, both RP-HPLC methods are useful tools to support clinical pharmacokinetic studies, for example, in the course of the development of IN formulations incorporating these drugs.

Despite multiple attempts to transfer to rat matrices the analytical conditions of the RP-HPLC method validated in human plasma for determination of LEV, PAZ, GAT, MOX and TRO – which have a broader antibacterial spectrum and are therefore more promising to face the diversity of the microbial community of CRS – difficulties were encountered with the appearance of specific matrix interferents that ruled out this possibility. So, the RP-HPLC method validated in human plasma for the determination of NOR, CIP and LOM was the best option found, together with the inclusion of LEV, to proceed with the development and validation of a bioanalytical method for quantification in rat biological matrices. Plasma, olfactory bulb and nasal mucosa rat matrices were selected, since the former two represent the relevant matrices to evaluate and compare the safety profile and

the latter to assess the potential efficacy benefits of the IN administration route. A few modifications of the aforementioned method were made that justified a partial validation of the RP-HPLC method for each rat matrix. The limited availability of rat nasal mucosa and olfactory bulb tissues due to their small size, reinforced the performance of a partial validation. The innovative step was the homogenization procedure of the small sized tissue samples whose feasibility required a high dilution of the drug. Moreover, this step was found to contribute to drug loss and hence to variability between samples; therefore the IS was added before homogenization of tissues. The RP-HPLC method described for rat matrices was successfully implemented and demonstrated to be linear over the calibration range, accurate, precise and selective. Partial validation results proved that it can be applied to reliably quantify LEV, NOR, CIP and LOM in plasma, olfactory bulb and nasal mucosa of rats.

All three RP-HPLC methods presented in the thesis were coupled with FD to take advantage of FQs fluorescence properties and thus enhance sensitivity and selectivity of the method. In addition, protein precipitation was a common simple sample treatment procedure and the mobile phase was essentially composed of water. These features, together with the technology used, make these bioanalytical methods economically attractive for most laboratories.

The experimental work at this stage led to the selection of LEV and CIP among the set of FQs included in the RP-HPLC-FD method partially validated in rat matrices. The choice was based on the fact that CIP and LEV are most commonly used FQs: the former is the most potent quinolone against *P. aeruginosa* and the latter has a broader antibacterial spectrum and belongs to a newer generation.

- Before the *in vivo* pharmacokinetic studies, preliminary experiments took place in order to optimize the IN administration methodology. The ideal situation for IN administration is the one that mimics the real conditions with minimal damage of nasal tissue. Having these goals in mind, the administration of drops with a micropipette to the entrance of nostrils was attempted; however, the dose administered was not sufficiently precise and accurate due to the unsteady behavior of the animal and subsequent loss around the snout. This pointed to the need of anesthesia, a procedure that is common in preclinical published work. At this stage, the theoretical ability of aerosol sprays to achieve

a uniform and widespread distribution of drug together with the advantage of dose precision was considered and practiced with simple solutions. The rats did not survive even after optimization of the volume administered and depth of the application site within the nasal cavity. Death was caused by respiratory distress as suggested by observation of colored solution occluding nasopharynx and impairing airflow of these obligate nose breathers (*Chapter III, section III.1.*). Due to these results and also taking into account the short residence time of tested solutions in nasal cavity, the use of a thermoreversible *in situ* gel was envisaged as a viable and promising option to proceed with *in vivo* studies. In fact, IN thermoreversible *in situ* gels have the dual advantage of being easily measured and administered as a liquid solution, undergoing transition into a viscous gel at the delivery site, by nasal physiological temperature, prolonging the residence time of the formulation in nasal cavity.

The intranasal administration of the *in situ* thermoreversible gel used in *in vivo* studies was performed by introducing, inside the nasal cavity, 1.0 cm of a flexible polyurethane tube attached to a microliter syringe with anesthetized rats placed in recumbent lateral position. This was the simplest way to firmly and precisely insert the catheter into nasal cavity for administration of the thermoreversible *in situ* gel. This administration technique, together with the *in situ* gelling ability of the optimized formulation, proved to be well tolerated by rats that survived throughout the whole study without any visible signs of mucosal damage and ensured an improved precision and accuracy of dosing.

The dose administered by IN route depends on the volume delivered and on the drug solubility in the *in situ* gel. A compromise must be found between the small size of the nasal cavity and the requirement to cover the highest possible area of nasal diseased tissue for the drug to exert its local effect. An excessive volume may cause drug formulation loss either by anterior leakage or posterior nasal-drip with subsequent swallowing or drainage to the lower respiratory tract. In mice, solution volumes less than 25 µL have been reported to be distributed primarily to the upper respiratory tract, whereas a 50 µL volume was predominantly deposited in the lower respiratory tract; for all doses in this range, minimal amounts were detected in the stomach (Southam et al., 2002; Turner et al., 2011). The final volume used was 22-27 µL (according to the weight of the rat – 80 µL/kg), which may ensure minimal drug formulation loss, considering not only the

larger size of the animal (compared to mice) but also the enhanced viscosity of the IN formulation. To reinforce this strategy, this volume dose was divided into both nostrils to obtain equal access of drug to both nasal cavity halves and as an attempt to enhance surface contact area and avoid the influence of nasal cycle on inter-administrations variability.

