David Silva Melo

TRAINING REPORT
ON HOSPITAL PHARMACY

Training report on Hospital Pharmacy executed in the Capital´s Region Pharmacy, under the guidance of Dr. Vagn Handlos, within the Master of Pharmaceutical Sciences from the Faculty of Pharmacy, University of Coimbra.

September of 2015

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UNIVERSIDADE DE COIMBRA

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Coimbra, September 2015.

Signature: _____

David Silva Melo

(Intern)

Signature: _____

Dr. Vagn Handlos

(Supervisor)

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1. Introduction

As part of my curricular training for the Integrated Master in Pharmaceutical Sciences from the Faculty of Pharmacy, University of Coimbra in Portugal, I had the opportunity to travel to Denmark to contact with different hospital pharmacy professional practices and learn more about a new (to me) healthcare system *"in situ"*. The training was planned and agreed upon the frame of European programme *Erasmus Plus* and it is equivalent to 280 hours, from the total of 920 hours required for the curricular training.

Right after finishing the first part of my training in community pharmacy, in Portugal, I headed north to Copenhagen to start the second chapter of my final training. The internship is inserted in the area of hospital pharmacy and lasted three months, from 5th of April till 6th of June, 2015.

The planning for the internship started to take place in December 2013, after the *Region Hovedstadens Apotek* (RAP) – The Capital´s Region Pharmacy - accepted me to collaborate with the organization, and by mid-2014 the backbone for the internship was completed.

My main supervisor and contact person was professor doctor Vagn Handlos, the senior scientist in Research and Development (R&D) department at RAP in Marielundvej, Herlev, the pharmacy headquarters where the central production is located. Among many things, it is worth mention that Vagn was the former Hospital Pharmacy Director at Rigshospitalet, from 1987 to 2005, and former Director of Education, Science and Research for the *European Association of Hospital Pharmacists* (EAHP), as for the new insights he has brought to me about hospital pharmacists in Denmark and in Europe are due to his large experience in the area.

I collaborated in a validation project from the quality control department aiming for the implementation of a new method for the quantification of Heparin in IV solutions, according to the new volume of European Pharmacopeia (8.3), Pharm. Eur. (8.3) supplement, which came into force on January 2015. The assay is classified as a Biological Assay and, being so, was expected to be a great challenge to validate since it was the first time Vagn, my supervisor, and I to work with this type of assay.

The project was divided in two parts, first the quantification of heparin potency using anti-factor IIa for testing the final product and, second, quantification using anti-factor Xa to analyse the raw material. During my stay, I only worked in the first part. The deadline for the project, established by the Danish Medicine Agency authorities, was 01/07/15. The culmination of the work developed resulted in a validation report that was sent out to the regulatory authorities.

The reason for RAP´s decision to take home the heparin assay was because, recently, they had encountered deviations in potency from the expected values in their heparin IV products. For quality control and batch released, the heparin products were analysed in an outside laboratory which still uses the previous method, using sheep

blood. In order to better understand if the differences in potencies were due to laboratory testing or production process, RAP made the decision to implement the method so that they could make their own analysis for their IV products. Also, from the economical point of view, taking home the assay has a very positive economical outcome for RAP.

Besides the work mentioned above, several opportunities were given to me to participate in many other areas such as having close contact and being constantly expose and updated about the activities in quality control (QC) and quality assurance (QA) departments and taken part in a project studying cytotoxic contamination in the workplace of hospital pharmacies in Denmark.

Also, I may add that I feel I had an excellent integration in both work and in the "Danish lifestyle". Vagn Handlos, along with the staff from QC and QA, always had the willingness to care and provide help for any problem I encountered, from automatic pipetting to bicycle punctures, and for that I am immensely grateful.

1.1. Report Format

To simplify the report I divided it in four parts.

The first part will be about the RAP organization. Here I make a brief overview about the services provided and the goals of the pharmacy.

Secondly, I approach the heparin project, starting with some theoretical details and up-to-date trivia about heparin and biological assays in order to better understand the drug and the analytical work developed. Right after, I describe my work within the heparin assay, however, here, I will not go into details about all the results and method improvements, but instead I´ll focus on my contribution and know-how from what I could learn from the analytical work developed. Therefore, I present a short list of challenges and problems faced and how we overcame them. The English version of the report is annexed in the end of the report and contains the data gathered and selected for the regulatory authorities.

Then, I talk about another project I got in contact with about cytotoxic contamination in the workplace of hospital pharmacies.

2. The Region Hovedstadens Organization

The *Region Hovedstadens* - Capital Region of Denmark - is a public authority headed by democratically elected politicians and a professional board of directors. The organization manages hospitals, performs research and services for the disabled and undertakes environmental duties. It collaborates with municipalities and the business community on developments concerning traffic, business and education. In total, there are 5 administratives *Regions* in Denmark: *Region Hovedstaden*, *Region Sjælland*, *Region Nordjylland, Region Midjylland* and *Region Syddanmark*.

Fig.2 - Region Hovedstaden logo.
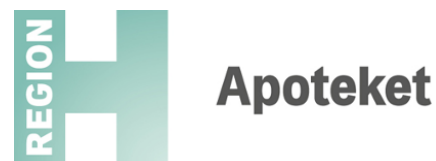
Fig.3 - Region Hovedstaden Apoteket (RAP) logo.

Fig.1 – Administratives Regions in Denmark.

2.1. Region Hovedstadens Apoteket

In total, there are 12 hospital pharmacies in Denmark. The Region Hovedstadens Apoteket (RAP), The Capital´s Region Pharmacy, is the hospital pharmacy for the Capital Region of Denmark.

It was established in 2007 as a result of a merger of the hospital pharmacies in the area of Copenhagen and Hillerød. The pharmacy delivers drugs and hospital pharmacy services, serving 2300 wards and other customers. The yearly turnover is about 360 million Euro (2011), and

Fig.4 - Map of Denmark and the hospital pharmacies.

has approximately 510 employees, including 75 pharmacists and 230 pharmacy technicians at all hospitals in the region.

RAP delivers registered drugs from own production facilities and clinical pharmaceutical services, including: clinical pharmacy, Top-Up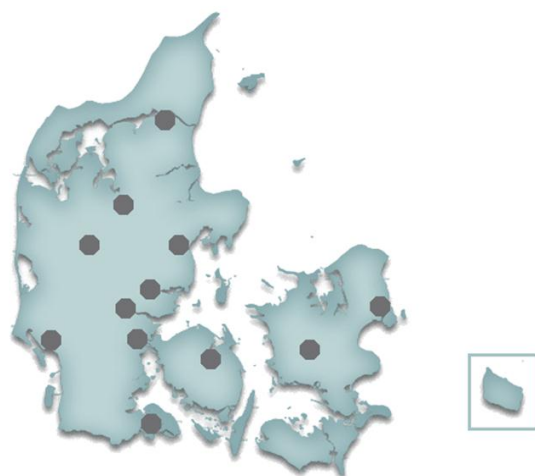 service, service production of antibiotics and cytotoxics and drug information. The centralized pharmacy functions can be found at:

- Marielundvej, Herlev: Logistics; central production; quality assurance (QA) and quality control (QC); IT department; administration.
- Rigshospitalet: central production.
- Frederiksberg hospital: Central production, unit dose dispensing.
- Bispebjerg hospital: drug information, production of reagents.
- Nordsjællands Hospital, Hillerød: decentralized service production.

The hospital pharmacy supports 3 centres for cancer treatment and prepares approximately 80.000 single doses of cytostatics per year for these centres.

The production departments produces approximately 2 million single containers of large volume parenterals, 300.000 patient oriented single doses of antibiotics, cytotoxics and TPN (total parenteral nutrition) per year together with 1.4 million ampules and 13 million tablets. The pharmacy is approved and inspected by the Danish Medicines Agency according to the European GMP regulation(1).

The pharmacy main products are not available from the industry and production logistics are based and updated according to hospital´s consumption data concerning previous years. Nevertheless, for some products, the pharmacy competes with the industry prices when selling drugs to hospitals, as the case for some IV solutions.

The pharmacy seeks to deliver safe and rational medication. Through combining purchase and hospital production, RAP tries to cover all customer´s needs. RAP exerts influence on the Capital Region drug consumption through formulary decision and invitation of tenders by the

Fig.5- Map of the Capital Region of Denmark and the hospitals of the region.

national organization Amgros A/S.

The drug information service is an independent platform accessible to health care professionals (pharmacists, nurses and doctors). The main goal is to give quality assured answers to questions from doctors and nurses and other healthcare professionals. Functions involve participating in drug and therapeutic committee work, drug consumption statistics, introducing new medication to the wards and pharmacy staff and literature service.

3. About Heparin

3.1. Brief History

Heparin, one of the oldest biological drugs still in widespread clinical use, is a naturally occurring glycosaminoglycan whose main function is to inhibit the coagulation of blood. It was discovered a century ago, in 1916, isolated from canine liver by Jay McLean, a graduate student of William H. Howell. However, there is some evidence that Maurice Doyon isolated heparin from dogs´ liver in 1911(2).

The next table can give a brief idea of heparin history throughout the century along with the important breakthroughs after the disputable discovery.

Table 1-Strutural history of heparin.

