The Development and Validation of a Quantitative Mass Spectrometric Method for the Analysis of Polypeptides in Human Plasma

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CONTENTS

I) Declaration

I declare that the work submitted in this thesis is my own, except where otherwise acknowledged, and has not been submitted for a degree or qualification at this or any other university.

Julie C Vincent

ii) Acknowledgements

I would like to thank and acknowledge my supervisor Professor Jane Thomas-Oates, whose support has inspired me towards in a career in mass spectrometry. I also am very grateful for the support and constant guidance of my industrial supervisor Dr. Phillip Turpin (Principal Method Development Specialist at Covance Laboratories Ltd) in both my research and in bioanalytical method development, which has lead me to an enjoyable and challenging career and a passion for mass spectrometry. Dr Turpin has not only acted as a supervisor to this project but as a mentor and his support throughout this project has been unparalleled e.

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iii) Publications (Posters)

Method development, optimisation and validation of a turbo ion spray LC-MS/MS bioanalytical assay for the quantitative analysis of arginine-8-vasopressin (AVP) in human plasma; Julie Vincent, Jane Thomas-Oates, Phillip Turpin ; Analytical Research Forum 2009

Method development of a bioanalytical assay for the quantification of arginine-8-vasopressin in human plasma including a performance evaluation and comparison of UHPLC-ESI-MS/MS with UHPLC-ESI-MS/MS; Julie Vincent, Mark Harrison, David Browne, Jane Thomas-Oates, David Bakes, Philip Turpin; BMSS 30TH Annual Meeting 7-10 September 2008

iv) Abstract

An assay for the quantitative analysis of the nonapeptide arginine-8-vasopressin has been developed. This utilised sample preparation techniques such as solid phase extraction in conjunction with ultra-high performance liquid chromatography. Methods to improve the limit of detection of AVP have been performed, utilising mass spectrometry. High field asymmetric ion mobility has been trialed to assess if the interface gave benefits. The limit of detection could not reach that of immunoassay techniques of 1 pg/mL but has been validated from 0.25-10 ng/mL.

Chapter 1 - Introduction

1.1 Introduction to bioanalysis and the pharmaceutical industry

Every year pharmaceutical companies spend millions of pounds on R&D to assess and develop potential new compounds hoping for the latest blockbuster drug that will help treat a disease and potentially bring them a healthy return on their investment.

Ten years ago, Edwards predicted a revolution for therapeutic peptides [1]. Since then, almost every pharmaceutical company has shifted some of their R&D of new chemical entities (NCEs) from being mainly focused on small molecules, to branching into the peptide market [2]. Peptides are important to the pharmaceutical and biotechnology industries due to their low toxicity and high potency, making them excellent drug candidates [3]. They can also be beneficial as biomarkers in disease [4], not to mention the fact that there are currently several therapeutic peptides with a market size of greater than $1 billion [5].

In 2004, an important report into the strategic analysis of the therapeutic peptide market was conducted by Frost and Sullivan [6]. The findings showed there to be more than 40 peptides on the market, nearly 300 in early stage clinical trials and over 400 in advanced stage clinical trials [6, 7]. Since then this market has continued to grow, with 67 peptides available to market in 2007 [2], and was expecting double-digit growth between 2003 and 2010 [8].

In this research, an important group of peptide compounds – the vasopressin family, in particular arginine-vasopressin – has been investigated. This was performed in the context of biological analysis (bioanalysis) as used in the pharmaceutical and contract research (CRO) industries for new medicines as required by law [9-13].

Bioanalysis is the analysis of drugs and chemicals in biological materials in order to gain information about them, which will enable drug discovery and development [14]. The route by which this is done is often not direct or straight forward, as there are many challenges along the way [15]. This research details many of the challenges faced in developing and validating a bioanalytical method and how to potentially overcome them.

The aim of bioanalysis is to assess the levels of a particular drug in biological fluid in different stages of the drug development process. The stages encompass discovery, where thousands of compounds are screened and the disease mechanisms analysed and pre-clinical (sometimes referred to as toxicological), where the effects of the drug in the body are measured (toxicology and metabolism). Once these stages have been assessed, application is submitted to the regulatory authority [9-11]. On approval, phase I, II and III trials commence dependent on the results of the previous stage. Phase I comprises low levels of the drug being dosed into a small number of healthy volunteers [16]. Phase II uses patients to see if the drug can treat the disease [16]. Phase III is a much

larger scale study of patients, which also uses a placebo in some patients; it is randomised and controlled to ensure a high standard of data, which will then be sent for approval to the regulatory bodies [16]. After approval, doctors monitor side effects in patients [16]. It is the samples generated in these trials which will be analysed using methods such as the one developed and validated in this research to quantify the amount of drug in each of the samples.

The process of bioanalysis starts with the target analyte which is used in the toxicological and clinical trials. This is used to dose the subjects. Samples of biological material are then taken at various intervals. These can be blood, plasma, serum or urine, among others [17]. These samples are then cleaned, analysed and the amount of target analyte in each sample compared to that of standards of known concentrations. To do this a method which is robust in the assessment of the analyte must be employed and to get a suitable method, a method development process of trials based on analyte characteristics is performed, optimized and then a final method is validated for use in the analysis of the target analyte.

The target analyte in this work was a nonapeptide hormone called arginine-8-vasopressin (AVP). It is 1.1 kDa in size and comprises nine amino acids with a disulfide cysteine bridge. It is being used as a model compound for basic peptides in the development of a bioanalytical assay to explore what benefits can be gained in bioanalytical method development by improving the assay.

AVP has many important roles in the body but its primary role was discovered 20 years ago by Verney and Du Vigneaud as being responsible for the osmotic constancy of body water [18, 19]. The importance of AVP in the renal system is widely researched and documented [18, 20-21]. AVP works to facilitate the "reabsorption of solute-free water from the collecting duct of the kidney" [22]. Further to this, patients with type 1 diabetes are resistant to the anti-diuretic effects of AVP; this is shown through them not being able to recruit aquaporin-2 and subsequently impaired urine regulation ability.

Secondary effects of AVP include its effects on the circulatory system [23-24], as a biomarker [25-26], and in psychological diseases [627-28]. Elevated AVP levels can be directly related to arterial pressure, cardiovascular surgery [20], chronic heart failure [29], hypertension and bradycardia [23].

As a biomarker for disease, AVP is quite generic and elevated levels can be seen in plasma in diseases such as alcoholism [30], potential tumours [25], diabetes [31] and when a subject is under psychological pain or distress [33]. In cerebral spinal fluid (CSF) it is seen in increased levels in patients with motor neurone disease [26]. Other important roles AVP plays are in myometrial contractions in human labour [32], in mood disorders [33], and in response to IVF superovulation treatments [34].

The method for analysis most commonly used for AVP is immunoassay [35-38, 33, 18, 25, 34] where the limits of detection are in the range 0.5-1 pg/mL plasma. At present there is limited vasopressin research using LC-MS/MS as the primary method for quantitative analysis. It is therefore of great interest to determine the limits of detection which can be reached and to determine whether these can compete with immunoassay statistics.

Immuno-affinity techniques are commonly used as a method of analysing peptides in biological fluids, as they have the potential to be highly selective due to very specific antibody-antigen binding [36-38], and sensitive. They require an antibody binding specifically to the target analyte or to sites on the molecule. These antibodies are generated in a host (animal) and it is this production of antibodies that is the time-limiting step in the process [17,39-40].

The advantages of immunoassay are that it is very sensitive, has a low limit of quantification [35-37, 33], does not rely too heavily on the characteristics of the analyte such as its ability to ionise or fluoresce [17], it can be automated to increase throughput, and it is generally very selective although it may not be able to differentiate between the analyte and a closely related metabolite [38].

The disadvantages of immunoassay are the amount of time it takes to set up [17], which can take from four months to a year, the selectivity of the assay where cross-reactivity testing needs to be very thorough to ensure that metabolite and

parent are distinguishable, matrix effects can occur in urine due to pH differences and the presence of polycarboxylic acids [17]. There can also be problems with false negatives if there is too high a level of antigen present [17].

In this work, the challenge was to investigate the levels of AVP that can be detected using state of the art quantitative mass spectrometric techniques, so that a method could be developed and validated suitable to analyse samples from pre-clinical and clinical trials.

Mass spectrometry is an important tool in bioanalysis because it is a highly selective technique [4, 32] that relies on the mass of the target analyte and the number of charges it possesses, which is especially significant when using electrospray ionisation where multiply charged ions may be formed [41-43]. A major benefit of mass spectrometry is its ability to differentiate between similar metabolites and the analyte, sometimes not possible in immunoassay [17]. However a major drawback is the limit of detection [35-38] that can be reached, especially at physiological levels, although this can be less of a problem for biomarkers such as AVP which has been shown to have elevated levels in disease states [4, 44-46].

There are many challenges in bioanalysis [15, 47, 48] some of which stem from the complex procedure of removing analyte from the biological matrix, effects of the matrix on the target analyte [48], and the compliance with government

regulations [9-11]. In the quest for cleaner samples, preparation procedures such as protein precipitation and solid phase extraction can be used in conjunction with liquid chromatography mass spectrometry for the removal of impurities and to extract and concentrate the analyte, and thus improve limits of detection.

In the cleaning of samples, matrix interferences need to be removed as these may interact with the analyte, causing inaccurate concentration readings, caused by enhancement or suppression of the detected signal. Removing the analyte from a biological matrix can be a complicated process. The main problem is removing the many proteins in plasma that may interfere with the analysis. Matrix effects are the suppression or enhancement of the detected analyte signal by components of the sample matrix, and are often compared by using pure standards in the presence and absence of biological matrix [49-59]. There are several ways to reduce matrix effects, the main one of which is to improve sample clean-up by using fractionation techniques [60-61].

1.2 Fractionation techniques

Fractionation techniques are important in bioanalysis due to the nature of the matrices and sample complexity involved e.g. blood, urine, plasma, and the often low levels of analyte that need to be detected and quantified. The most important factors are to isolate the analyte [62-64] and remove any interfering substances [17, 62, 64-65].

There are many stages in bioanalysis where fractionation techniques are employed. These can range from the centrifugation of un-clotted blood to give plasma, through to sample preparation via column chromatography or ultra high performance liquid column chromatography (UHPLC). The focus of the techniques applied in this work is on preparing plasma samples read for high/ultra high performance liquid chromatography-mass spectrometric analysis.

Fractionation techniques are employed prior to quantitative analysis as the levels of interferents in the sample are frequently too high to be removed solely by an HPLC step. This may be due to high levels of proteins in the sample [17,66-67] or the sample may need to be concentrated to improve limits of detection [65, 68]. The most common techniques employed in bioanalysis are protein precipitation, liquid-liquid extraction and solid phase extraction [17, 64], each of which have different benefits, drawbacks and suit abilities for different types of analytes.

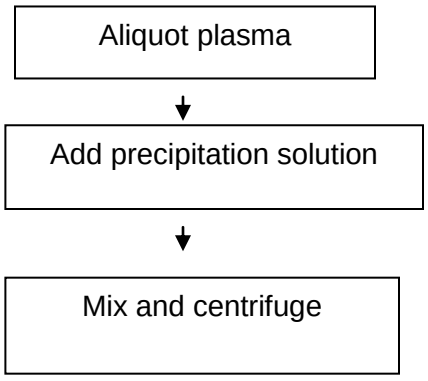
1.2.1 Protein precipitation

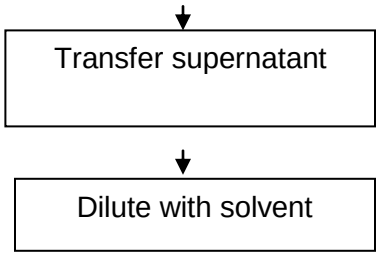
Protein precipitation (PP) is the simplest sample preparation technique utilised, as it focuses only on the removal of proteins from plasma. Proteins need to be removed from the sample due to their incompatibility with subsequent separation techniques such as HPLC [17, 64, 67] There are a variety of ways to remove proteins from a sample using heat, salts, organic acids or solvents [17, 64, 67].