Based on pre-existent experience in the research group, PF-127 was selected as the gelling agent for the IN formulation (Serralheiro et al., 2015, 2014), since it is one of the most widely used thermosensitive materials in topically delivered pharmaceutical preparations (Li and Bartlett, 2014). The percentage of PF-127 was optimized to ensure gelation at the nasal physiological temperature (≈ 34 °C) and 21.6% (w/v) of drug-free PF-127 yielded a sol-gel transition temperature of 29-31 °C. As a result, a starting 24% (w/v) PF-127 was selected and used to incorporate FQs (CIP and LEV) leading to a final PF-127 concentration of 21.6% (w/v), which is within the range of commonly reported IN thermoreversible *in situ* gels (Bhandwalkar and Avachat, 2013; Majithiya et al., 2006; Perez et al., 2012; Rao et al., 2017). The concentration of the incorporated FQs – 3 mg/mL (0.3% w/v) – was based on values reported in literature for several *in situ* ophthalmic gels containing PF-127, designed for topical delivery of some FQs, to guarantee complete solubilization of both CIP or LEV in this vehicle (Abdelkader and Mansour, 2015; Ahmed et al., 2014; Nesseem, 2011). The dose volume and the concentration of IN formulation determined the drug dose to be administered in the *in vivo* studies (0.24 mg/kg). To our knowledge this is one of the lowest doses cited in literature for airway (IN and pulmonary) delivery of FQs in rats.

- *In vivo* pharmacokinetic studies were performed using IV administration as the conventional delivery route to be compared with IN administration. Despite the fact that IV antibiotics are not routinely recommended in the management of CRS, this route was chosen and oral administration discarded, because absolute oral bioavailability of CIP and LEV in rats is only 20% and 59%, respectively, much lower than in humans (70% and 100%, respectively). Moreover, when testing a new route of administration it is recommended and easier to compare it with IV administration where bioavailability is 100%, thus avoiding the limiting factor of intestinal absorption.

Time course of CIP and LEV concentrations in nasal mucosa, olfactory bulb and plasma of healthy adult Wistar rats (weighing 280–340 g; 10-11 weeks old) was investigated after IN and IV administration.

Bacteria present in most cases of CRS can be within biofilms, adherent to the surface of nasal and sinus epithelial layer, or in their planktonic form, when released from the biofilm to nasal or sinus secretions, also invading fragile mucosal underlying tissue. The decision to quantify CIP and LEV only in nasal mucosa and not in nasal secretions of rats was based on two arguments: firstly, absorption or aspiration collection procedures hardly yield sufficient nasal secretion volume for bioanalysis in the small animals used, whereas nasal lavage would lead to unpredictable dilution factors, impairing an accurate drug concentration determination and interpretation; secondly and most important, negative bacterial culture of nasal secretions does not reflect the eradication of bacteria in nasal mucosa biofilms, nor does it measure (or predict) the drug concentration in tissue (where planktonic bacteria also exist). Moreover, in collected nasal secretions drug from gel formulation would be quantified. In conclusion, nasal mucosa tissue is the most important infection site when studying CRS and thus the most adequate biophase to be used in pharmacokinetic studies.

To produce adequate information with respect to potential adverse effects/toxicity, plasma and olfactory bulb were selected as the appropriate rat matrices. Plasma concentrations dictate the propensity to develop systemic adverse effects, since drugs are distributed to organs and body tissues via systemic circulation. Owing to the direct connection between nose and brain, IN administration may represent a higher risk of developing CNS adverse effects, as compared with systemic administration routes. This is reinforced by the fact that CNS, more specifically olfactory bulb, is directly exposed to the nasal cavity environment where the IN formulation is applied. Several studies have shown that when drugs are intranasally administered, concentrations attained in the olfactory bulb are generally amongst the highest CNS concentrations observed (Chow et al., 1999; Colombo et al., 2011; Kaur and Kim, 2008; Wong and Zuo, 2013). This was the driving argument that led us to infer CNS adverse effects from the concentrations observed in the olfactory bulb.

The pharmacokinetic results obtained for CIP and LEV administered by IN and IV routes have been reported and interpreted in detail in the previous chapter (*sections III.2.*

and III.3.). An integrative and comparative discussion will now be presented focusing on the main aspects related to concentration-time profiles and exposure pharmacokinetic parameters.

- After IN administration, both FQs showed a heterogeneous deposition pattern in nasal mucosa, attaining markedly higher concentrations and/or exposure parameters in ANM than in PNM, in contrast with the homogeneous pattern observed after IV administration. These results suggest that IN application of the thermoreversible *in situ* gel containing, either CIP or LEV (0.24 mg/kg), was in the anterior region of the nose of rats and only a fraction of its volume reached the posterior region. This is reflected by the lower exposure of PNM to both drugs and especially to LEV, where concentrations were only measured at three time points.