1918	Howel and Holt named the substance heparin.
1929	Best (who also discovered insulin) introduces heparin into clinical medicine at the university of Toronto.
1933-1936	Charles and Scott of Connaught laboratories working under Best finalized the method for large scale production of heparin from bovine lung.
1935	Murray conducted the first human trial of heparin for the prevention of post-operative thrombosis.
1935	Heparin was found in mast cells.
1950s	Proteoglycans where described.
1960s-1970s	Main structural features of heparin elucidated.
1978	Heparin proteoglycan finally identified.
1960s-1970s	Main structural features of heparin elucidated.
1950s	Proteoglycans where described.
1960s-1970s	Main structural features of heparin elucidated.
1980	Heparan sulphate described in detail.
1990s	Critical role of heparin sulphate in embryonic development and cell growth recognized.

(3)

3.2. Anticoagulant Action of Heparin

In vivo, free flowing of blood depends on the balance of pro-coagulant and anticoagulant processes. A complex network of serine proteases, acting in an amplification cascade, converts pro-enzymes to their active form. Factor IIa (fIIa), known as thrombin, is the final serine protease that cleaves fibrinogen to form fibrin which, together with a platelet plug, is the basis of a clot. This coagulation cascade is activated when there is an injury to the vasculature, so that the serine proteases are exposed to pro-coagulant stimuli such as tissue factor and collagen. Several natural or endogenous anticoagulant proteins, which include antithrombin (AT), heparin co-factor II (HCII), protein C inhibitor (PCI) and tissue factor pathway inhibitor (TFPI), are also in place to regulate the formation of thrombin. These inhibitors are found at a higher total concentration than the proteases and under normal physiological condition act to keep the clot local to the wound by mopping up any proteases straying

into the rest of the vasculature. Antithrombin, HCII and PCI are members of the structural class of proteins known as "serpins" (serine protease inhibitors). There are two common features in the inhibitory mechanism of serpins: first, there is usually a requirement for conformational change in both the protease and the protease inhibitor and second the reaction is often greatly enhanced by the presence of glycosaminoglycans such as heparin, heparan sulphate (HS) and dermatan sulphate (DS). Unlike direct enzyme inhibitors, heparin has no anticoagulant activity on its own. It acts in a catalytic fashion to activate the inhibitor and to stabilise the inhibitor–enzyme complex; after the permanent, stoichiometric inactivation of the enzyme by the serpin, heparin is released and can act again, as long as free serpin is available(4).

Heparin has anticoagulant action in several ways. The most significant of these is through its potentiating action on the serpin antithrombin, best known as the major heparin co-factor in the inhibition of the coagulation proteases, particularly factors Xa and IIa. Although antithrombin alone is able to inactivate proteases, the reaction is accelerated several thousand times in the presence of heparin. The interpretation of this rate enhancement is controversial because of the difficulty in attributing it either to protease-heparin or to an antithrombin-heparin interaction (4, 5).
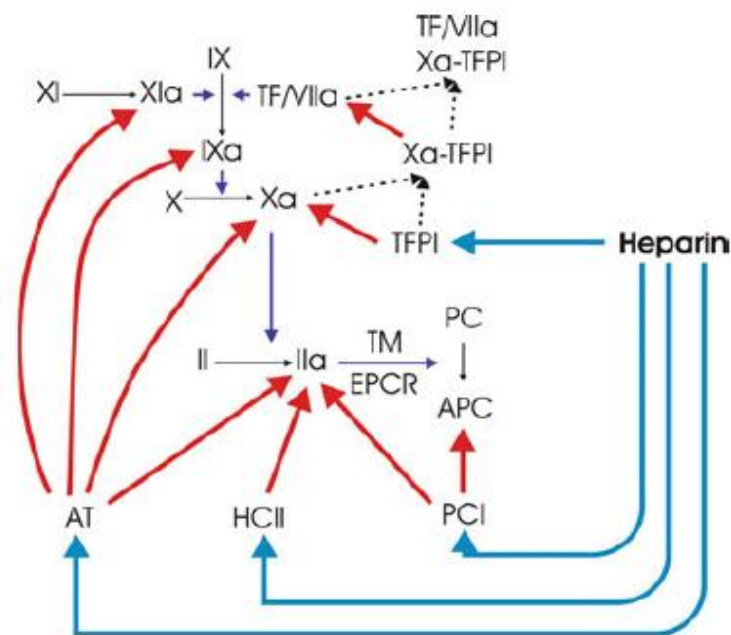
Fig.6- Diagram representation of the interactions of heparin with the natural anticoagulant systems.
Blue arrows represent the potentiating interactions of heparin with coagulation inhibitors; Red arrows represent the inhibition of coagulation enzymes; Black arrows represent the conversion of proenzymes to their active forms, and purple arrows represent the effects of the enzymes. Dotted arrows indicate the formation of complexes.

3.3. Heparin Sodium

Heparin sodium is the sodium salt of sulfated glycosaminoglycans present as a mixture of heterogeneous molecules varying in molecular weights.
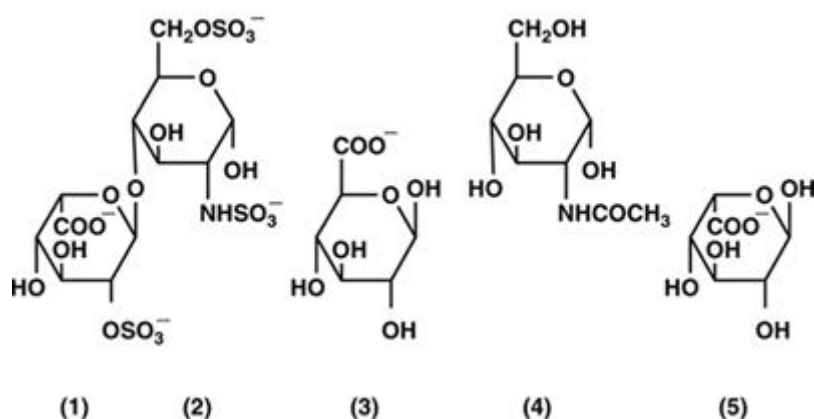
Fig.6- Structural formula (representative subunits of Heparin sodium.

Although others may be present, the main sugars occurring in heparin are: (**1**) α-L-iduronic acid 2-sulfate, (**2**) 2-deoxy-2-sulfamino-α-D-glucose 6-sulfate, (**3**) β-D-glucuronic acid, (**4**) 2-acetamido-2-deoxy-α-D-glucose, and (**5**) α-L-iduronic acid. These sugars are present in decreasing amounts, usually in the order (2)> (1)> (4)> (3)> (5), and are joined by glycosidic linkages, forming polymers of varying sizes. Heparin is strongly acidic because of its content of covalently linked sulfate and carboxylic acid groups. In heparin sodium, the acidic protons of the sulfate units are partially replaced by sodium ions(6).

It is present in mammalian tissues and is usually obtained from the intestinal mucosa or other suitable tissues of domestic animals used for food by man. Unfractionated heparin as a pharmaceutical is heparin that has not been fractionated to sequester the fraction of molecules with low molecular weight.

Two types of heparins are commonly used as anticoagulants – unfractionated heparin (UFH) and low molecular weight heparins (LMWHs). UFH has been used for the prevention and treatment of thrombosis for several decades. UFH has variable anticoagulant effects and pharmacological properties and also has limited bioavailability and highly variable anticoagulant response. LMWHs are derived from UFH by depolymerization. Each LMWH product has a specific molecular weight distribution that determines its anticoagulant activity and duration of action. LMWHs are associated with a predictable dose–response and have fewer non-haemorrhagic side-effects.

3.4. Production Scandal

In 2007-2008 there was a big problem regarding heparin production. Severe side effects, some leading to death (149 deaths, according to the *Food and Drug Administration*) were detected to batches of contaminated unfractionated heparin imported from China. This crisis led to the recall of much of the heparin on the market and could have been a much greater problem had there not been sufficient amounts of non-contaminated product to meet the needs of dialysis and surgery patients. The

contamination was traced to an adulteration of the crude heparin precursor with an oversulphated chondroitin sulphate somewhere in the process between the slaughterhouse where heparin was collected from pig intestines and the pharmaceutical manufacturing site. While ULMWH was not contaminated, it could not be relied on to alleviate this crisis because of its high cost, difficulty to produce in sufficient quantities to meet worldwide needs, and limited utility for kidney dialysis. The inspection of foreign suppliers and upgrading the pharmacopeial monographs has reduced the likelihood of a similar crisis in the future but the increased demand on heparin, as modern medicine is applied to more of the world, and the limited number of pigs (1 pig provides about 3 doses of unfractionated heparin or 1 dose of LMWH) pose constraints on the supply of this critical drug. Since the crisis, the cost of heparin API has increased 10 times(6).

3.5. Biological Assays

Bioassays are methods employed to estimate the effect of a given substance in living matter, and therefore they are frequently used in the pharmaceutical industry. Biological assays are complicated experiments with several factors, there is not a general formula to estimate how many experiments are needed in order to assure that the confidence interval of the estimate has a certain size.

Biological methods are described for the assay of certain substances and preparations whose potency cannot be adequately assured by chemical or physical analysis. The principle applied wherever possible throughout these assays is that comparison with a standard preparation so as to determine how much of the substance to be examined produces the same biological effect as a given quantity, the *Unit*, of the standard preparation and on the substance to be examined be carried out at the same time and under identical conditions. Variability occurs in bioassay factors (mice, analysts, other reagents, etc.) that can influence potency assessment independent of the tested substance's level of biological activity.