The most commonly used method for protein removal is to use solvents (organic) due to their being readily available, inexpensive and the technique being simple [66], they are also volatile and so can be readily removed from the sample if necessary. When organic solvents are added to plasma (at least 1:3 plasma: solvent) [17,66, 69], the di-electric constant of the solution is lowered, which results in an increase in the electrostatic interactions between protein molecules [17]. The organic solvent molecules can also displace water molecules, which can increase the electrostatic interactions and further the protein aggregation [17]. The ultimate aim in PP is to denature the proteins so that they precipitate out of solution and can be removed by centrifugation (>3000g) [17,69-71].

The benefits of this technique lie in the fact that it can be easily automated in a 96 well plate format, resulting in it being cheap and allowing high sample throughput.

Figure 1 – Protein precipitation procedure



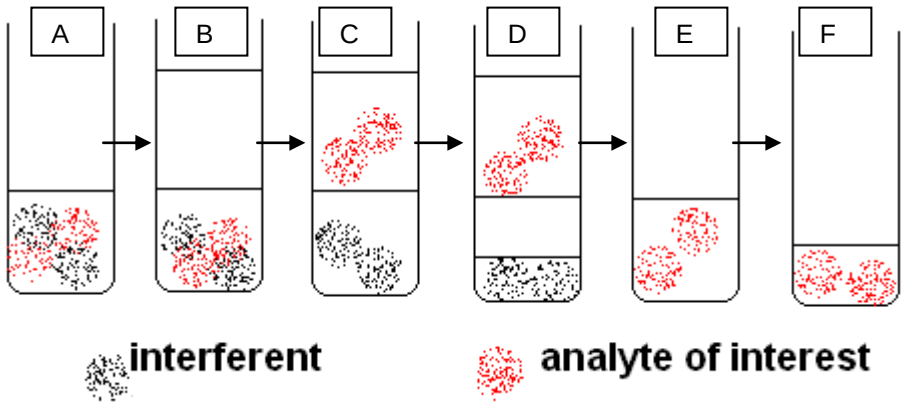


Although PP is often the method of choice and a primary step in bioanalytical method development, it only removes protein and so the sample may require further clean up e.g. solvent treatment does not remove phospholipids and these may interfere with detection [72-73] and cause matrix effects [65, 68, 72-73]. Slightly more complex methods such as liquid-liquid extraction using analyte properties to achieve a more thorough clean up may be preferable.

1.2.2 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a separation technique based on the use of two immiscible solvents [64] (generally an aqueous and an organic), and is dependent on polarity and the solubility of the analyte in each of the solvents [66]. The main influences in LLE are the pH at which the analyte is un-ionized [64], as this technique is generally not suitable for isolation of charged analytes [66], the volume of solvents used, which should be at least five times the aqueous sample volume and well mixed [17], and the compatibility of the analyte with each of the phases –e.g. non-polar analytes extract best into non-polar solvents [67]. If the analyte moves into the organic layer, the solvent may be suitable to be evaporated and the sample reconstituted in a solvent of choice, depending on the type analysis required (figure 2).

Figure 2 - Liquid-liquid extraction



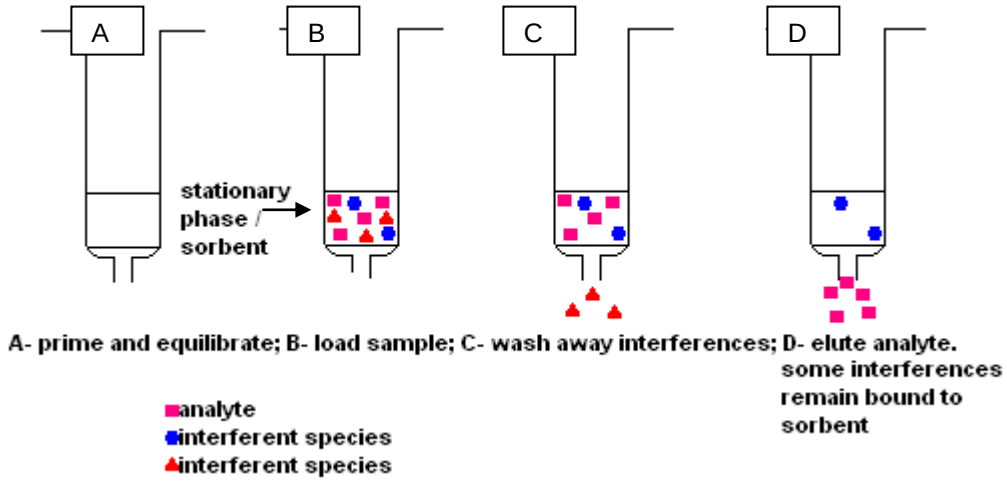
A: sample in plasma matrix, B: addition of extraction solvent, C: mixing phases followed by phase separation, D: centrifugation, E: removal of supernatant, F: evaporation and reconstitution in appropriate solvent.

Although LLE may benefit sample clean up as it is not just based on removing proteins but on the analyte's chemical characteristics, however it is unsuitable for the analysis of polar or ionized species.. Other techniques such as solid phase extraction (SPE) can offer improved clean-up and has up to 50 times the separating power of a simple LLE [62].

1.2.3 Solid phase extraction (SPE)

SPE uses a solid support, in the form of a stationary phase , which the analyte can interact with and bind to selectively. The process involves steps to prime the solid support and equilibrate it, so that it is prepared for the sample. The sample is then loaded and passes through the SPE column, where the analyte interacts with the sorbent. Elution of interferent species takes place and then separately of the analyte itself with specifically tailored washes. After elution, the sample is evaporated and reconstituted in a medium appropriate for LC-MS/MS analysis. A diagram of the processes in SPE is given in figure 3.

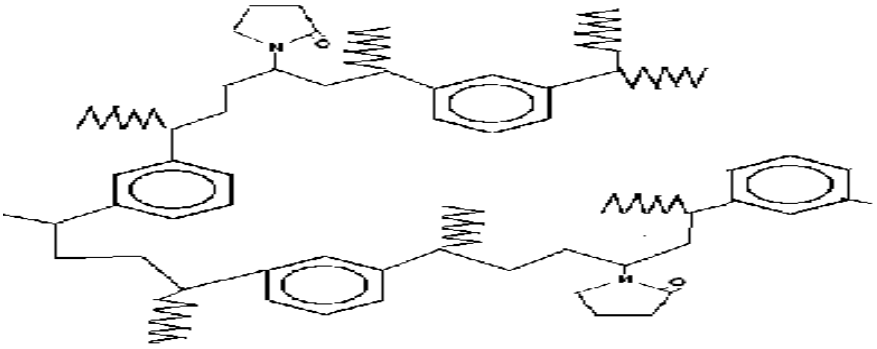
Figure 3 - SPE



SPE gained popularity in bioanalysis in the early 80s as a replacement for LLE as it is compatible with a larger variety of compounds [62,64, 66]. The main reason for this was that it is not only compatible with polar compounds but also zwitterions such as amino acids, as there are many different stationary phases which can be used [66]. The mechanism of SPE depends on using a chromatographic solid phase in the form of a solid sorbent and a mobile phase. The mechanism for SPE is dependent on the type of stationary phase used and this determines which mobile phase(s) can be used. The two main mechanisms are reversed phase and ion exchange, although due to the polar nature of the analytes typical of bioanalysis [68, 74], mixed mode SPE is often used.

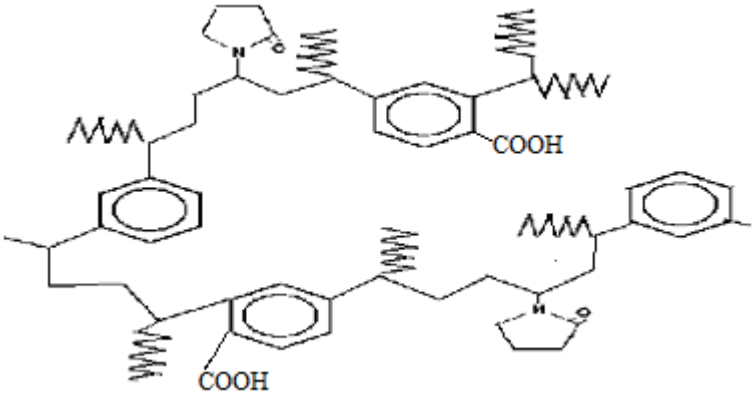
Reversed phase (RP) SPE generally uses a C18 or C8 type packing. However there are polymer sorbents such as polystyrene di-vinyl benzene (Waters Oasis HLB) [6875-77] which have become increasingly popular. The structure of this polymer can be seen in figure 4. This method development, as previously mentioned takes place in the form of a gradient profile, where different ratios of solvent are trialed to determine the best sample clean up (Figure 3, c) and recovery (Figure 3, d).

Figure 4 –HLB reversed phase sorbent structure, *adapted from* [18]



Mixed mode SPE is an important technique that utilises different interactions in the same stationary phase. An example of this is Waters Oasis mixed mode weak cation exchange sorbents for very basic compounds (WCX). When using an ion exchange sorbent it is important that the sample is loaded at the correct pH for the analyte to be ionized [17,68,75,78] and subsequently interact with the charged species on the stationary phase [75,79]. Once the analyte is bound, it may be transferred between the charged groups and benzene ring using the ionic and RP interactions to assist sample clean up. The stationary phase structure for this mixed mode sorbent is given in figure 5.

Figure 5- Waters Oasis WCX sorbent chemistry, *adapted from* [18]



Once the process of removing interferences from the biological sample has been completed using primary separation techniques, it is then introduced to a secondary more advanced separation technique such as HPLC, interfaced with a detector for quantification.