Moreover, a plateau was observed in the concentration-time profile of CIP in ANM and PNM, whereas in the case of LEV a more rapid and continuous decay of concentration values was observed, particularly in the anterior region. This points to a more prolonged delivery of CIP compared to LEV into nasal mucosa tissue.

Taking into account that the same vehicle was used for both drugs and considering that thermomechanical properties of this *in situ* gel with CIP or LEV are not expected to be significantly different, one possible explanation for the observed behavior of both drugs is a difference in drug release profile which is determined not only by polymer concentration but also by drug polymer solubility. In fact, LEV is far more soluble in water than CIP and therefore it is likely to be also more soluble in this type of hydrogel and diffuse more rapidly from the formulation. *In vitro* drug release studies of CIP (Abdelkader and Mansour, 2015) and LEV (Saher et al., 2016) from *in situ* ophthalmic gels containing 20% (w/v or w/w) of PF-127 at pH 5.0 and 7.4, respectively, have shown a faster release of LEV from formulation with 70% of LEV released versus 20% of CIP after 2 h of the *in vitro* study. The faster release of LEV leads to a higher concentration available for tissue penetration which is consistent with the higher C_{max} (81.75 µg/g *versus* 43.83 µg/g) observed at 10 min in the anterior region (where the *in situ* gel was applied) and to smaller concentrations available at subsequent times, which do not compensate the elimination processes from tissue and therefore justify the non sustainable concentration in the nasal mucosa, in opposition with

the sustained concentration observed in CIP. In fact, a higher extent of ANM and also PNM exposure to CIP was observed when compared to LEV.

With the dose administered intranasally (0.24 mg/kg), only vestigial concentrations of CIP were found in plasma during the entire study and low concentrations of LEV were observed at the first three time points. In olfactory bulb, only one sample was quantified above the LLOQ, 10 min after IN administration, both of CIP and LEV. Therefore, IN administration of the prepared thermoreversible gels at the referred dose is a safe procedure with no risk for systemic and CNS adverse effects.

To enable comparison between IN and IV administration routes, dose-normalized values were used to eliminate the differences between doses delivered by each route (0.24 mg/kg for IN route *versus* 10 mg/kg for IV route).

The dose-normalized exposure parameters obtained for nasal mucosa, C_{max}/dose and $AUC_{0\text{-}inf}$/dose (or AUC_{all}/dose), were consistently higher by IN route than by IV route – one and two orders of magnitude higher in PNM and ANM, respectively – both for CIP and LEV. This confirms the potential of CIP and LEV thermoreversible *in situ* gels in delivering drugs to biophase, in particular to ANM and more limited to PNM, via IN administration in comparison with the IV route.

In plasma, C_{max}/dose (determined from vestigial/low values) and C_0/dose parameters for IN administration are approximately 12- and 2-fold lower than for IV administration for CIP and LEV, respectively, evidencing the advantage of a lower systemic exposure for IN delivery of the *in situ* gel. With respect to olfactory bulb, comparison of C_{max}/dose values shows that moderately higher values (5- and 3-fold higher) were obtained after IN administration than after IV administration when CIP and LEV were delivered. Based on these results, it can be concluded that IN delivery of CIP and LEV with the *in situ* gel leads to lower systemic exposure than IV administration. However, the higher IN C_{max}/dose in olfactory bulb may suggest a direct transport of the drug from the nasal cavity to brain, hence to a higher risk of CNS adverse effects which are the second most frequently reported for FQs. This is an alert to carefully select the administered dose and the administration technique to obtain maximum effectiveness with minimum brain risk. Notwithstanding, it is not a real drawback because, anatomical and physiological characteristics of the nasal cavity and physicochemical properties of the drug in the

formulation, limit the dose that can be intranasally administered to lower values than typical doses administered intravenously. In the present *in vivo* studies, negligible and minimal exposure in plasma and olfactory bulb was demonstrated using a 41.7-fold lower IN dose than IV dose (0.24 mg/kg vs 10 mg/kg) of CIP and LEV.

The work described in this thesis is the first step to obtain key information about CIP and LEV concentration levels at the biophase as well as about systemic and brain exposure after IN delivery. Bearing in mind that the expectations raised by the dose-normalized results in nasal tissue are clearly higher in the ANM (than in PNM), it is expected that, with low doses, topical IN delivery of CIP and LEV will still be an advantageous alternative and safe approach to be implemented in the management of refractory CRS.

Future Perspectives:

The lower systemic and brain exposure demonstrated with lower doses administered by IN route, could open the way to test other FQs of third- and fourth-generations that have been withdrawn from the market due to their systemic toxicity.

In the future, this exploratory work should be pursued with microbiological pharmacodynamic studies with animal infection models of CRS presenting biofilms, to assess the true effect of CIP and LEV dose to eradicate mucosal biofilm and infection using this topical approach.

Research in chronic biofilm-related infections is under development and, although several procedures and parameters for determination of antibacterial activity against biofilm bacteria have been implemented (e.g. minimum biofilm-eradication concentration), their standardization is still missing, as well as pharmacokinetic/pharmacodynamic breakpoints to predict therapeutic success.

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