In order to mitigate these influences, a measure of potency of a test material relative (compared) to a standard material can be obtained in one assay, in which levels of the bioassay factors that contribute to biological response are common to the assessments of test and standard materials. A frequently invoked perspective is that relative potency is the degree to which the test preparation is diluted or concentrated relative to the standard. The result from a relative potency assay quantitates the test compound's capacity for achieving the same specific response relative to the effect observed for the Standard compound.

### 3.6.	Heparin anti-factor IIa assay principle and summary.

Thrombin (Factor IIa) is the terminal enzyme in the classical coagulation cascade, where it plays roles in both the fibrin deposition and platelet activation processes of blood coagulation. UFH and LMW-H inhibit blood coagulation via initial binding of these molecules to Antithrombin (AT), followed subsequently by the AT-heparin complex inhibiting Factor IIa.

In buffer, this same "Anti-IIa" activity of Heparin can be followed enzymatically by means of a chromogenic peptide substrate which, upon hydrolysis by residual factor IIa, provides a chromophore (405nm) whose measure is inversely proportional to the initial amount of heparin present in the test sample. An indication of the amount of heparin present the sample is then obtained.

Schematically, the procedure is carried out as follows:

AT + heparin -> [AT-heparin]

[AT-heparin] +[Thrombin] IIa (know excess) -> IIa (residual) + IIa-AT-heparin (inactive)

IIa (residual) + IIa Substrate (colorless) -> pNA chromophore (405nm) + peptide

The International Unit is the activity contained in a stated amount of the International Standard for UFH. Heparin sodium Biological Reference Product (BRP), calibrated in International Units by comparison with the International Standard, is used as the reference preparation.

The basic procedure, according to the Pharm. Eur. Monograph, involves preparing 4 standard dilutions and 4 sample dilutions, within a specified concentration range, using a tris-EDTA buffer pH 8.4, R1. Then, the reagents are added to the heparin dilutions, followed by a reaction time of 1 minute, incubated at 37°C. Each potency determination is carried out on 16 standard dilutions and 16 dilutions of the sample, adding 2 to 4 blanks. The dilutions treatment with the reagents is done in a specific order as indicated in the monograph. As the previous scheme indicates, the potency is determined though the thrombin excess, which is measured spectrophotometriclly. This analysis was performed by automated testing equipment, the ACL TOP 300 hemostasis testing system, which was set-up accordingly, in order to save time and eliminate errors in manual work.

For data treatment the monograph states the use of "the usual statistical methods for parallel-line assays (5.3)" and so, the *Combistat* software version 5.0.0., delivered by the European Directorate for the Quality of Medicines (EDQM) was used.

4. Role and contribution for the Heparin anti-factor IIa assay

4.1. First steps

In order to train and get used to using automatic, I started by determining the systematic error for each one of the auto-pipettes and checking if the deviation from the nominal number was within the pipette official calibration.

The procedure went as follows: a fixed water volume, similar to the volumes used in the assay, was auto-pipetted into a beaker and weighted using a precision balance. The water density was calculated, as a function of temperature, in order to obtain the real volume pipetted using the water mass weighted after pipetting. The average volume was based on 15 measurements and the algorithm used for determining water density was based on:

$$p = 1000(1 - (T+288.9414)/(508929.2*(T+68.12963))*(T-3.9863)^2)$$

$T - C^o$
$P - kg/m^3$

Algorithm taken from McCutcheon, S.C., Martin, J.L, Barnwell, and T.O. Jr. 1993. Water Quality in Maidment, D.R. (Editor). Handbook of Hydrology, McGraw-Hill, New York, NY (p. 11.3).

Table 2 - Results obtained for the pipette testing.

Test	Pipette ID	Range (ul)	Vi(ml)	Vr. Av. (ml)	SD	SD (%)	RSD (%)	Ref. RSD (%)
A1	D0842177E	500-5000	1.5	1.495	0.001841	0.12	-0.35	+-0.8
B1	D0842177E	500-5000	1	0.993	0.00153	0.15	-0.73	+-0.8
C1	D0842177E	500-5000	0.6	0.597	0.000655	0.11	-0.53	+-0.8
A2(a)	K1200416U	100-1000	0.15	0.1489	0.000757	0.51	-0.76	+-0.8
A2 (b)	F0500215E	100-1000	0.15	0.1485	0.00032	0.22	-0.98	+-0.8
B2	7054413	5-120	0.1	0.099	0.00043	0.44	-1.42	+-1.33
C2(a)	14022207	5-120	0.06	0.059	0.00013	0.23	-1.95	+-1.33
C2(b)	11137961	5-120	0.06	0.059	0.00012	0.20	-1.36	+-1.33
C2(c	7054413	5-120	0.06	0.059	0.00026	0.44	-1.56	+-1.33

Vi – nominal volume set to be pipetted.
Vr. Av – Real volume average.
SD – Standard deviation.
RSD – Relative standard deviation. Yellow – acceptable; Red – unacceptable.
Ref. – reference value for the systematic error taken from the official calibration.

The main interpretation for the results can be the error associated with my pipetting technique, which can contribute to the differences seem in the method when different executed by another operator.

4.2. Materials

The EDQM Heparin sodium BRP for chromogenic assays was purchased. The chromogenic assays for the determination of heparin were performed on an ACL TOP 300 coagulation analyzer from Instrumentation Laboratories. The heparin anti-IIa assay was run using the Kinetichrome Anti-IIa Test kit from Provision Kinetics.

4.3. Developing and optimizing the method.

When I entered the project I was put up-to-date with the project status. My main responsibilities were to do the analytical work and data treatment. The instrument set-up, reagents and the first version of the procedure where already prepared. The quality control department was about to start the analytical procedure and gather the data, but improvements still had to be done. Some of problems we faced, along with the solutions are listed below:

- The buffer volume used for preparing the diluted solutions from the first preparation guide was too large, so it was reduced 10 times and tested for linearity behavior.

- The diluted solutions concentrations were altered several times, as we tried to find the best concentration range for linearity. During this period we experienced difficulties with dispersed measurements from the robot and on a few occasions we obtained strange results. This indicated possible dilution problems and great of effort was inputted to resolve the problem and several measures were adopted, such as making sure all the dilution factor were below 100; saturate the pipette tip by picking up and rejecting the volume 3 times; working at the same concentration distance ratio at log-scale; developing dilutions schemes for all heparin products.

- The report from the ACL TOP 300 was optimized in a way that we could treat the data and have the potency determination immediately, avoiding printing or manual work, thus diminishing errors and saving a considerable amount of time after each run.

4.4. Results outcome

The results gathered were used to prove the accuracy, repeatability, reproducibility and specificity of the method and also to develop the acceptance criteria for the statistical model used. The data processing was done according to the work framework delivered by Amgros A/s, the regulatory authority, in order for the validation to be approved.

The necessary data was therefore organized and a validation report was written down and set to the authorities for evaluation approval. In the beginning of June the

feedback was sent with a positive response, so RAP can perform the Heparin anti-IIa assay.

5. Other projects

5.1. Cytotoxic contamination in the workplace of hospital pharmacies

Hospital staff can be exposed to the genotoxic effects of cytotoxic drugs. Occupational exposure can occur when control measures are inadequate, meaning the toxicity of cancer drugs can present significant risks to those who handle them.

Exposure may be through skin contact, skin absorption, inhalation of aerosols and drug particles, ingestion and needle stick injuries resulting from drug preparation, drug administration, handling patient waste, transport and waste disposal or spills. The personnel more likely to be involved in these activities are nurses, responsible for drug administration, hospital pharmacists and laboratory staff, responsible for drug preparation, also including medical officers and cleaning, maintenance and waste disposal staff.

With this in mind, since 2005, RAP is testing Danish hospital pharmacies and taking measures to reduce the level of contamination and increase staff safety. Most the changes are about staff behavior making sure everyone is aware of the implications of working with cytotoxic drugs.

I took part in the project collecting samples from 2 hospitals pharmacies, Ålborg and Herning, and I also had the chance to visit the analysis laboratory.

The process involves a swab test of all facilities to determine the surface contamination, swabbing using an acrylic frame of 20x20cm. The testing was done for Cyclophosphamide (CP) and Iphosphamide (IP). The analysis is done using LC-MS-MS in the Division of Occupational and Environmental Medicine in the Department of Laboratory Medicine in Lund University, Sweden. RAP decided not to perform the analysis for safety reasons since the pharmacy doesn´t have, nor considers having, one instrument dedicated only to perform these kind of testing.

The laminar air flow (LAF) is well establish and has a generalized use but the efficiency of the LAF chamber has generated a discussion, particularly for cyclophosphamide, which can vaporize at room temperature, originating particles smaller than the porous of HEPA filter (High Efficiency Particulate Air). Also, gloves can be permeable to cytostatics, with the permeation intensity being dependent of time and glove thickness. For example, a study tested the permeability to 2 types of gloves to cyclophosphamide and verified that the first sign of permeation occurred after 10 minutes for latex gloves and 20 minutes for PVC gloves(7).

Next, is showed the results from Ålborg, confronted previous testing (March 2012):

Table.3 – Results from Ålborg's Hospital Pharmacy from March 2012 and April 2015.