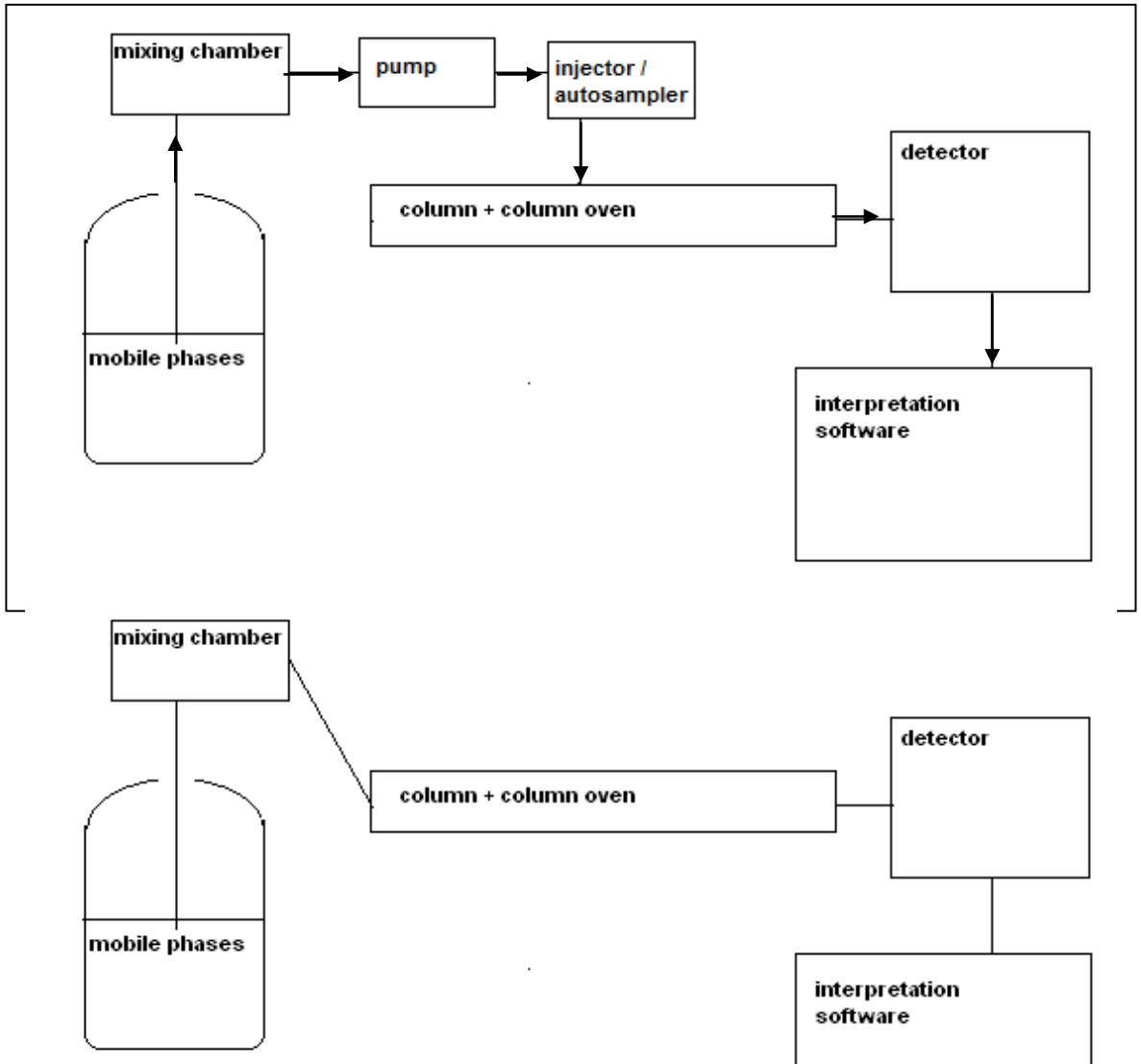
1.3 Separation techniques

High performance liquid chromatography (HPLC) is an analytical chromatographic technique which separates molecules based on their physico-chemical characteristics [63,80] using a stationary phase for which analytes have different affinities to [81-82]. It works at high pressures of 6000 psi and modern ultra high performance liquid chromatography can work at up to 15000 psi [83]. This means that smaller particles can be used in the stationary phase along with higher flow rates, reducing sample analysis time.

1.3.1 The HPLC system

The principle components of an HPLC system are the solvent reservoir, the pump(s), injection valve, column (and column oven), the detector and recorder. A schematic is given in figure 6.

Figure 6 – Schematic of an HPLC system



The solvent reservoirs not shown on diagram supply the mobile phases and needle washes. The mobile phases are used to control the elution of analyte from the column [69,83-84]. The washes are used to clean the needle and loop after each injection in order to minimise sample carryover [17,84].

The pumps used in HPLC need to be 'pulse-free' and to give a steady continuous flow at high pressures otherwise a steady signal will not be detected. This can be done using dual-piston pumps [66]. In UHPLC, two serial flow pumps are used and enable mixing at high pressure. [85-86]

The column itself consists typically of a stainless steel body which has a polished interior filled with stationary phase [69,87,88]. The internal diameter (i.d) of an analytical HPLC column is generally 4.6 mm and the length 25 cm [88]. In UHPLC this is typically reduced to an i.d of 2.1 mm and length 5 cm. The diameter of the packing material particles is especially important. This is because the smaller the particles, the tighter the packing can be, resulting in more particles in a small space [87] and the more regular the path of the analyte through the column [reduces peak broadening – van Demeter]. This means that there is a larger surface area for the sample to interact with but also small gaps between particles through which the phase flows, causing higher back pressures [87]. This is especially important for UHPLC columns where the particles are sub 2 μm in size. The column oven allows the temperature can be controlled and varied as required.

The type of detector used in quantitative bioanalysis is most commonly a mass spectrometer as it is sensitive and selective [75,89]. This is discussed in more detail later in this chapter, once the fundamental principles of chromatographic separations have been explored.

1.3.2 Chromatography principles

Chromatography is the movement of mobile phase over a stationary phase where analytes are separated based on their differential affinities for the stationary and mobile phases, causing retention and separation [90]. There are five main types of molecular forces which govern the interactions between the analyte and the phases [91]. These are 1, Van der Waals forces which occur between non-polar molecules [92-93]; 2, dipole-dipole interactions [94] which result from a distorted electron cloud; 3, hydrogen bonds between proton donors and acceptors [95]; 4, dielectric and finally 5, electrostatic interactions [91] which are charged species interactions e.g. ion-ion interactions [90].

1.3.2.a Distribution

The first important factor in chromatography is the distribution of analyte between stationary and mobile phase [96]. This can be described as the concentration of analyte in the stationary phase divided by the concentration of analyte in the mobile phase [17, 84]. The equation for this is $k=c_s/c_m$ where the distribution co-efficient is referred to as k and the concentration in the stationary and mobile phases c_s and c_m respectively. The value of k is important in chromatography as a large value of k

reflects a high proportion of analyte in the stationary phase compared to the mobile phase, thus retention is greater and elution slower. A small value of k reflects a small amount of analyte in the stationary phase and a larger amount in the mobile phase resulting in less retention and fast elution from the column [84].

1.3.2.b Retention

The extent to which an analyte is retained on a column is called its retention and it is determined by the amount of time the analyte takes from injection to it leaving the column and being detected. This is the retention time t_R. The rate at which the sample travels from injector to detector is described by the retention (or capacity) factor k' [64,87-88,97]. Calculation of k' for all analytes is the retention time minus the time taken for the mobile phase to pass through the column (t_m), divided by the time taken for the mobile phase to pass though the column (t_m). The equation is $k' = (t_R - t_m)/t_m$.

1.3.2.c Selectivity

Separation of components of a mixture is important in bioanalysis due to the complex matrices which are analysed. Therefore the analytes need to be retained selectively so that they do not co-elute [88]. The selectivity factor (α) is dependent on the retention factor k' of each analyte (a and b) as $\alpha = k'_a / k'_b$.

1.3.2.d Column efficiency

The efficiency of the column is an important consideration as in chromatography there is a needs for chromatographic peaks which are sharp, separate and resolved with as short a run time as possible so as to maximize throughput. There are two models for calculating column efficiency; the first model based on theoretical plates has by some been replaced by the rate theory [90, 98].

1.3.2.e Plate theory

The theoretical plate model was set out by Nobel laureates Martin and Synge in 1954 [90,98]. The idea is that the column is made up of a series of theoretical plates [82,90] and at each of these plates there is a phase distribution as described in eqn 1. As the mobile phase flows through the column, so does the analyte, which is in the mobile phase and so flows from one plate to the next [17]. An important factor in plate efficiency is plate height which needs to be as small as possible. One way of lowering it is to use smaller particles of stationary phase [90]. Height equivalent to the theoretical plate (HETP) can be calculated using equation 1; this equation has two unknowns, the HETP and the number of plates. The number of theoretical plates [90,98] can be calculated from equation 2. The modern version of this theory is the rate theory and it forms the basis of the van Deemterequation, which is given in equation 3.

Eqn 1: HETP = column length / number theoretical plates

Eqn 2: number of theoretical plates = $\frac{5.55 \times (\text{retention time})^2}{(\text{peak width at half height})^2}$

Eqn 3: $HETP = A + B / \mu + C\mu$

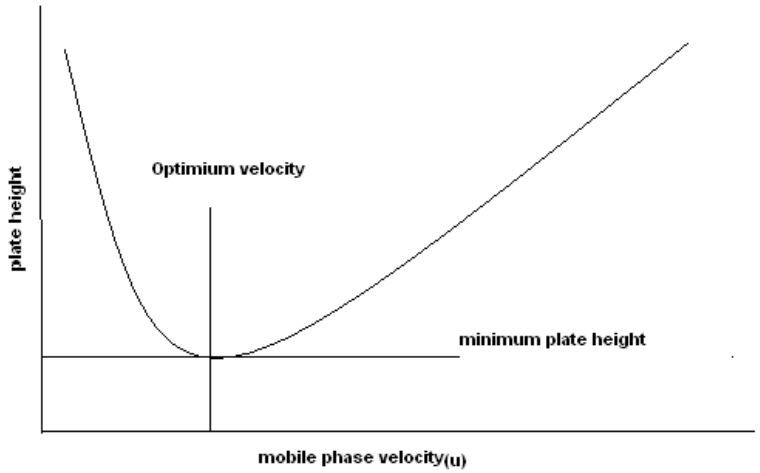
1.3.2.f Rate theory

The basic principles of rate theory are that it takes different factors into consideration such as the time it takes for the partition equilibrium of the analyte between mobile and stationary phases [90]. It is described by the van Deemterequation (3). The factors affecting the HETP include the consideration of eddy diffusion [90,99-103] (A term in equation 3), where molecules may not all take the same route through the stationary phase particles [90]. Longitudinal diffusion [90,99-103] is described by the B term in equation 3 and reflects the speed (linear velocity) at which the analyte flows through the column. This is important as it can impact on peaks becoming wide and diffusing at the edges of the peak (band broadening), which is caused when the flow rate is too slow and the analyte itself does not form a sharp peak but a broad one. The barrier to mass transfer is the C term in equation 3. It is of importance because when the velocity of the mobile phase is too high, but the analyte has a high affinity for the stationary phase, the analyte in the mobile phase will quickly pass through the column due to the flow rate. If there is a greater analyte interaction with the stationary phase, it will take

longer to elute the analyte which can result in subsequently broad peaks [90,101-103]. The linear velocity of the mobile phase in equation 3 is μ [90].

A van Deemterplot can be used to describe the relationship between plate height and mobile phase flow rate as illustrated in figure 7. This shows the minimum plate height and thus the optimum velocity of the mobile phase.

Figure 7- van Deemterplot, adapted from [90]



1.3.2.g Resolution

The resolution is a measure of how well two peaks separate [17]. The separation of two peaks can be described in terms of their resolution as given in equation 5, where t_R is the retention time, w the peak width at base and a and b analytes in the mixture. Baseline resolution of two peaks occurs when R≥1.5.

Eqn 5: $$R = \frac{2(t_{ra} - t_{rb})}{W_a + W_b}$$

The resolution can be influenced by the number of theoretical plates in the column (N), the selectivity factor and the retention factor of the analytes to be separated [94,103]. These factors can be combined to give equation 6. In this equation there is the retention factor (k') which can be altered by changing mobile phase conditions, the number of theoretical plates (N), which can be increased by lengthening the column or reducing the stationary phase particle size, and also the selectivity factor (α) which can be altered by changing mobile or stationary phases and column temperature [101]. Other changes that can be made to improve the resolution include: changing the mobile phase flow rate, using a gradient elution and ensuring the packing and stationary phase particle size are uniform [82].

Eqn 6: $R=(\sqrt{N}/4)\{(a-1)/a\}\{(1+k'_b)/k'_b\}$

1.3.3 Separation mechanisms

There are many different types of chromatography used in HPLC. The chemistries can be similar to some of those exploited in SPE, such as reversed phase or ion exchange, however a gradient elution can be applied which can allow for faster analysis for a wide variety of compounds with different properties. The main mechanisms utilized in HPLC are adsorption in normal phase (NP) chromatography, and partitition in reversed phase chromatography (RP) and hydrophilic interaction liquid chromatography (HILIC).

Adsorption in NP chromatography works by utlising the adsorptive properties of the stationary phase (often silica); suggested mechanisms for this type of

chromatography are based on analyte molecules displacing solvent molecules that are bound to the stationary phase, and also sorption of the analyte molecules onto the layer of solvent molecules which are adsorbed onto the stationary phase, without any kind of displacement [17,84,101, 103]. Key interactions in the mechanism of NP chromatography include dipole-dipole and dipole induced dipole interactions [94,104-105] as well as hydrogen bonding. In NP chromatography, the stationary phase is polar, the mobile phase is non-polar and the least polar components of the sample mixture are eluted first [84].