	Mar-12		Apr-15	
Area	CP pg/cm2	IF pg/cm2	CP pg/cm2	IF pg/cm2
Blank	<lod	<lod	< 0,25	< 0,25
Locker cabinet in the clean room	1,7	<lod	< 0,25	< 0,25
Office desk	1,88	<lod	< 0,25	< 0,25
Floor on the clean side of locker room	1,92	<lod	< 0,25	< 0,25
Helping person desk 1	9,9	<lod	< 0,25	< 0,25
Laminar air flow desk 1		<lod	< 0,25	< 0,25
Floor between LAF and the helping person 2	8,68	1,61	0,48	0,85
Helping person desk 2	2,9	<lod	-	-
Laminar air flow desk 2	4,58	<lod	-	-
Floor between LAF and the helping person 2	13,75	0,21	3,75	0,4
Shelf in the store under CP	<lod	<lod	< 0,25	< 0,25
Desk next to the welding machine	2,15	<lod	< 0,25	< 0,25
Floor at the exit door	3,41	<lod	1,5	< 0,25
Baxter CP container exterior	Not tested	Not tested	14,8	37,7
Baxter CP container exterior	Not tested	Not tested	37,7	32,8

*lod – less than limit of detection 0,01.

The latest contamination are lower than the previous testing made 3 years ago for both CP and IF. Improvements in the handling of cytostatics and better cleaning could justify the contamination decrease. However, on the latest testing, by error, the pharmacy cleaned the facilities before the swab.

During the latest test we searched for contamination on the cytostatic surface container. Although the producer is expected to guarantee that no contamination occurs during the packaging we found presence of both CP and IF in the CP container exterior, which might indicate contamination in the packaging process.

Taken part in the project has raised my attention for the influence of materials, especially plastic, used for handling cytostatics. Choosing the right material is crucial to safety in the workplace. The important outcome is to change behaviors and raise awareness to safely handle dangerous medicine, for the worker and patients benefit. Simple changes can make big differences. For example, PVC is permeable to CF and the releases after being absorbed is problematic. To minimize contamination at the desk the PVC keyboard hand supports were removed from the desks in all hospital pharmacies.

6. Discussion

Looking back into my mobility program I feel a profound professional and personal growth. Being responsible for executing the technical work for the heparin testing improved my analytical skills and I also learned to cope better with failure, not giving in and look for solutions when faced with unexpected problems.

The choice of doing the training at RAP enable me to contact and use state-of-the art instruments and materials. From the QA and QC departments I got a clear image on how to work in a team, the value of individual work and the positive outcomes of collaboration between departments. In order to achieve maximum productivity all staff opinions and suggestions can be relevant and are always taken in consideration.

With several visits to different hospitals and hospital pharmacies, coupled with long discussions about the pharmaceutical sector in Denmark, I have great knowledge on how the Danish healthcare system works beyond hospital pharmacy.

Working in an English based environment was a valuable experience. The adaptation was fast and there also room for improving the technical terms which exceeds classrooms and are better learned on the job. Although Denmark is not an English speaking country, the majority of people are fluent in English and communication at RAP was never a problem.

My role in RAP was a small part of a larger, and still going, project. The validation for anti-factor IIa assay is finished and now a technician must be trained to carry out the testing of the production samples. The validation for anti-factor Xa is the next step and afterwards, depending on the sample analysis from production, RAP will determine if the production process is in need of improvements.

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ANNEX

Validation Report

Validation of Ph. Eu. Monograph

2.7.5 Assays of Heparin,

used for the quantitative determination of heparin in the Danish Hospital Pharmacy (SAD) area

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0. Objective

To verify if the implementation of the monograph 2.7.5, *Assay of Heparin*, from Pharm. Eur. 8.3 is done correctly. The assay will be used for releasing heparin intravenous (IV) solutions produced by the Capital´s Region Pharmacy, RAP (*Region Hovedstadens Apotek*), in form of national hospital pharmacy registered products (SAD).

1. Introduction

The RAP manufactures unpreserved IV solutions of Heparin *Sygehus Apotekerne I Danmark* (SAD) - The hospital Pharmacies in Denmark - in potencies of 100, 500, 1000 and 5000 IU/ml. The IV solutions with the potency of 1000 and 5000 IU/ml are packed in glass ampoules, while 100 and 500 IU/ml are packed in both vials and ampoules. All products are unpreserved and only contain *Water for Injection, Pharm. Eur*. and *NaCl, Pharm. Eur.*, as excipients. Heparin sodium is added in an amount corresponding to 5% excess to all products, when compared to the specified strength.

The report describes the suitability/verification for Pharm. Eur*. analytical method 2.7.5*, Assay of Heparin*, for determining anti-factor IIa activity in Heparin SAD IV solutions. The analytical method is described in *Heparin Sodium monograph, Pharm. Eur. 8.3*, which contains reference to the analysis description in chapter 2.7.5.

1.1. Background for the planning of the assay (Anti-Factor IIa activity).

In January 2015 Pharm. Eur. 8.3 came into force, updating the monograph 2.7.5, *Assay of Heparin*, for the quantitative determination of heparin. The method is based on the reaction between heparin and anti-thrombin and subsequent addition of thrombin and measurement of a color reagent. The previous method used sheep blood and coagulation time was the main component/principle of detection.

The work behind the report is not planned nor executed as a full validation, as described in *ICH guidelines*. In fact, according to the *ICH guidelines*, which *RAP* follows, this is not required because the pharmacopeia method has already been validated. This verification is planned and carried out to prove the method is suitable for the assay of Heparin SAD IV solutions, through determining method´s accuracy, repeatability and reproducibility. In addition, considering the formulated products specificity containing NaCl as an excipient, which is not considered in the monograph, which is based on the potency of the raw material only.

Until now (June 2015) RAP has used an external laboratory, *CiToxLAB*, who determines the potency of the raw material. This laboratory has, according to a contract, performed analysis for the pharmacy until January 1 of 2015 using the official method.

The method of analysis in 2.7.5 describes two assays for determining heparin potency, one based on anti-factor IIa activity and another based on anti-factor Xa activity. The *Heparin sodium Pharm. Eur. monograph*, under "*Definition*", states that potency is expressed as anti-factor IIa activity, which is also the potency determined in the monograph assay. The potency of Heparin SAD is therefore expressed as anti-factor IIa activity. Verification of RAP´s use of

anti-factor Xa determination will be described in a separate report. Anti-Factor Xa results are included along with anti-factor IIa results in the identification of Heparin sodium, Pharm. Eur..

The method suitability verification for Heparin SAD assay is focused on testing the product with the highest potency, 5000 IU/ml, and the one with the lowest potency, 100 IU/ml, as they are considered to be representative of the entire range, see section 1.2.

1.2. Briefing about potency determination.

The analysis begins preparing the dilution series, consisting of 4 solutions - in a narrow concentration range - Pharm. Eur. specifies the range between 0.03 - 0.005 IU/ml. In order to do so, we prepared a dilution series, for both standard and sample, in the same concentration range. The difference between testing potency on the IV solution of 5000 IU/ml and 100 IU/ml is only in the dilution series for the solutions concentration, down to the same 4 concentrations. This process is simple but of great importance for analysis results. Thus, we created a dilution scheme for all Heparin SAD products, not only to avoid error in manual work but also to optimize accuracy of the dilution series. An example, that is only indicative, is shown next:

Diluted Solutions Preparation Scheme for Heparin 5000 IU				
Standard (delivered from EDQM)		IU/ml	Batch #	
Heparin Sodium (BRP, Ph. Eur.)		1000		
Diluent (R1):	Tris/EDTA buffer pH 8,4, ph.Eur 8.3			

1 - Standard Mother solution

1 - Standard Mother Solution				
Name	cf(IU/ml)	vf (ml)	ci (IU/ml)	vi (ml)	Dilution factor
SA1	12.12	50	1000	0.606	82.5

2 - Intermediate Standard Dilution				
Name	cf(IU/ml)	vf (ml)	ci (IU/ml)	vi (ml)	Dilution factor
SA2	0.5054	10	12.12	0.417	24.0

3 - Test Mother Solution IU

Heparin Sodium RAP #	IU/ml	
_ _ _ _ _ _	5000	

3 - Test Mother Solution				
Name	cf(IU/ml)	vf (ml)	ci (IU/ml)	vi (ml)	Dilution factor
TA1	60	10	5000	0.120	83.3

4 - Intermediate Test Dilution 1				
Name	cf(IU/ml)	vf (ml)	ci (IU/ml)	vi (ml)	Dilution factor
TA2	1.2	25	60	0.500	50.0

5 - Intermediate Test Dilution 2				
Name	cf(IU/ml)	vf (ml)	ci (IU/ml)	vi (ml)	Dilution factor
TA3	0.5054	10	1.2	4.212	2.4

6 - Diluted Standard and Test solutions				
Sx, Ty	cf(IU/ml)	vf (ml)	ci (IU/ml)	vi (ml)	Dilution factor
1	0.0300	10		0.594	16.8
2	0.0200	10	0.5054	0.396	25.3
3	0.0133	10		0.264	37.9
4	0.0090	10		0.178	56.2

The anticoagulant effect, which defines heparin potency, is determined *in vitro* by adding an excess of anti-thrombin IIa to the 4 dilutions (s1, s2, s3, s4 of the heparin IV solution and the 4 dilutions of the heparin standard BRP (Biological Reference Preparation). Then, the excess of anti-thrombin reacts with an added excess to the excess of thrombin and, finally, the thrombin excess is determined spectrophotometrically, at 405nm, after reacting with a color

reagent (substrate). The measurement result is expressed in terms of change in optical density potency per. min., ΔOD.