Reversed-phase chromatography is common in bioanalysis due to its robustness and reproducibility. A common mechanism for some reversed-phase systems is partitioning which works by hydrophobic interaction [84]. The stationary phase is made up of carbon chains on a silica support, the more carbon atoms in the chain, the better the retention of hydrophobic analytes as there is increasing hydrophobicity of the stationary phase [103,106]. Subsequently a common chemistry used is the C18. In reversed-phase chromatography, the stationary phase is non-polar, the mobile phase polar and subsequently the most polar components are eluted first [84].

HILIC is a separation technique which uses a hydrophilic stationary phase. There are many different types of stationary phases for HILIC such as zwitterionic phases, however this section focuses on using bare silica due to its reproducibility [103]. The stationary phase is saturated with a layer of water molecules and these surround the silica [103]. The main proportion of the mobile phase is an organic solvent,

typically acetonitrile. There are many aspects to the mechanisms that occur in HILIC, starting with the partitioning, where the analyte can move between the organic phase and water phase which surrounds the silica [103], where the analyte is retained until the amount of water in the mobile phase exceeds the level required to saturate the silica. Another important factor is the Coulombic interactions and hydrogen bonding resulting from the stationary phase, and the water molecules, which are bound to the phase [107-109].

Benefits of HILIC include increased ESI-MS sensitivity due to the high organic solvent [17]. The elution order is in terms of analyte hydrophilicity, with the least hydrophilic analytes eluting first.

A detector is required to measure the signal produced by the analyte in order to quantify it. The types of detector can be varied, but the most common type used today is a mass spectrometer.

There are many different types of mass spectrometers, however the type often used in quantitative bioanalysis is a triple quadrupole [77, 110-111, 75]. This is due to the selective modes of operation such as multiple reaction monitoring (MRM) which make efficient use of the duty cycle by focusing on selected ions, therefore increasing sensitivity [17,112].

1.4 Mass Spectrometry

Mass spectrometry has been a long established technique in the analytical world since the first mass spectrometer was developed in 1897 by J.J. Thomson who used it to measure anode and cathode rays [113]. Since this date, a range of significant developments have been made such as the time of flight mass analyser by W. Stephens in 1946, and the electrospray ionisation source in 1969 by Dole [114] which, although not developed for MS was further developed and utlised for MS by Fenn in 1984 [115]. Other developments include the quadrupole and quadrupole ion trap [116] in 1953 by W. Paul, the development of collision induced dissociation (CID) in 1968 [117] by K.R. Jennings and F.W. McLafferty, and tandem mass spectrometry in 1966 by J.H. Futrell [118]. Other significant developments in terms of how we practice MS today include the matrix assisted laser desorption/ionisation (MALDI) source by F. Hillenkamp and M. Karas in 1985 and Tanaka (1988), and the Orbitrap in 1999 by A. Makarov.

A further improvement was the coupling of liquid chromatography (LC) with mass spectrometry. This enabled higher throughput and increased efficiency in sample analysis [17]. This however meant that the reagents for LC must be compatible with mass spectrometry and vice versa.

As the focus of this thesis is the analysis of biomolecules in biological fluids, it is important to examine the most commonly used approaches for such applications. The most commonly used type of mass spectrometer is the quadrupole; this is

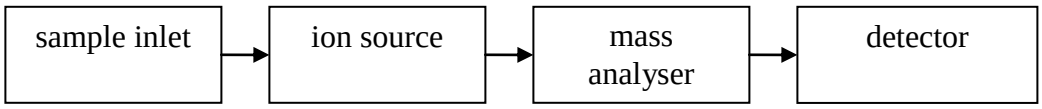
because they are highly sensitive and can be used to focus on just one or a few species at a time. The technique of MRM has been applied to small molecules for many years, and more recently to peptides and proteins. The quantitative analysis of peptides and proteins was traditionally the focus of immuno-affinity techniques, such as ELISA [17] but has been of interest to mass spectrometrists due to the benefits which can be gained from using mass spectrometry over immuno-affinity analysis, such as efficiency, sample throughput, selectivity, faster set-up times, lower costs and the structural information, not available from immuno-affinity techniques.

Immuno-affinity techniques have been used as an analytical technique since the mid 1950s [17] due to the low levels of quantification that can be reached. They rely on production of an antibody. This is a costly and time consuming process. The alternative of using LC-MS/MS has the potential to reach the same detection limits as immuno-affinity [119].

The mass spectrometer

A mass spectrometer is made up of four main parts. These are the sample inlet, the ion source, the analyser and the detector, all of which have a different design dependent on the application; a general schematic is given in figure 8. In bioanalysis, the samples generally come from an HPLC system and therefore are already in solution in the mobile phase. This thus needs to be compatible with the ion source.

Figure 8 – general schematic of a mass spectrometer



Commonly used ion sources in bioanalysis include electrospray (ESI) and atmospheric pressure chemical ionisation (APCI).

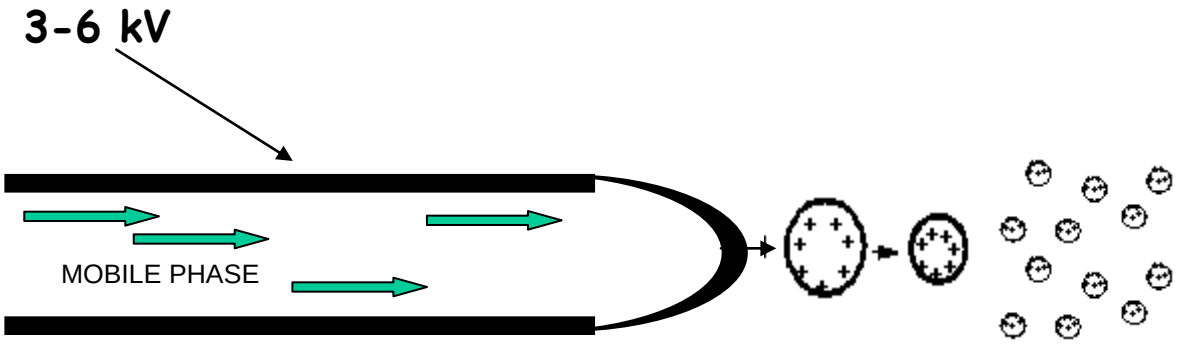
1.4.1 Electrospray ionisation

ESI was primarily developed as a technique independent of mass spectrometry. It was first implemented in 1968 by Dole, for polymer analysis [120]. Its potential was realised by Fenn, Yamashita and Alexandrov for use with mass spectrometry in 1984 [121-125]. The source was then further developed [126] to give the typical format we work with today.

In ESI, the ions in solution travel down a capillary which has a voltage of several kilovolts applied to it [127]. It can operate without heat, although most modern systems such as the API 5000 (figure 9 - Applied Biosystems, Canada) and TSQ Quantum Ultra QQQ (ThermoFisher Scientific, UK) use heat. The result of the TurboIonSpray is a fine spray of charged droplets where there can be multiple charges and the charge on these droplets is determined by the polarity of the electric field [17].

Figure 9 – API 5000 Triple quadrupole mass spectromter with TISP source [128]

Figure 10 – ESI model adapted from [19]



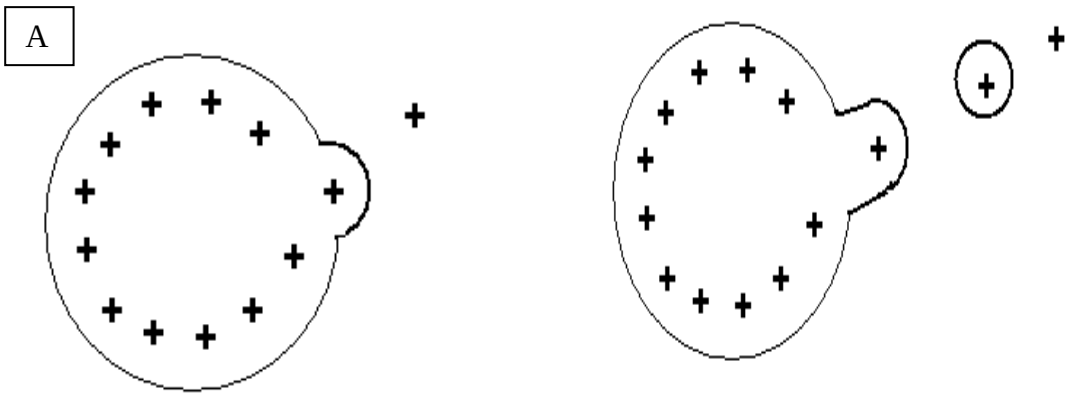
As the mobile phase containing the analyte passes through the capillary, the charge causes a spray effect due to the voltage on the capillary with a Taylor cone at the end of the capillary where a jet of liquid is emitted at a threshold voltage [129] and this results in the formation of charged droplets, which occurs when the droplets leave

the charged tip. They travel into the first vacuum stage, driven by an electric field. A nebulization gas such as nitrogen is often used to help aid droplet formation and the solvent evaporates until the Raleigh [130] limit is reached. At this point, the drop is unstable [120] and breaks, in a process described as columbic fission (essentially the repulsive forces between the charges become greater than the surface tension on the droplet) [128].

In ESI there are two different theories to the mechanism. However it has been suggested that it doesn't really matter which one is correct as they both lead to the same result [129]. The process of ion evaporation in which the ion leaves the droplet (figure 11A) is one of the processes by which desolvated ions are proposed to be formed. Alternatively a small droplet containing an ion leaves the larger droplet (figure 11B). These are more technically referred to as the ion evaporation model [131] and the charge residue model [132]. In the ion evaporation model, the field strength at a droplet's surface becomes strong enough, when a small enough droplet size is reached, to encourage an ion to leave the droplet [131]. In the charge residue module, it is suggested that there are many steps of fission which gradually cause the droplet to get smaller and smaller until there is only one ion left. The ions are then accelerated into the mass analyser by application of an electric field.

Figure 11 – a) ion evaporation model, the ion leaves the droplet b) The charge residue model, where a small droplet leaves the larger one adapted from *[129]*

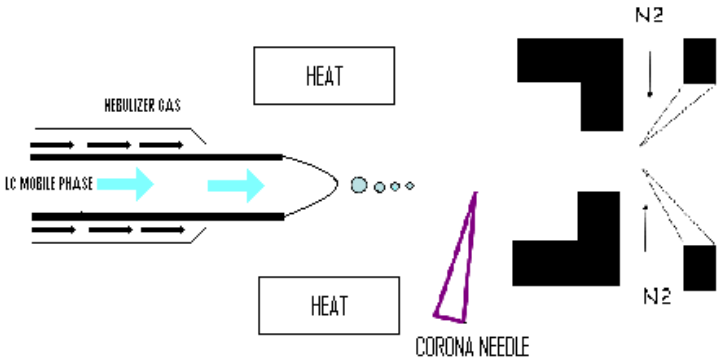
B



1.4.2 Atmospheric pressure chemical ionisation (APCI)

APCI is another technique which can be used in combination with LC to ionise biomolecules; a schematic is given in Figure 12. It works by using a heated nebulizer [17]. The nebulizer gas makes heated droplets which rebound off the hot surface of the capillary and vapourise in a manner referred to by Covey [129] as nucleate boiling, by conductive heat transfer via the surface of the liquid. At optimal conditions (when the temperature in the capillary is hot enough) the Leidenfrost effect occurs. This is when a film layer builds up between the solvent and the walls of the capillary and subsequently the droplets rebound before impact on the capillary walls. As the droplets flow out of the capillary, the corona discharge needle which is highly charged causes solvent molecules to be ionized. These gaseous solvent ions react with neutral analyte molecules to give charged species (ions), which are then accelerated into the mass spectrometer [133-134]. Although used in bioanalysis, this technique does always not provide as much sensitivity as ESI [17].

Figure 12 – APCI schematic, *adapted from [129]*



1.4.3 Mass analyser

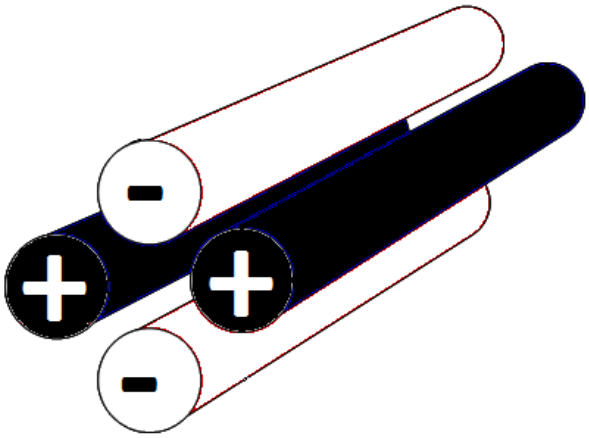
Mass analysers provide the selectivity element for mass spectrometry; different styles of mass analyser have different benefits e.g. high resolution, fast scan times, low levels of detection. The mass analyser used in an experiment depends on what results are required. Quadrupoles are the most appropriate mass analyser for use in quantitative mass spectrometry, due to their selectivity as a mass filter in SIM and MRM modes rather than scanning mode. The quadrupole mass analyser is not suitable for all mass spectrometric applications, due to the duty cycle being poorer in full scan mode (i.e. to scan a range of different *m/z*) as it only has a short amount of time to transmit each ion, whereas if it was to focus on just one or a few specific *m/z* species, then it acquires those data for a greater proportion of the total cycle time.

Quadrupole mass analyser

A quadrupole is composed of four identical rods, ideally hyperbolic in cross section but often cylindrical due to ease of manufacture [17], arranged as in figure 6. The

pairs are electrically connected so that the two opposite rods have the same charge as shown in figure 13 negative for the vertical rods and positive for the horizontal rods.

Figure 13 – quadrupole rods



Pairs of rods, of opposite polarities, have an RF voltage 180º out of phase from each other. This RF voltage (radio frequency) is fixed at 816 kHz and establishes harmonic ion trajectories. The rods also have a direct current applied.

In order for ions to pass through the quadrupole, they need to have the correct trajectory. This is determined by the RF and DC voltages, figure 15 shows the optimum transmission settings. To understand this more fully, it is easiest to look at how light and heavy ions move when subjected to electric fields. If a light ion enters the quadrupole with a positive charge, it will be drawn towards the negative rods, the rods are constantly and quickly switching polarities and the ion therefore moves towards whichever rods have negative charge. Small ions can change direction quickly and therefore avoids collision with the rods as is shown in figure 14A where the low mass ions are transmitted. Figure 14 illustrates this and also the ions filtered

by the positive rods (B) these combine to give the transmission diagram c where the window is 1 m/z (unit resolution)

Figure 14 – Low mass filter transmission diagram for negative rods (A) and High mass filter transmission diagram for positive rods (B) and a combined diagram (C)

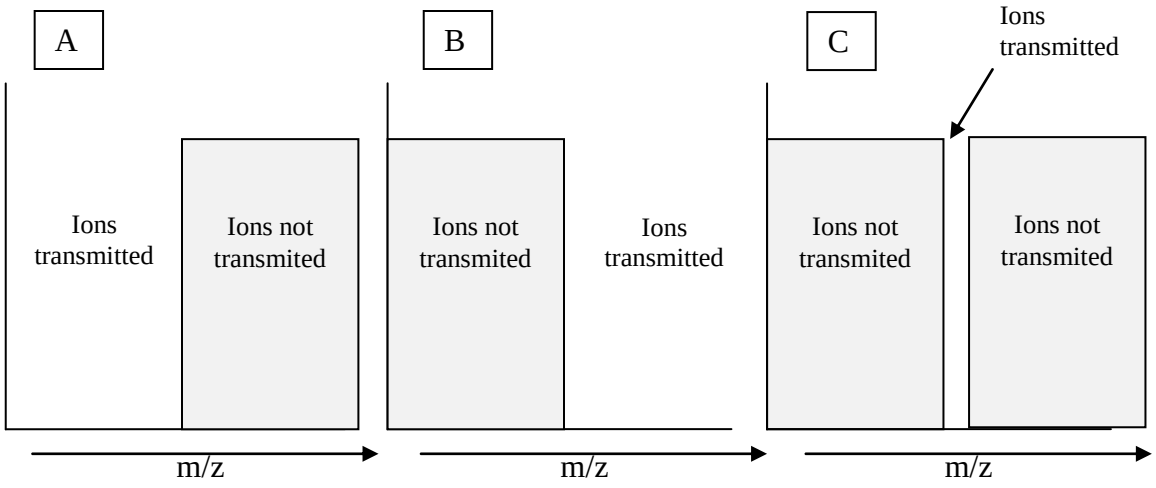


Figure 15– stable ion trajectories *adapted from [135]*

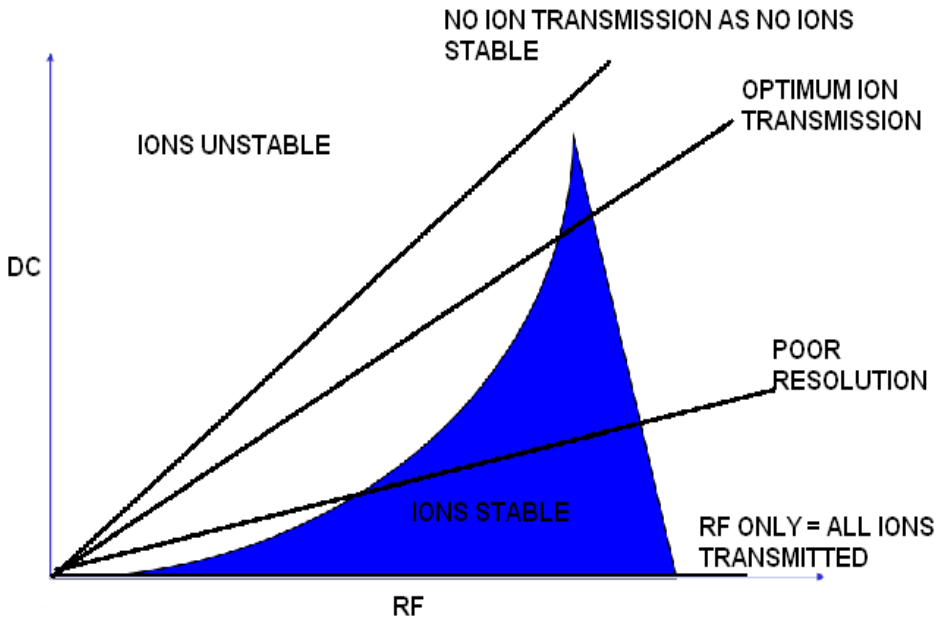


Figure 16 – cross section of a single quadrupole showing a stable trajectory of ions [26]

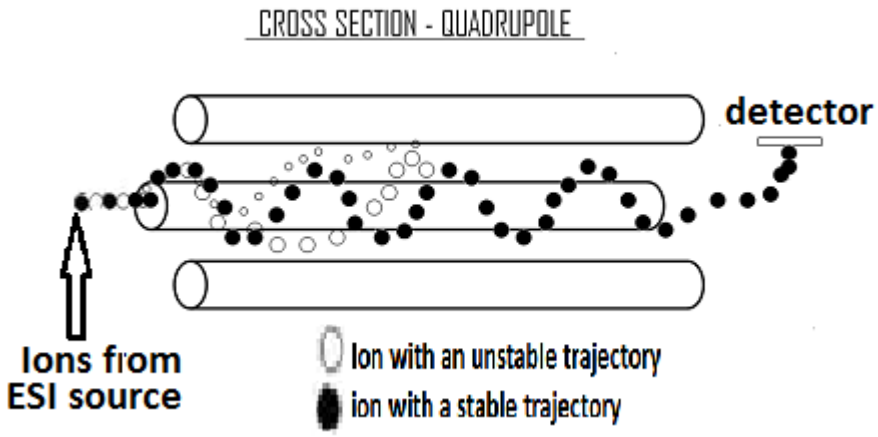
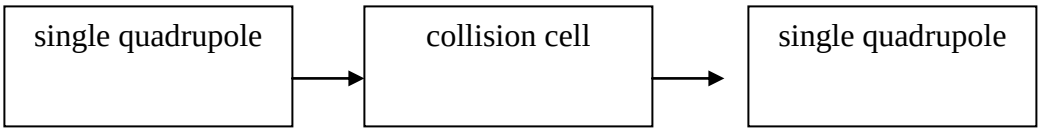


Figure 15 also illustrates that in RF-only mode, where there is no dc voltage applied, all ions are transmitted. This effect is used on some mass spectrometers as an ion focusing device. Figure 16 shows a quadrupole in which the dc and RF voltages have been optimized for an ion with medium *m/z* shown in black.

Although single quadrupole instruments are available, they have mostly been replaced by more selective instruments such as triple quadrupoles. These are the most commonly used instruments for quantitative bioanalysis [17]. They comprise a single quadrupole (Q1 and Q3) as mass filters with a collision cell in between (figure 17).

Figure 17 – triple quadrupole (QQQ) layout



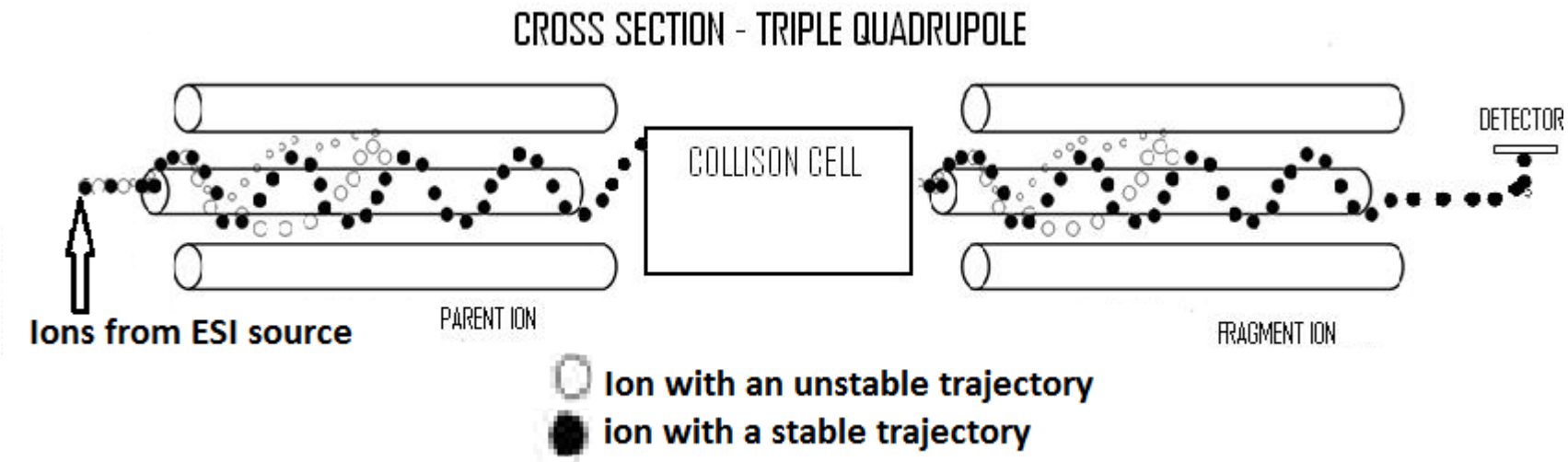
Multiple reaction monitoring mode (MRM), is a technique where multiple sets of ions can be analysed in Q1 and Q3 and still optimize the duty cycle. In this case, there are two transitions with a similar *m/z* can influence each other's spectra; this is referred to as cross-talk. Cross talk can be minimized by implementing a time gap

between analyte scans allowing for the collision cell to clear prior to the next transition being analysed.

1.4.4 Collision cell

The collision cell also consists of four (or sometimes six or eight) rods contained in a vacuum. Ions are accelerated into the collision cell, thus gaining some additional kinetic energy. This kinetic energy is converted into internal (vibrational) E when the accelerated ions subsequently collide in the collision cell with molecules of a neutral gas, typically nitrogen or argon and fragmentation results. This is collision induced dissociation.