After adding the reagents to the heparin dilutions there´s a reaction time of 1 min., incubated at 37$^\mathrm{o}$C. Each analysis contains, approximately, 36 individual determinations performed in one single run, as described in the pharmacopeia, containing sequences of both samples and standards. Each potency determination is carried out on 16 standard dilutions and 16 dilutions of the sample, adding 2 to 4 blanks.

1.3. Equipment used for performing the analysis.

After studying market products and taking on the advice of Elaine Gray, National Institute for Biological Standards and Control, NIBSC (England), it was decided to purchase a *Mini Robot Systems ALC TOP 300* from *Instrumentation Laboratories*.

For statistical treatment of analytical data we worked with the *CombiStat*s software, version 4.0.0.0, delivered by EDQM. The software was used for the methods described in *Pharm. Eur., Section 5.3.,* the monograph referring the use of: "the usual statistical methods for parallel-line assay (5.3)".

This statistical tool was set up and used according to the monograph.

2. Parameters related to test results and how each parameter is defined in the monograph 2.7.5, in conjunction on how we follow the monograph requirement´s.

The method must be ensured that´s performed under conditions where the major analysis parameters are within monograph limits. The parameters affecting the analytical results we evaluated or examined can be found below.

2.1. Concentration range, linearity.

The concentration range from 0.03 – 0.005 IU/ml is established by the monograph, meaning that there are 4 dilutions from BRP standard prepared and used inside this concentration range. The 4 test solutions concentrations must be located in the same area. As an example, if the change in the sample ΔOD is significantly outside (>10%) from the standard analytical result, you should repeat the analysis with the correct sample dilutions. Initially, it seemed that the analytical answer (ΔOD), when plotted against the logarithmic concentration, showed no linear relationship, although being inside the pharmacopeia specified concentration range.

A study of linearity, as a function of concentration, showed the range 0.030 - 0.009 IU/ml was linear, therefore it´s used for the validation. The pharmacopeia mentions the range should be within 0.030 to 0.005 IU/ml, which contain our selected range. Thus, the concentrations used are 0.030; 0.020; 0.0133 and 0.009 IU/ml, so there is a linear increase in concentration at the logarithmic scale. The statistical model for calculating the activity uses a logarithmic data representation, see Figure 1.

Therefore, we assure the analysis is carried out in the pharmacopoeia concentration range and with logarithmic growth the concentration.

2.2. Temperature incubation, 37°C

According to the monograph, incubation temperature for the sample is set to 37°C. This means the incubation must be carried out in the temperature range of 36.5 – 37.4^{o}C (*Pharm. Eur. General Notices 1.2: "Rules for rounding the Quantities"*).

In the *ACL TOP 300*, temperature is set to 37,00^{o}C and alarm limits are set at 36.70 and 37,30^{o}C, lower and upper limits respectively. This way, in case the analysis temperature is outside the upper and lower limits, an alarm is activated and the ongoing analysis interrupted.

2.3. Incubation

Incubation time for anti-thrombin and sample is set up to "at least 1 min.", and the time for the second incubation with thrombin is indicated as exactly.

The practical meaning of those limits, in terms of time period, is not specified in the pharmacopeia. The *ACL TOP 300* is set to 60 sec. with the limits from 60.000 to 65.000 seconds. The machine uses clock time. It is estimated that this time period is sufficient to satisfy the pharmacopeia requirements.

2.4. Determination of end-point (Abs min-1, Δ OD).

For quantitative determination of heparin in the sample we use the spectrophotometric determination of light absorption, caused by thrombin/chromogenic substrate complex, at 405nm.

Pharm. Eur. specifies two principles for the determination. Here, the "*kinetic method*" was selected, where the sample absorption of light is recorded as a function of time, and the slope of the linear portion of the absorption curve is the actual analysis result (Abs min-1 (Δ OD)).

The *ACL TOP 300* calculates the absorption curve slope against time measurements from the first 5 seconds, are compared to measurements from the last 5 seconds. If the difference is greater than 0.5%, then, the measurement is rejected and showed as "Failed" in the result report.

The time course of the light absorption is recorded from 10 to 50 sec. is seen to be linear within the given uncertainty. Before and after this period, the curve is not linear.

2.5. pH of buffer R1.

The supplier of the buffer set buffer pH to 8.4, with limits from 8.35 to 8.45, at 20°C. There is no specified limit to the pH in the pharmacopoeia.

2.6. Reagents and stability.

The method´s reagent R5 (anti-thrombin III), R2 (human thrombin) and factor IIa specific substrate are provided by the company *Provision Kinetics®*, Arlington Wisconsin, USA, under the trademark *Kinetichrome®*. They are delivered as a dry set, containing the three reagents, intended for reconstitution before use.

The reagents are specially designed for the methods described in Pharm. Eur. and USP. Before reconstitution, the reagents have a self-life of more than one year, when stored at 2-8°C. Reagents are stored in a monitored refrigerator. Reconstitution is performed under the instructions from the supplier.

After reconstitution, reagents are stable during the next 48 hours at 15-19°C. After inserting reagents into the *ACL TOP 300* robot, they are kept at 15.00^{o}C (upper limit - 15.50^{o}C; lower limit - 14,50^{o}C). After the daily use of the instrument, we keep the reconstituted reagents in the refrigerator at 2-8°C.

The reconstituted reagents are used within 48 hours after the reconstitution.

2.7. Data processing.

The monograph states that the sample potency is determined against Heparin Sodium BRP and expressed in IU/ml. The calculations are performed "*using the usual statistical methods for parallel-line assays*" according to Pharm. Eur., Chapter 5.3. This chapter contains statistical models and description of calculations which can be used by users "*whose primary training and responsibilities are not in statistics, but who have responsibility for analysis or interpretation of the result of these assays, often without the help and advice of a statistician*". Precisely the situation RAP finds itself in.

The model described in Chapter 5.3, section 3.2, "*The parallel-line assay*" (PLA) is used as described in section 3.2.1 and the related commentary in section 7.6.

For the data analysis we worked with the *CombiStats* software. The program has been prepared by Pharm. Eur. and, as described above, is specially provided for non-statisticians users. The user creates a relevant version of the model and builds a template for data entry, calculus and results placement.

Fig. 1 shows, as an example, one graphic for analysis results values (ΔOD) versus log concentrations and **Table 1** demonstrates the completed *CombiStats* report for Heparin SAD 100 IU/ml.

Our program set-up was verified by John Hogwood from the National Institute for Biological Satandards and Control, NIBSC - England. The last table indicates the analysis result showed as "*Estimated Potency*" in IU/ml, associated with the confidence interval named "*Upper limit*" and "*Lower limit*", which is part of the analysis result.

Substance	Heparin
Method	Ph. Eur.
Assay number	1
Technician	Vagn H.
Date of assay	04.06.15

Remarks: Standard prepared in 04.06.15 by Vagn H.
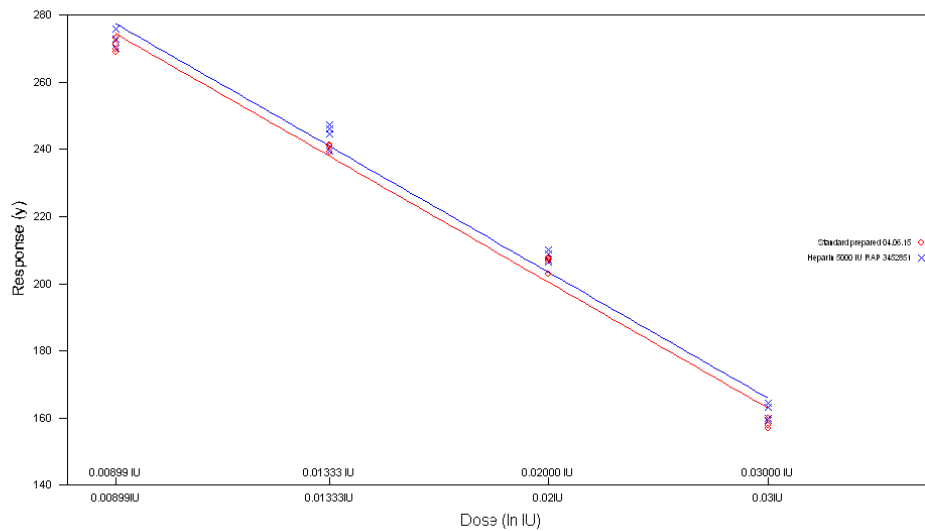
Figure 1. Graphic representation from analysis result values (ΔOD) versus log concentration for test (blue) and standard (red), Heparin SAD 5000 IU/ml.

Substance	Heparin
Method	Ph. Eur.
Assay number	2
Technician	David M
Date of assay	20.05.15

Remarks: Standard MIX I vs Standard MIX 2

Standard				
Id.	SA Mix I			
	With intermediate sol. I			
Ass. pot.	1010 IU/ml			
Doses	(1)	(2)	(3)	(4)
0.0300 IU	137.55	140.83	140.98	139.33
0.0200 IU	196.65	191.31	193.24	194.90
0.0135 IU	228.50	229.47	226.84	228.71
0.00900 IU	264.40	267.62	269.14	267.71

Sample 1				
Id.	SA Mix II			
	with intermediate sol. II			
Ass. pot.	100 IU/ml			
Doses	(1)	(2)	(3)	(4)
0.0300 IU	140.28	143.67	142.92	144.74
0.0200 IU	190.86	194.27	190.52	189.99
0.0135 IU	231.31	232.01	229.62	223.05
0.00900 IU	263.24	265.60	263.46	269.66

Model: Parallel lines
Design: Completely randomised
Transformation: $y' = y$
Variance: Observed residuals

Common slope(factor) = -102.688 (-104.307 to -101.069)
Correlation | r |: 0.995279

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability	
Preparations	1	0.122512	0.122512	0.021	0.885	
Regression	1	67656.7	67656.7	>1000	0.000	(***)
Non-parallelism	1	13.7609	13.7609	2.394	0.135	
Non-linearity	4	491.622	122.905	21.384	0.000	(***)
Standard	2	336.574	168.287	29.280	0.000	(***)
Sample 1	2	155.048	77.5241	13.488	0.000	(***)
Quadratic curvature	1	378.950	378.950	65.934	0.000	(***)
Lack of quadratic fit	3	112.671	37.5572	6.535	0.002	(**)
Treatments	7	68162.2	9737.46	>1000	0.000	(***)
Residual error	24	137.939	5.74744			
Total	31	68300.2	2203.23			

Sample 1			
Id.	SA Mix II		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	98.4291	100.121	101.841
Rel. to Ass.	98.4%	100.1%	101.8%
Rel. to Est.	98.3%	100.0%	101.7%

All samples	Standard SA Mix I	Sample 1 SA Mix II
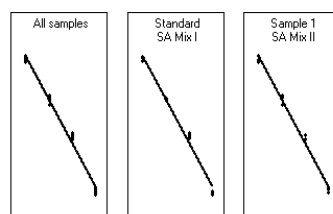
Executed by: Calculated by: Approved by:

2.7.1. Criteria for acceptance or rejection of analysis results.

In the PLA, the statistical model used, there are three parameters, all of which appears in the *CombiStats* report, to assess whether a given analysis is "*statistically valid*" (section 3.2.4):

1. "*The linear regression term*" parameter is found as regression in the table. The value is expressed as probability and must be <0.05;
2. "*The term for the non-parallelism*" must be >0.05;
3. "*The term for the non-linearity*" must be >0.05.

Compliance with conditions 1 and 2 can be correlated with a well conducted and reported heparin assay, whereas condition 3 was never met. Also, in our results always we found values for p<0.001 for well conducted assays.

Chapter 5.3 of the pharmacopeia was developed before the automated equipment, as *ACL TOP 300* was available on the market. The work methods were manual and the number of individual samples included in a single heparin analysis was, and remains, high (36) leading to great uncertainty in the analysis compared to what we obtain today using the automated assay for anti-factor II. This is reflected in the value of probability of *non-linearity* (p), increasing with greater uncertainty.

Figure 2 exemplifies one of our analyses with the treated PLA model (red plot). The blue graph in the figure represents the same analysis results with added noise in the form of spread values greater than the original ones. P for the initial measurement is <0.0001, and 0.07 for the blue graph. Observations of the same nature were published in conjunction with USP´s introduction of anti-IIa method. The conclusion from these observations is that with little uncertainty on the measurement results, which can be achieved using the automated equipment of *ACL TOP 300*, the probability of *non-linearity* (p) value is small, although the analysis is still valid. This is observed in the validation, where all analysis we have assessed as valid have the probability for "*non-linearity*" parameter at 0.0001, below 0.05.

This situation is foreseen in the Pharm. Eur., Chapter 5.3, section 7.6, where "*Non-parallelism of dose response curves*" is treated. It mentions: "*underestimation of non-linearity for valid analyses*", just what we see in our work. In these cases the Pharm. Eur. recommends a solution "*on a case by case basis with the help of statistical expertise*". On this note, we have contacted a statistician at *NNE Pharmaplan* who advised to use the correlation quotient and slope as parameters for acceptance criteria when validating the analysis. The statistician is behind the calculations in *Fig. 2*.

Therefore, we have chosen to use the correlation quotient (R) and slope value (a) from the linear representation obtained through the relationship between the ΔOD vs logarithm concentration as the 3rd and 4th parameter for analysis acceptance/rejection. This is, as mentioned, in accordance with our statistician's recommendations and was also recommend by John Hogwood (NIBSC).
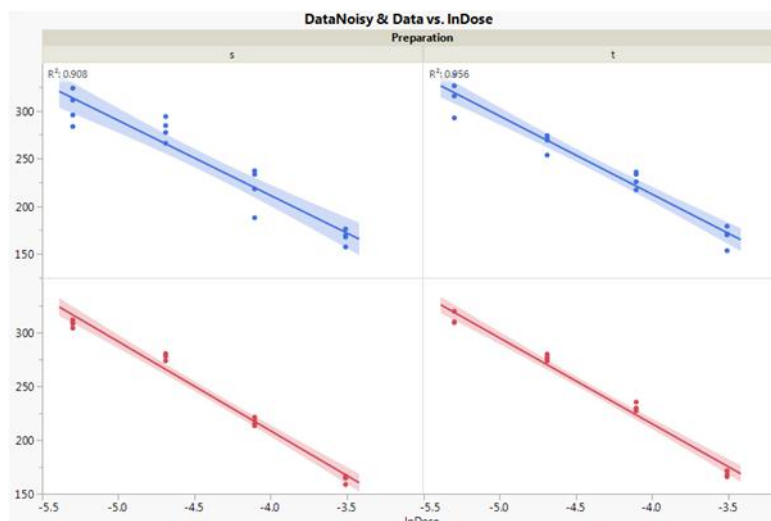
Figure 2. The red curve is the view of the analytical results, ΔOD, against log concentration of original data (s: standard; t: test). The Blue curve represents our "*values added*" noise. Blue representation of "*non-linearity*" p = 0.07, red p <0.0001 (T. Zelikman, NNE Pharmaplan, May 2015).

To ensure proper use of the statistical model, we used a standard example from the *CombiStats* manual and an in-house example Elaine Gray, the principal scientist for NIBSC, had verified. Elaine Gray and her PhD student, John Hogwood, have kindly assisted RAP setting up the program.

To verify the individual analyzes reliability, the following parameters specified in the *CombiStats* report are used:

1. "The linear regression term" "regression in the table. This value is expressed as the probability and must be <0.05;

2. "The term for the non-parallelism" probability must be >0.05;

3. "Correlation", r, must be >0.98

In addition, the acceptance limits for "Common slope factor" parameter is yet to be determined since there currently is sufficient statistics material to accurately determine the limits. Limits will be given and justified by a later addendum to the report.

2.8. Accuracy.

To determine accuracy, all the analysis where done with a BRP heparin standard from Pharm. Eur. For each assay, sample and standard dilutions were prepared from the BRP standard, so that the analysis reflects the overall dilution uncertainty, all the determining the ΔOD against log concentration. As standard, we used Heparin Sodium BRP batch 3.6, from EDQM.

2.8.1. Accuracy of the method.

The method´s accuracy is calculated by determining the content of heparin in the Heparin BRP standard (1000 IU/ml) from EDQM. Standard is used as both sample and standard. Each analysis "run" covers all stages from the initial samples to the 4 dilutions of standard and

sample solutions loaded into the robot and analyzed so that all sources of error of dilution to the final measurement of ΔOD are included in the uncertainty.

					Potency (IU/ml)			Potency (%)relative to assay		
Results Overview for Heparin BRP 1000 IU										
Operator	Date	RUN	Standard Batch #	Sample Batch #	Lower limit	Estimate	Upper limit	Lower limit	**Estimate**	Upper limit
Vagn.H	13.05.15	1	SAmix1	SAmix1	991,5	**1002,6**	1013,7	99,1	100,3	101,4
David.M	13.05.15	2	SAmix2	SAmix2	983,0	**997,6**	1012,3	98,3	99,8	101,2
David.M	20.05.15	3	SAmix1	SAmix2	988,3	**1010,9**	1033,9	98,8	101,1	103,4
David.M	20.05.15	4	SAmix1	SAmix2	984,3	**1001,2**	1018,4	98,4	100,1	101,8
David.M	20.05.15	5	SAmix1	SAmix2	988,2	**1006,1**	1024,3	98,8	100,6	102,4
David.M	03.06.15	6	SAmix2	SAmix1	963,7	**985,2**	1007,2	96,4	98,5	100,7
				Mean	983,2	**1000,6**	1018,3	98,3	**100,1**	101,8
				SD	10,03	**8,79**	9,58	1,00	**0,88**	0,96
				%RSD	1,02	**0,88**	0,94	1,02	**0,88**	0,94

					Common Slope Factor			|r|	Non-paralellism	Regression
Operator	Date	RUN	Standard Batch #	Sample Batch #	Lower limit	Estimate	Upper limit			
Vagn.H	13.05.15	1	SAmix1	SAmix1	-104,095	**-103,037**	-101,979	**0,99652**	0,433	<0,000
David.M	13.05.15	2	SAmix2	SAmix2	-104,722	**-103,300**	-106,144	**0,99586**	0,14	<0,000
David.M	20.05.15	3	SAmix1	SAmix2	-104,318	**-102,383**	-100,248	**0,99373**	0,126	<0,000
David.M	20.05.15	4	SAmix1	SAmix2	-104,307	**-102,687**	-101,068	**0,99528**	0,135	<0,000
David.M	20.05.15	5	SAmix1	SAmix2	-103,752	**-102,060**	-100,368	**0,99619**	0,38	<0,000
David.M	28.05.15	6	SAmix1	SAmix1	-104,469	**-102,374**	-94,941	**0,99649**	0,669	<0,000
				Mean	-104,277	**-102,640**	-100,791	**0,996**		
				SD	0,33	**0,46**	3,60	**0,00**		
				%RSD	0,32	**0,45**	3,57	**0,11**		

Table 2. Results obtained for accuracy.