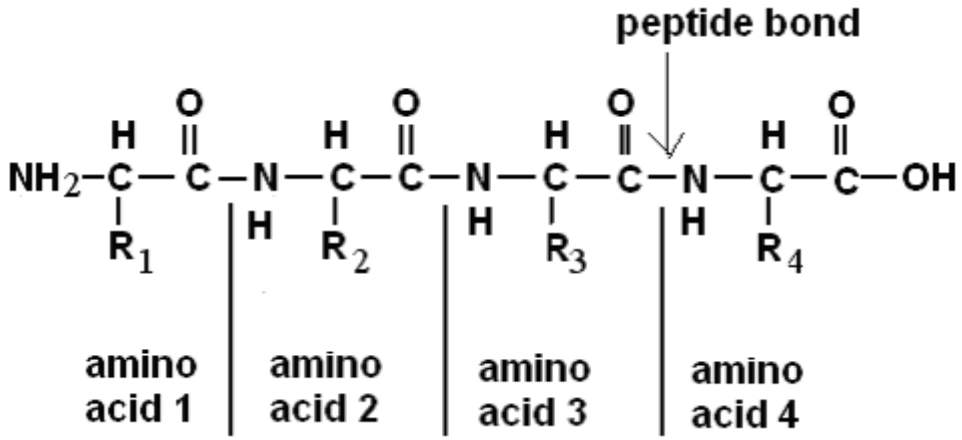
Figure 18- Cross section of a triple quadrupole instrument adapted *from [26]*



1.4.5 Peptide fragmentation

Peptides are chains of amino acids joined by peptide bonds (amide linkages) (figure 19). The weakest bond in the peptide backbone is the amide bond and this is generally the first bond to break when a peptide ion is subjected to CID.

Figure 19 – tetrapeptide (four amino acids joined by peptide bonds)



The mechanism of how peptide ions fragment is based on the mobile proton mechanism as proposed by Gaskell and Wysocki [135]. The two most common peptide fragments in positive ion mode CID spectra are b and y ions (figure 20). The formation of b and y ions by the mobile proton mechanism is summarised in figure 20. Figure 21, outlines the two routes which are taken in peptide bond fragmentation from singly protonated peptide molecules to produce b and y ions.

Figure 20 - the fragmentation of peptides to give b and y ions, adapted from [136]

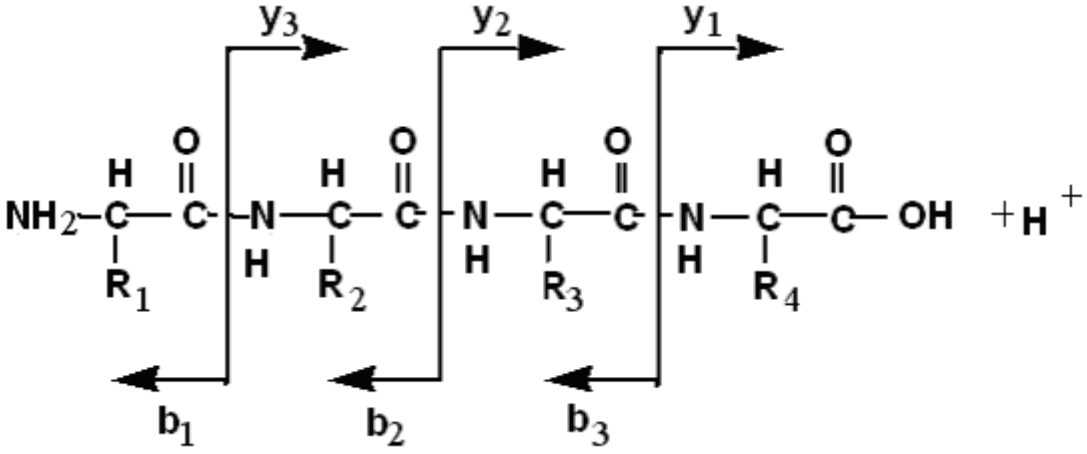
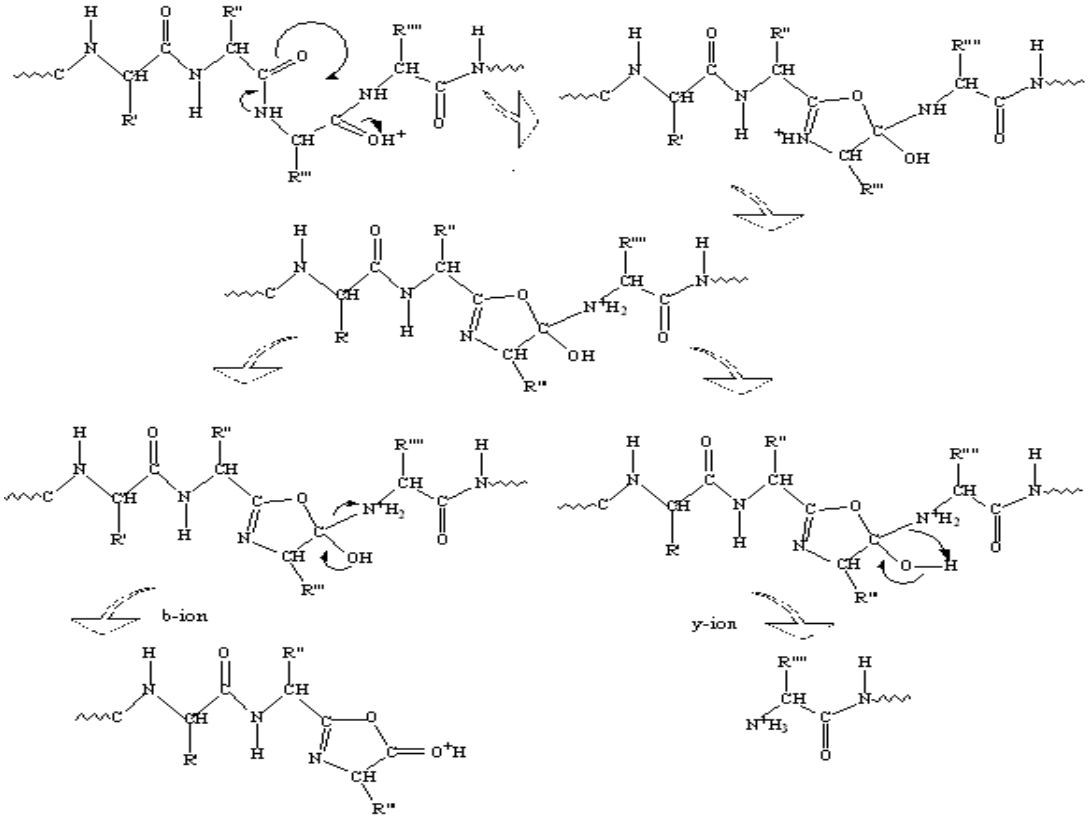


Figure 21 – the mobile proton theory of peptide fragmentation, adapted from [136]



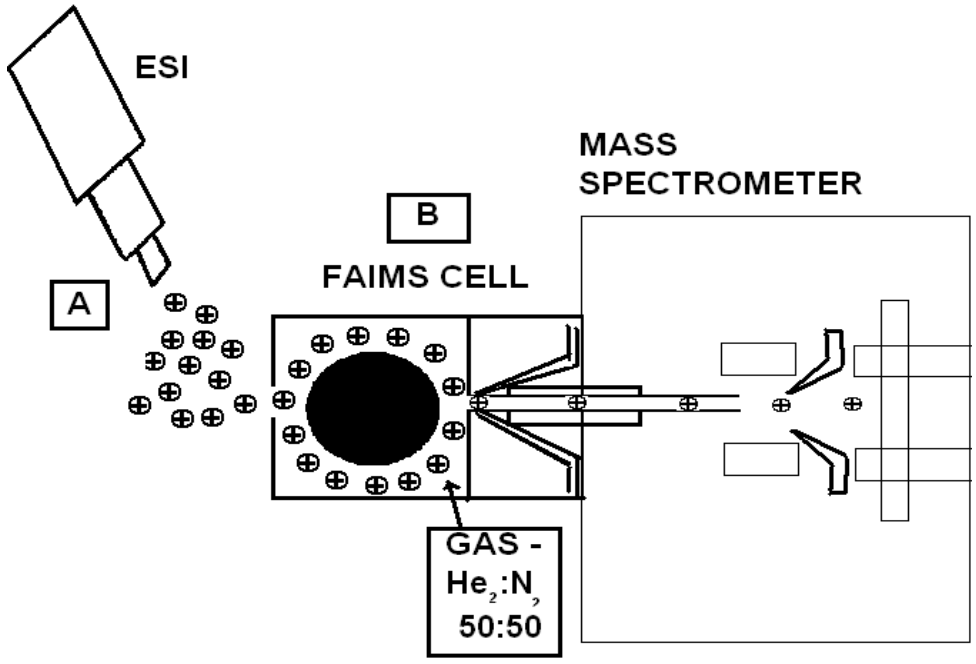
1.5 High field asymmetric waveform ion mobility spectrometry (FAIMS)

Increasingly, quantitative bioanalytical assays are exploring new ways to improve their sensitivity [137]. The challenge with many signal-enhancing techniques is to achieve this without an increase in the background noise. The TSQ Quantum Ultra triple quadrupole (QQQ) mass spectrometer can be used in conjunction with FAIMS technology as a means of enhancing sensitivity. This technology is designed to improve the signal-to-noise ratio [38] by reducing the chemical and endogenous matrix component noise [37,39] whilst transmitting analyte ions. This approach has shown significant benefits in bioanalytical assays such as improved signal-to-noise ratio, improved limits of quantification and assay robustness [140-142]. Other benefits of FAIMS include its ion focusing mechanism [143] that offers high sensitivity and the fact that it operates at atmospheric pressure and ambient temperature, which offers ease of operation [143]. FAIMS also has the potential benefit of being able to separate isomers such as leucine and isoleucine [144-145]

FAIMS can reduce chemical and endogenous background noise [146] by adding an extra selectivity factor. In FAIMS, ions are separated by their differential ion mobility as they pass through low and high electric fields. The selection of transmitted ions is regulated by the compensation voltage, which is compound dependent [146-147], and thus offers a means of separating analyte from background ions, thereby improving detection and reducing noise.

A diagram of the FAIMS interface is given in figure 22. Part A is the ESI source where the ions are formed. Part B is the FAIMS cell. The cell is made up of two concentric cylinders [143] (figure 23), which are electrodes that have a voltage applied to them (figure 3).

Figure 22- FAIMS interface, adapted from [13]



The FAIMS cell is filled with a mixture of helium and nitrogen gas. As the ions pass into the cell, dispersion (DV) and compensation (CV) voltages are applied to the inner electrode. Ions oscillate due to a combination of these voltages applied in a waveform. The DV is set at a constant value and determines the magnitude of the waveform [148]. The CV alternates between a high field (k_h) for one unit of time and then a low field (k) of opposite polarity [149] for two units of time to give an asymmetric waveform type function [149] (figure 24).