- Results are validated with the statistical values for "non-parallelism", "regression" and "correlation (r)" within the limits: >0.05; <0.05; >0.98, respectively.
- The Lower and Upper limit (983.2 to 1019.3 IU/ml) indicates the results confidence interval (P=0.95), which must be within in the range of 800-1250 IU/ml.
- The nominal value for the sample potency is 1000 IU/ml and the measured value 1000.6 IU/ml, differing 0.1% from the nominal value.

Conclusion: The method is found to be sufficiently accurate.

In ***Table 2***, the values for slope factor and correlation quotient (r) are included, belonging to the linear correlation of assay response against log concentration, used for acceptance/rejection of analysis results.

2.9. Repeatability.

Repeatability determination was performed through a repetition of analysis for Heparin SAD unpreserved IV solutions, using the products with the potency of 100 and 5000 IU/ml. Each analysis "run" covers all stages from the initial samples to the 4 dilutions of standard and sample solutions loaded into the robot and analyzed so that all sources of error of dilution to the final measurement of ΔOD are included in the uncertainty.

Results Overview for Heparin 100 IU

Operator	Date	RUN	Standard Batch #	Sample Batch #	Potency (IU/ml)			Potency (%)relative to assay		
					Lower limit	Estimate	Upper limit	Lower limit	Estimate	Upper limit
David.M	13.04.15	1	SA1	RAP 4382961	103,7	**107,6**	111,7	103,7	**107,6**	111,7
David.M	14.04.15	2	SA1	RAP 4382961	99,4	**101,9**	104,4	99,4	**101,9**	104,4
David.M	15.04.15	3	SA1	RAP 4382961	97,8	**101,0**	104,2	97,8	**101,0**	104,2
David.M	16.04.15	4	SA1	RAP 4382961	100,6	**103,4**	106,2	100,6	**103,4**	106,2
David.M	20.04.15	5	SA1	RAP 4382961	101,0	**102,8**	104,8	101,0	**102,8**	104,8
David.M	20.04.15	6	SA1	RAP 4382961	95,3	**106,3**	118,7	95,3	**106,3**	118,7
David.M	04.05.15	7	SA1	RAP 4382961	96,9	**100,2**	103,6	96,9	**100,2**	103,6
David.M	05.05.15	8	SA1	RAP 4382961	99,8	**102,4**	105,1	99,8	**102,4**	105,1
				Mean	100,5	**103,3**	106,3	100,5	**103,3**	106,3
				SD	2,16	**2,56**	3,14	2,16	**2,56**	3,14
				%RSD	2,15	**2,47**	2,95	2,15	**2,47**	2,95

Operator	Date	RUN	Standard Batch #	Sample Batch #	Common Slope Factor			|r|	Non paralellism	Regression
					Lower limit	Estimate	Upper limit			
David.M	13.04.15	1	SA1	RAP 4382961	-78,8570	**-77,8570**	-75,3489	**0,99892**	0,680	<0,000
David.M	14.04.15	2	SA1	RAP 4382961	-81,1224	**-79,9223**	-78,7222	**0,98837**	0,811	<0,000
David.M	15.04.15	3	SA1	RAP 4382961	-81,9061	**-80,3474**	-78,7888	**0,98641**	0,856	<0,000
David.M	16.04.15	4	SA1	RAP 4382961	-80,7591	**-79,4452**	-78,1313	**0,98568**	0,640	<0,000
David.M	20.04.15	5	SA1	RAP 4382961	-77,8779	**-77,0071**	-76,1364	**0,98696**	0,669	<0,000
David.M	20.04.15	6	SA1	RAP 4382961	-82,5642	**-77,5302**	-72,4961	**0,98722**	0,203	<0,000
David.M	04.05.15	7	SA1	RAP 4382961	-82,3683	**-80,6970**	-79,0257	**0,98610**	0,274	<0,000
David.M	05.05.15	8	SA1	RAP 4382961	-82,8353	**-81,5334**	-80,2315	**0,98424**	0,201	<0,000
				Mean	-80,105	**-78,916**	-77,426	**0,989**		
				SD	1,68	**1,42**	1,58	**0,01**		
				%RSD	2,09	**1,80**	2,04	**0,55**		

Table 3. *Results obtained for repeatability using Heparin SAD 100 IU/ml.*

- Results are validated with statistical values for "non-parallelism", regression" and "correlation (r)" within the limits: > 0.05; <0.05 and > 0.98, respectably.
- The Lower and Upper limit (98.9 to 103 IU/ml) indicates the result confidence interval (P=0.95), which must be within the range 80-125 IU/ml.
- The nominal value of the sample potency is 100 IU/ml, the measured value is 103.3 IU/ml, differing 2.56% from the nominal value. The analysis RSD is 2.47%.

					Results Overview for Heparin 100 IU					
					Potency (IU/ml)			Potency (%)relative to assay		
Operator	Date	RUN	Standard Batch #	Sample Batch #	Lower limit	Estimate	Upper limit	Lower limit	**Estimate**	Upper limit
Vagn H.	23.06.15	1	SA 02EOM1	RAP 4382061	97,0	**99,8**	102,8	97,0	**99,8**	102,8
Vagn H.	23.06.15	2	SA 02EOM1	RAP 4382061	97,5	**100,2**	102,9	97,5	**100,2**	102,9
Vagn H.	23.06.15	3	SA 02EOM1	RAP 4382061	99,8	**101,5**	103,2	99,8	**101,5**	103,2
Vagn H.	23.06.15	4	SA 02EOM1	RAP 4382061	100,8	**102,2**	103,6	100,8	**102,2**	103,6
Vagn H.	23.06.15	5	SA 02EOM1	RAP 4382061	99,4	**101,8**	104,3	99,4	**101,8**	104,3
Vagn H.	23.06.15	6	SA 02EOM1	RAP 4382061	102,7	**104,4**	106,1	102,7	**104,4**	106,1
				Mean	98,9	**101,1**	103,4	98,9	**101,1**	103,4
				SD	1,62	**1,05**	0,62	1,62	**1,05**	0,62
				%RSD	1,63	**1,03**	0,60	1,63	**1,03**	0,60

					Common Slope Factor			|r|	Non paralellism	Regression
Operator	Date	RUN	Standard Batch #	Sample Batch #	Lower limit	Estimate	Upper limit			
Vagn H.	23.06.15	1	SA 02EOM1	RAP 4382061	-103,463	**-100,778**	-98,093	**0,99393**	0,889	<0,000
Vagn H.	23.06.15	2	SA 02EOM1	RAP 4382061	-103,965	**-101,425**	-98,884	**0,99185**	0,803	<0,000
Vagn H.	23.06.15	3	SA 02EOM1	RAP 4382061	-101,326	**-99,809**	-98,289	**0,99525**	0,321	<0,000
Vagn H.	23.06.15	4	SA 02EOM1	RAP 4382061	-100,913	**-99,636**	-98,359	**0,99425**	0,497	<0,000
Vagn H.	23.06.15	5	SA 02EOM1	RAP 4382061	-102,462	**-100,236**	-98,011	**0,99195**	0,897	<0,000
Vagn H.	23.06.15	6	SA 02EOM1	RAP 4382061	-100,357	**-98,901**	-97,445	**0,99550**	0,329	<0,000
				Mean	-102,081	**-100,131**	-98,180	**0,994**		
				SD	1,45	**0,89**	0,47	**0,00**		
				%RSD	1,42	**0,89**	0,48	**0,16**		

Table 4. *Results obtained for repeatability using Heparin SAD 100 IU / ml.*

- Results are valid with the statistical values for "non-parallelism", "regression" and "correlation (r)" within the limits: > 0.05; <0.05 and > 0.98, respectively.
- Lower and Upper limit (98.9 to 103.4 IU/ml) indicates the result confidence interval (P=0.95), which must be within the range 80-125 IU/ml.
- The nominal value of the sample potency is 100 IU /ml, the measured value is 101.1 IU/ml, differing 1.1% from the nominal value. The RSD of the analysis is 1.03%.