The CV is the selectivity factor, and affects the trajectory of ions through the FAIMS cell in different ways dependent on their mobility. Subsequently only ions for which the CV is optimised will have a stable trajectory through the FAIMS cell. This is best described in figure 3, where the movement of ions through the electrode is determined by the field and its effect on the mobility of the ions. Selectivity is achieved because the ions behave differently in the high and low fields [150-151]

Figure 23- FAIMS cell electrodes

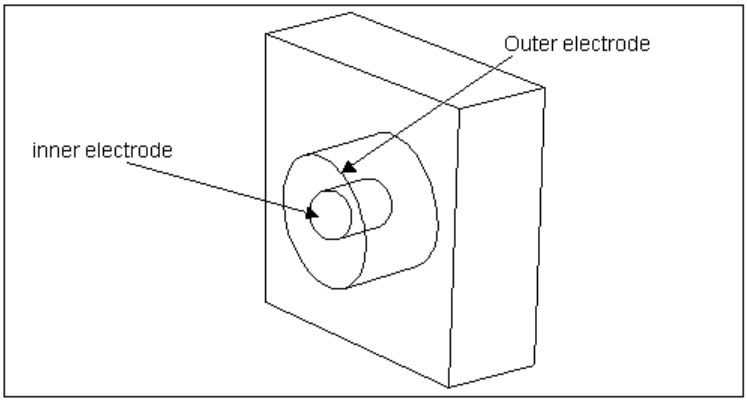
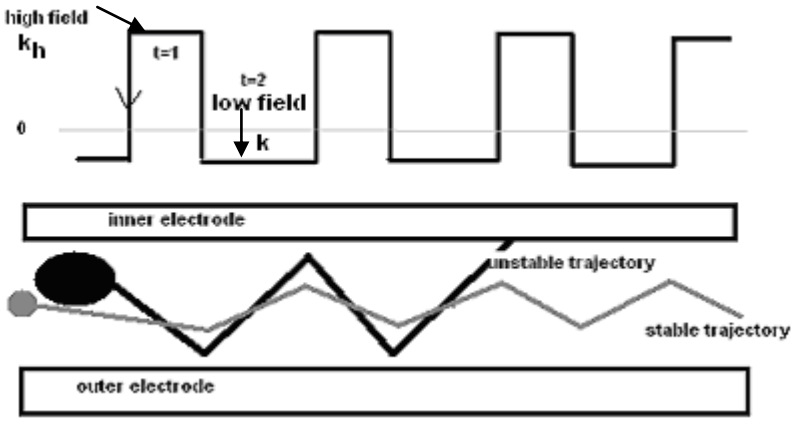


Figure 24- the asymmetric waveform, *adapted from [*149]



Bioanalytical assays involve the steps of fractionation, separation and quantification techniques. The importance of using mass spectrometry to quantification is that the selectivity which can be used for MRM analysis. Important additions to the method can include an internal standard, which should behave in the same way as the analyte. This can be used as an analyte to internal standard ratio.

1.6 **Internal standards for quantitative analysis**

Internal standards are important in mass spectrometry as they are designed to mimic the behavior of the analyte and thus account for any variation in sample extraction and ionization efficiencies of individual samples. Commonly, either a structural analogue of the analyte or a stable isotope-labeled internal standard is used. Analogue internal standards are used because of their structural similarity to the analyte, although there are likely to be differences in LC retention time and this may not account for ionisation variability [17] due to differences in co-eluting matrix components.

A stable isotope-labeled internal standard is the preferred choice and the most suitable isotopes are ^{13}C and ^{15}N; although ^{2}H can be used, it exhibits some additional effects due to the size of the deuterium over hydrogen which give it different special characteristics and subsequently a slightly earlier elution time when assessed by RP-LC. However as this can be marginal it is only important that the deuteriums are not able to hydrogen exchange with the solvents.

1.7 Detector response comparisons in quantitative analysis

In assessing a suitable instrument to give the lowest limits of detection and best performance, the best way place to start is without involving matrix effects and simply preparing standards of the analyte at known concentrations and assessing the response on different mass spectrometers using the same conditions.

The techniques detailed in fractionation, separation and quantification help to improve the selectivity and sensitivity of an assay. They support the fundamental foundations of regulated bioanalysis, its guidelines and its regulations and this is the important aspect of bioanalysis is the regulations and compliance with those regulations. The regulations set out by government agencies such as the food and drug administration (FDA) in the USA, the medicines and health regulatory authority (MHRA) in the UK and the European medicines agency (EMEA) in the EU [9-11]. These, among others are crucial to the bioanalyst in knowing what is required of the work they perform. These guidelines [9-11,152-153] provide the structure, and they ensure that the work carried out on new chemical entities (NCEs) is of a standard to justify this drug reaching market [12, 13]. In order to fulfil these guidelines, a method must be deemed "validated" or to have fulfilled specific validation criteria set out by regulatory bodies. These criteria show that the assay is robust and suitable for use. It is achieved by analysing a set of quantitation standards and AVP spiked frozen plasma standards. Several of these analyses can be performed, generally on different days, to show repeatability in the form of inter-day and intra-day precision and accuracy.

1.8 Validation

A bioanalytical method validation is a series of experiments designed to test an assay and see if it is suitable for use in the quantitative analysis of clinical/ pre-clinical samples – in the case of the assay being developed in this work, the aim was to devise an assay for generic evaluation of elevated levels of AVP in human plasma samples. The purpose of validation is to give a method that is reliable and repeatable and fit for purpose, where it can quantify the analyte with appropriate accuracy and precision [154]. Specific validation criteria are required for each analyte to be assessed [60], and are referred to in the FDA guidelines for bioanalytical method validations as fundamental parameters [9]; these encompass accuracy, precision, selectivity, sensitivity, reproducibility and stability [9]. These criteria can be interpreted in many different ways so much so that groups of industry analysts have joined forces to develop publications which explain them [60-61]. The FDA guidelines for bioanalytical method validations [9] and the Crystal City guidelines [60-61] among others are major influences on the design of validation and the assessments which were made.

Quantification of an analyte for a validation assessment is performed with a series of experiments, including quantitation standards, sometimes referred to as calibration standards, which are used to generate a calibration line. The calibration standards are samples of the relevant biofluid, for example human plasma, to which have been added known concentrations of analyte. These samples are extracted, after addition of an internal standard. The peak area from the extracted ion (XIC) chromatogram

for the analyte is divided by the peak area of the XIC for internal standard, to give the peak area ratio. The peak area ratio is then plotted against the known concentration of analyte in the standard. A line of best fit is then drawn between these points on a graph. The linearity coefficient R^2 of this line shows how close the data points are to the line is also measured to ensure that there are no outliers included that may adversely bias the line, which is then used to determine the levels of analyte in samples where the concentration is unknown. To validate a calibration curve, sample matrix and analyte spiked frozen standards may be measured against it.

1.8.1 Linearity and range

Linearity is the "extent to which any effect is proportional to its cause" [155]. In this case, the area ratio of analyte to internal standard and the nominal concentration of analyte are directly proportional. This proportionality may vary and therefore may not be a 1:1 ratio but a curve. The best and simplest overall linear fit across the validation experiments run over the five days in the validation is the one that is recommended for sample analysis [61].

1.8.2 Statistical outliers

For the assessment of statistical outliers, a test must be used to decide if a value is anomalous and can therefore be deemed a statistical outlier and excluded from the calibration data set. In deciding this, FDA guidelines must be taken into consideration; these stipulate that a minimum of five replicates [9] of the sample

matrix AVP spiked frozen plasma standards are required to determine accuracy and precision. If there is one obvious outlier in the data set, a statistical test such as Grubbs test [156] can be applied. This is also the case for two outliers, but if two outliers were present then the FDA criteria of a minimum number of five that must pass acceptance out of six measured could not be met and subsequently the experimental data would not be reported but repeated.

Figure 25- Grubbs test for statistical outliers [20]

$$\text{Grubbs} = \frac{\text{I mean – value to be assessed I}}{\text{standard deviation}}$$

The Grubbs test can only be used when there are six or more data points as it is based on a normal Gaussian distribution [157-158]. It works by calculating the maximum and minimum values that fall within 95% of the distribution from the values of the other five data points and then determines the probability of the sample in question being an outlier. The values are compared to literature values and have predefined limits. If a value falls outside of these limits, then it is excluded.

1.8.3 Accuracy

The accuracy of a method can be defined as being "the degree of closeness of the observed concentration to the nominal or known concentration" [159]. To determine the accuracy of an analytical method, the guidelines recommend testing at least five replicates of spiked frozen level of analyte for each concentration [159] and at least three different concentrations of analyte in plasma [159] in the calibration range.

AVP spiked frozen plasma standards are used in this assessment, which can be sample matrix aliquots containing known concentrations of the analyte, which have been stored in the same way as the anticipated samples. These should be analysed as six replicates and the peak area ratios compared to the line of best fit resulting from the calibration standards to ensure that analyte is stable in the sample matrix and that the quantitation standards give accurate concentration data. The overall aim is to make sure that the AVP spiked frozen plasma samples give results that are within 100±15% of the concentrations they are known to be spiked at when compared to the calibration line (the line of best fit created from the quantitation standards).

1.8.4 Precision

When generating scientific data, the precision of replicate measurements is important as it refers to the closeness of a set of measurements [160] and can be expressed as the coefficient of variation (CV) [160]. The criteria for assessing precision are determining whether the values obtained are 100±15% of the known spiked concentration [9]. This is tested using the AVP spiked frozen plasma standards from which XIC peak area ratios are compared to the calibration curve (line of best fit of the calibration points) at the lowest concentration quantified, a point three times the lowest, a median value and a point at about 25% from the upper limit of detection and the upper limit of quantification.. The purpose of this is to show that the results are consistent and reproducible.

The coefficient of variation (CV) is also referred to as the relative standard deviation (RSD) as it is the standard deviation / mean *100. This offers a means of assessment of the intra-day and inter-day data. The acceptance criteria are generally that no concentration measurement should exceed CV>15% except for at the LLOQ which is the lowest limit which can be quantified (with a S:N >5:1) where CV< 20% is recommended by the regulatory agencies [159].

1.8.5 Stability

In the assessment of stability of an analyte, there are important factors for consideration in a bioanalytical assay based on the treatment of patient biofluid samples and the conditions to which they may be subjected. The importance of this is the integrity of the data produced. For this purpose, stability standards, prepared by spiking the analyte into the biofluid at known concentration and freezing serve the purpose, not only to validate the calibration curve but also to prove that the initial freezing and defrosting of samples does not impact on the amount of analyte quantified in the biofluid. Once this is determined, it can be furthered by performing freezing and storage stability more than once; this is done in a validation as there may be the need to re-analyse samples and to do this it is important to know that the analyte is stable when this is done. Analyte stability in biofluids stored at room temperature reflects the maximum time samples could be defrosted and then stored at room temperature and maintain stability. The standards are extracted, the peak area ratio from the XICs calculated and the concentration taken from the calibration

line. This value is then compared to the known concentration and the accuracy and precision calculated.

1.8.6 Matrix effects

Matrix effects are changes in the amount of analyte detected in the presence of matrix. The matrix is often complex biological material which contains the target analyte e.g. plasma, urine. Its components can interfere with the analyte peak to give enhancement or suppression of the analyte signal. Matrix effects are examined during method validation to ensure there is no influence on the reproducibility or linearity of the assay as well as the potential sensitivity [161-162]. Matrix effects have been observed in fluorescence and electron capture techniques [163-164], however they are more commonly associated with LC-MS. In LC-MS, both atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) assays have been shown to be susceptible to matrix effects [165-167]. This is more apparent for ESI than APCI [165] and this is thought to be related to their mechanism of operation. The mechanism by which matrix effects influence the MS signal is not yet fully understood although suggestions of this being related to competition for charges and droplet surface accessibility between target analyte ions and matrix components, have been made [165,166-168].

The FDA requests that steps are taken to limit matrix effects [159] and thus in assessing matrix effects, matrix samples from different donors can be used, as suggested by Karnes *et al* [169]. These blank matrix extracts are spiked with analyte

and internal standard and the peak area ratios taken from the XIC compared to that obtained from a standard solution in the absence of matrix, to determine the amount of change to the analyte and internal standard MS signals occurring in the presence of matrix. This approach is consistent with that used by Nadiong et al [170].

In assessing the impact of matrix effects in a validation, a matrix factor (MF) is used. This is a ratio of the analyte response when matrix is present to the analyte response when matrix is absent [61]. The Crystal City II guidelines report that an MF of 1 indicates that the matrix does not impact on the analyte response but that an MF>1 indicates ionisation enhancement and consequently MF<1 analyte suppression [61].

1.8.7 Selectivity

The selectivity of an assay is its ability to detect and detect only the target analyte. In tandem mass spectrometry, it is not possible to assume that there are no other species in the sample that would give both precursor and product ions of the same *m/z* as the analyte, although it is unlikely due to the selective nature of this *m/z* discriminating process in MRM mode.