					Results Overview for Heparin 5000 IU RAP 3452851					
					Potency (IU/ml)			Potency (%)relative to assay		
Operator	Date	RUN	Standard Batch #	Sample Batch #	Lower limit	Estimate	Upper limit	Lower limit	**Estimate**	Upper limit
David.M	08.06.15	1	02DMG3	RAP 3452851	4847,4	**4927,5**	5008,9	96,9	**98,6**	100,2
David.M	08.06.15	2	02DMG3	RAP 3452851	5084,7	**5244,2**	5409,2	101,7	**104,9**	108,2
David.M	22.06.15	3	02DMG3	RAP 3452851	4953,01	**5043,48**	5135,63	99,10	**100,90**	102,70
David.M	22.06.15	4	02DMG3	RAP 3452851	4862,3	**4950,8**	5040,9	97,20	**99,00**	100,80
David.M	22.06.15	5	02DMG3	RAP 3452851	5015,0	**5114,4**	5215,9	100,30	**102,30**	104,30
David.M	22.06.15	6	02DMG3	RAP 3452851	4711,65	**4827,78**	4946,52	94,20	**96,60**	98,90
				Mean	4912,3	**5018,0**	5126,2	98,2	**100,4**	102,5
				SD	100,72	**128,98**	160,33	2,04	**2,58**	3,21
				%RSD	2,05	**2,57**	3,13	2,08	**2,57**	3,13

					Common Slope Factor			|r|	Non paralellism	Regression
Operator	Date	RUN	Standard Batch #	Sample Batch #	Lower limit	Estimate	Upper limit			
David.M	08.06.15	1	02DMG3	RAP 3452851	-95,20	**-93,80**	-92,40	**0,997**	0,982	<0,000
David.M	08.06.15	2	02DMG3	RAP 3452851	-96,02	**-93,37**	-90,71	**0,991**	0,343	<0,000
David.M	22.06.15	3	02DMG3	RAP 3452851	-100,04	**-98,38**	-96,75	**0,994**	0,858	<0,000
David.M	22.06.15	4	02DMG3	RAP 3452851	-101,82	**-100,15**	-98,49	**0,994**	0,371	<0,000
David.M	22.06.15	5	02DMG3	RAP 3452851	-100,04	**-98,27**	-96,49	**0,992**	0,621	<0,000
David.M	22.06.15	6	02DMG3	RAP 3452851	-101,82	**-99,59**	-97,36	**0,992**	0,719	<0,000
				Mean	-99,15	**-97,26**	-95,37	**0,994**		
				SD	2,87	**2,94**	3,08	**0,00**		
				%RSD	2,90	**3,02**	3,23	**0,21**		

Table 5. *Results obtained for repeatability using Heparin SAD 5.000 IU/ml.*

Samples were prepared as indicated for the experiment, described in Table 3.

- Results are valid with statistical values for "*non-parallelism*", *regression*" and "*correlation* (r)" within the limits: > 0.05; <0.05 and > 0.98, respectably.
- The Lower and Upper limit (4912.3 to 5126.2 IU/ml) indicates the result confidence interval (P = 0.95), which must be within the range of 4000-6250 IU/ml.
- The nominal value of the sample potency is 5000 IU/ml, the measured value is 5018 IU/ml, differing 0.4% from the nominal value. The analysis RSD is 2.57%.

					Results Overview for Heparin 5000 IU RAP 3452851						
						Potency (IU/ml)			Potency (%)relative to assay		
Operator	Date	RUN	Standard Batch #	Sample Batch #	Lower limit	Estimate	Upper limit	Lower limit	**Estimate**	Upper limit	
Vagn H.	12.06.15	1	02EOM1	RAP 3452851	4969,0	**5105,9**	5246,6	99,4	**102,1**	104,9	
Vagn H.	12.06.15	2	02EOM1	RAP 3452851	4903,7	**5009,5**	5117,4	98,1	**100,2**	102,3	
Vagn H.	12.06.15	3	02EOM1	RAP 3452851	5039,7	**5118,1**	5197,6	100,8	**102,4**	104,0	
Vagn H.	15.06.15	4	02EOM1	RAP 3452851	4926,6	**5024,5**	5124,4	98,5	**100,5**	102,5	
Vagn H.	15.06.15	5	02EOM1	RAP 3452851	4870,1	**4960,6**	5052,8	97,40	**99,20**	101,10	
Vagn H.	15.06.15	6	02EOM1	RAP 3452851	4975,9	**5055,2**	5135,5	99,5	**101,1**	102,7	
				Mean	4947,5	**5045,6**	5145,7	99,0	**100,9**	102,9	
				SD	60,18	**59,92**	67,63	1,20	**1,21**	1,34	
				%RSD	1,22	**1,19**	1,31	1,22	**1,19**	1,30	
		RUN	Standard Batch #	Sample Batch #	Common Slope Factor			|r|	n paralelli	Regression	
Operator	Date				Lower limit	Estimate	Upper limit				
Vagn H.	12.06.15	1	02EOM1	RAP 3452851	-96,056	**-93,709**	-91,363	**0,99301**	0,919	<0,001	
Vagn H.	12.06.15	2	02EOM1	RAP 3452851	-98,952	**-97,070**	-95,188	**0,99467**	0,344	<0,001	
Vagn H.	12.06.15	3	02EOM1	RAP 3452851	-98,193	**-96,798**	-95,402	**0,99434**	0,119	<0,001	
Vagn H.	15.06.15	4	02EOM1	RAP 3452851	-93,005	**-91,347**	-89,690	**0,99571**	0,642	<0,001	
Vagn H.	15.06.15	5	02EOM1	RAP 3452851	-94,125	**-92,533**	-90,941	**0,99632**	0,894	<0,001	
Vagn H.	15.06.15	6	02EOM1	RAP 3452851	-94,664	**-92,967**	-91,270	**0,99751**	0,754	<0,001	
				Mean	-95,832	**-94,071**	-92,309	**0,995**			
				SD	2,35	**2,35**	2,39	**0,00**			
				%RSD	2,45	**2,50**	2,59	**0,16**			

Table 6. Results obtained for determining repeatability using Heparin SAD 5.000 IU / ml.

- Results are validated with statistical values for "non-parallelism," "regression" and Correlation (r) within the limits > 0.05; <0.05 g> 0.98, respectively.
- The Lower and Upper limit (4947.5- 5145.7 IU/ml) indicates the result confidence level (P = 0.95), which must be within the range of 4000-6250 IU / ml.
- The nominal value of the sample potency is 5000 IU/ml, the measured value is 5045 IU / ml, differing 0.9% from the nominal value. The analysis RSD is 1.19%.

Conclusion: The values in Table 3, 4, 5 and 6 show the method is repeatable as the relative deviation of measurement results is less than 5%.

2.10. Reproducibility.

The reproducibility is determined by the accuracy, which is obtained when the analysis is performed by two different people on different days.

Table 7 shows a summary overview for the relative standard deviations of the analysis presented in tables 3, 4, 5 and 6.

Relative deviation RSD%

	Batch #	Data David M.	Data Vagn H.
Heparin 100 IU	4382061	2.47	1.03
Heparin 5000 IU	3452851	2.57	1.19

Table 7. Reproducibility evaluation in the form of analysis results deviation, based on two preparations analyzed by two operators.

The difference between results of the two operators and the two preparations is small. At the same time, the relative deviation is low compared to the expected relative spread (<5%) in the group of biological assay potency results, in which the heparin assay belongs.

Conclusion: The method is reproducible.

2.11. Requirements for blank amidolytical activity.

The blank amidolytical activity is listed in the pharmacopoeia as an analytical result of a single sample measuring the ΔOD where, instead of test or standard, is added reagent R1. During the period of analysis we performed 336 measurements, obtaining the following results: Mean ΔOD = 344.6, SD = 5.80 and SD% = 1.7%.

The pharmacopoeia requirement reference is: "*activity at the beginning and at the end of the procedure*…" "*…do not differ significantly*". However, the deviation is not defined. USP states a limit in terms of RSD <10% for the amidolytic activity measured before and after the completion of an analysis of 32 samples. The values obtained are well within these limits.

Conclusion: The requirements for Blank Amidolytical activity are met**.**

2.12. Specificity.

Influence of added excipients on the test results:

The pharmacopeia method has been developed for determining heparin activity in the raw material, and without added excipients used for formulating a finished product. Heparin SAD is formulated as unpreserved injections, the only substance added is NaCl, 9 g/L (6.8 to 9.0 g/L) in order to reach an isotonic solution.

Since the pharmacopeia analysis is performed on highly diluted solutions, the maximum concentration of NaCl in the final sample solutions is be 3 ppm derived from SAD products. This should be seen in relation to the content of NaCl in the analysis main solvent tris EDTA buffer with 1% NaCl. NaCl from the sample is, therefore, a negligible proportion of the total quantity of NaCl in the sample solution.

Based on this, it is estimated that the heparin assay of ready formulated injection, diluted with tris EDTA buffer, does not give different results when compared to a potency of equipotent amount of heparin in tris EDTA buffer.

Therefore, the influence on the analytical result of sodium chloride in the IV solution is not further investigated.

3. Conclusion.

The pharmacy´s heparin IV potency can be determined by the implemented method since relevant analytical parameters, such as accuracy, repeatability, and reproducibility, are within the demands of the method as:

- The specific method is shown valid for a sample containing 1000 IU/ml;
- The method is proved accurate (repeatable and reproducible) when used for Heparin IV SAD products with the lowest and highest potency, 100 and 5000 IU/ml;
- The test for "blank amidolytical activity" is fulfilled. Method is specific because the SAD – products, beside heparin and water, is only added NaCl in minor amounts compared to the NaCl in the assay reagents.
- All the results gathered upon analysis comply with the pharmacopoeia requirements for potency (containing 90-110% heparin; 80-125% Cl), as well statistical parameters for assessing the analysis suitability.

4. Acknowledgements