The selectivity of the MRM transition is in addition to that offered by the sample preparation used, such as SPE, which removes interferents, and LC, where the analyte needs to be retained by the stationary phase selectively. It is also important to consider the matrix and use a biofluid pool from the appropriate organism, usually

human. Using a number of blank biofluid samples in the assay and screening the XIC for peaks at the retention time of the analyte and internal standard makes it possible to determine whether another species at the same retention time as the target analyte/internal standard and undergoing the same transition is detectable.

1.8.8 Recovery

Recovery assessments are made during validation to determine the amount of analyte recovered during sample extraction. The amount of analyte expected to be present at the end of the extraction is assessed. This takes into consideration sample volumes used and any transfer steps. For sample preparation, sample matrix with and without analyte may be extracted. A standard solution of analyte in injection solvent serves to spike the blank matrix with analyte after the extraction. The recovery is calculated by dividing the XIC peak area from the extracted matrix spiked with analyte before extraction, by the analyte peak area from the XIC for the matrix which is spiked after the extraction. This value is then multiplied by 100 to give a percentage recovery.

1.8.9 Limit of quantification (LOQ)

The challenges of improving sensitivity in LC-MS/MS assays are well researched. Methods range from concentration steps to altering the separation chemistry in SPE and liquid chromatography (LC). The mobile phases used in LC can also impact on the sensitivity, especially if a high percentage of organic solvent is present as the analyte is ionised, due to more efficient desolvation in the ion source.

The limit of quantification is defined as "the lowest concentration of analyte that can be quantified and measured with an accuracy and precision of less than 20%" [17]. Analytical method validation guidelines require the signal to noise ratio of the analyte and internal standard XIC peaks must also be greater than or equal to 5:1 [59].

1.9 Aims

The research in this thesis is aimed to develop and validate a quantitative bioanalytical method for the analysis of arginine-8-vasopressin in human plasma that can compete with current detection limits in immunoassays of 1 pg/mL. This level is important because it is the endogenous level of AVP in human plasma. The levels found in disease states as previously mentioned are however much higher. If a limit of detection of 1 pg/mL, was not reached, an assay with a limit of quantification of 0.5 ng/mL would be appropriate as a tool to screen for elevated biomarker levels.

In developing a quantitative bioanalytical method with the lowest potential LLOQ using the most advanced instrumentation and techniques available to a bioanalytical assay I hope to also become trained in the skills which will enable me to develop bioanalytical methods. This will allow me to assess different fractionation, separation, mass spectrometric and complementary techniques and will also allow analysis and breakdown of the validation requirements of a bioanalytical assay using analytical protocols.

Chapter 2 -Methods

2.0 Methods

2.1.1 Chemicals

Acetonitrile, propan-2-ol, phosphoric acid, acetic acid, ammonium acetate and ammonium formate, were obtained from ThermoFisher Scientific, UK and were all of highest available grade. Methanol, acetone, and water were HPLC grade (Rathburn, UK). Blank human plasma (EDTA was the anticoagulant at 2 mg/mL) was used to prepare the calibration and AVP spiked frozen plasma standards. AVP was obtained from Sigma Aldrich, UK and AVP-D5 from Lardon fine chemicals, Sweden. All other chemicals and reagents were obtained from VWR international limited (Poole, UK). 30 mg HLB and WCX 96 well SPE plates were obtained from Waters, Manchester, UK. Instrumentation included a Waters Acquity system (Waters, Manchester, UK) and API 5000 triple quadrupole mass spectrometer (Applied Biosystems / MDS Sciex, Canada). Columns used included Waters BEH C18 5 cm * 2.1 mm * 1.7 μm and Waters Acquity HILIC C18 5 cm * 2.1 mm * 1.7 μm.

2.1.2 Peptide Analytes

The structures and molecular weights of the peptides analysed in this work are given in table 1.

Table 1 – AVP and AVP-D5

Arginine-8-vasopressin (AVP, MW=1084)

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

Deuterated Arginine-8-vasopressin (AVP-D5 MW=1089)

Cys-Tyr-Phe(D5)-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

2.1.3 AVP concentrated and dilute stock solution preparation

1 mg analyte and internal standard (AVP, AVP-D5), were accurately weighed and dissolved in 1 mL methanol:water:TFA 50:50:0.1 v/v/v. This was split into 200 µL sub aliquots in 0.5 mL plastic microfuge tubes and stored at -20°C. From this a dilute stock solution at 2 µg/mL solutions were prepared by adding 20 µL of the concentrated stock solution to 9.98 mL methanol:water:TFA 50:50:0.1 v/v/v. This was used on the day of preparation and not stored.

2.1.4 Mass spectrometry optimization (infusion) solution preparation

10 µL secondary analyte stock solution was added to 9.99 mL 50 mM ammonium formate: acetonitrile: formic acid (50:50:0.5 v/v/v).

2.1.5 Plasma preparation

Human plasma K_2EDTA was received from Biochemed (Winchester, VA). The blood was treated with 2 mg/mL anti-coagulant K_2EDTA to prevent the blood from clotting and was mixed and centrifuged to remove the plasma within 5 minutes of sampling. This was stored at -20°C.

2.1.6 Quantitation standards

Quantitation standards were prepared in human plasma K_2EDTA. The analyte was spiked into the plasma using the dilute stock solution. The standards were prepared daily in plasma.

2.17 Stability assessment samples

Stability assessment standards were prepared using human plasma K_2EDTA. The analyte was spiked into the plasma using the dilute stock solution. After spiking, these samples were frozen at -20°C. This differentiated them from calibration standards. They are referred to throughout as AVP spiked frozen plasma standards.

2.2 Method development -fractionation techniques

Quantitation standards were used to assess the suitability of fractionation techniques which were designed to optimize the purity of the final sample extract. Two designs were employed, which were reversed-phase and mixed-mode weak cation exchange.

2.2.1 Reversed Phase

An SPE plate (HLB 30 mg) was primed with 0.5 mL methanol then conditioned with 0.5 mL water. 0.5 mL human plasma spiked with 2 μg/mL AVP was diluted with 0.5 mL 10:90 acetonitrile: water containing 2 μg/mL AVP-D5 (the internal standard), mixed, and then loaded onto the plate unless otherwise stated.

SPE Profile 1- formic acid

Elution was performed using 500 μL 2% formic acid and varying the percentage methanol in the solution from 5-100 %l. The extracts were analysed on LC-MS/MS as detailed in the final method (section 2.6) and the peak area ratio for AVP: AVP-D5 determined.

2.2.2 Mixed-mode weak cation exchange

An SPE plate (WCX 30 mg) was primed with 0.5 mL methanol then conditioned with 0.5 mL water. 0.5 mL human plasma spiked with 2 μg/mL AVP was diluted with 0.5 mL 10:90 acetonitrile: water containing 2 μg/mL AVP-D5 (the internal standard), mixed, and then loaded onto the plate unless otherwise stated.

SPE Profile 2- ammonium hydroxide

Elution was performed using 500 µL 5% concentrated ammonium hydroxide solution and varying the percentage methanol in the solution from 5-100%. The extracts were analysed on LC-MS/MS and the peak area for AVP determined

2.2.3 Validation method

An SPE plate (WCX 30 mg) was primed with 0.5 mL methanol then conditioned with 0.5 mL water. 0.5 mL human plasma spiked with 2 µg/mL AVP was diluted with 0.5 mL 10:90 acetonitrile: water containing 2 µg/mL AVP-D5 (the internal standard), mixed, and then loaded onto the plate. The plate was then washed with 0.5 mL H_2O: MeOH (95:5 v/v) to remove aqueous soluble impurities whilst retaining the analyte then 0.5 mL H_2O: MeOH: NH_4OH (20:80:5 v/v/v) and then a third wash of 0.5 mL acetone was then used to remove lipid impurities in the plasma whilst retaining the analyte. Two separate elutions of 0.5 mL H_2O:MeCN:acetone:HCOOH (20:30:50:0.5 v/v/v/v) were performed and collected together .

2.3 UHPLC

UHPLC separation techniques were employed in both reversed phase and HILIC mode. Standards were prepared by spiking analyte directly into the solvent representative of the gradient starting conditions for each of these analyses.

2.3.1 Standard solution preparation

A stock solution was used to make standard solutions (in the absence of matrix). The spiking scheme and concentrations are given in Table 2. It was performed in a dilution series of your concentrated stock, diluted 1 in 1000 in a volume of 10 mL.

Table 2 –AVP spiking scheme

Concentration of standard (pg/mL)	Concentration of stock used for preparation and amount (µL)	Volume solvent (mL)	Final volume (mL)
1000000000	This is the concentrated stock solution		
10000000	100 µL concentrated stock solution	9.90	10
100000	1000 µL of 10000000 ng/mL	9.00	10
10000	1000 µL of 100000 ng/mL	9.00	10
1000	1000 µL of 10000 ng/mL	9.00	10
100	1000 µL of 1000 ng/mL	9.00	10
10	1000 µL of 100 ng/mL	9.00	10
1	1000 µL of 10 ng/mL	9.00	10

2.3.2 Reversed-Phase Chromatography

An Acquity UHPLC system with integrated auto sampler and column oven was used. The column was an Acquity C18 peptide column of length 10 cm, internal diameter 2.1 mm, particle size 1.7 µm with a pore size of 130 Å.

The mobile phases used consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Gradient elution was performed with starting conditions 10% mobile phase B for 0.5 min; this was then ramped to 25% B over 1.2 min and held there for 0.8 min. The column was then flushed by ramping to 95% B over 0.5 min and holding for 0.5 min. After 0.1 min it was returned to starting conditions and held for 0.4 min.

2.3.3 HILIC Chromatography

An Acquity UHPLC system with integrated auto sampler and column oven was used fitted with an Acquity BEH HILIC column of length 10 cm, internal diameter 2.1 mm, particle size 1.7 um. The mobile phases used were phase A = 10 mM ammonium formate with 0.5% (v:v) formic acid and B = MeCN . Gradient elution was performed with starting conditions 80% B for 0.25 minutes; ramping to 65% B over 2.75 minutes. Holding for 0.5 minutes. The column was then flushed by ramping to 50% A over 0.5 minutes and holding for 1.0 minutes. After 0.1 minute it was returned to starting conditions and held there for 0.4 minutes.

2.4 Method Development - Optimization of Mass Spectrometry Parameters

2.4.1 Fragmentation of Peptides by CID

Peptides were manually infused at 10 μL/min using the integral syringe driver. Detection was performed using an API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex (Foster City, CA)). The ions monitored were *m/z* 543.46; *m/z* 545.5, m/z for the $[M+2H]^{2+}$ ions for each of the peptide analyte and the internal standard. The TurboIonSpray© source temperature was 650 ˚C, with gas settings of 50 psi (G1) and 70 psi (G2). The curtain gas was set to 20 psi and the collision gas setting was cad=5.

2.4.2 Collision Energy Setting Optimisation

$[M+2H]^{2+}$was selected in Q1 and fragmented at a range of collision energy settings to give product ion spectra for each analyte. Q3 scanned *m/z* 200-1000 to record the product ion spectra.

2.4.3 Quantitative Analysis – Limit of Detection Assessment

Detection was performed using API 5000 and 3000 triple quadrupole mass spectrometers (Applied Biosystems/MDS Sciex (Foster City, CA)). The TurboIonSpray© source temperature was 650 ˚C, with gas settings of 50 psi (G1) and 70 psi (G2). The curtain gas was set to 20 psi and the collision gas setting was CAD=5. The transitions monitored for AVP were *m/z* 543.46 to 328.2. The dwell

time used for each transition was 100 ms with a pause time of 5 ms. Standard solutions of AVP covering the concentration range 0.1 ng/mL to 1 mg/mL were analysed using both RP and HILIC columns using 5 µL full loop injections.

2.5 Method development - FAIMS

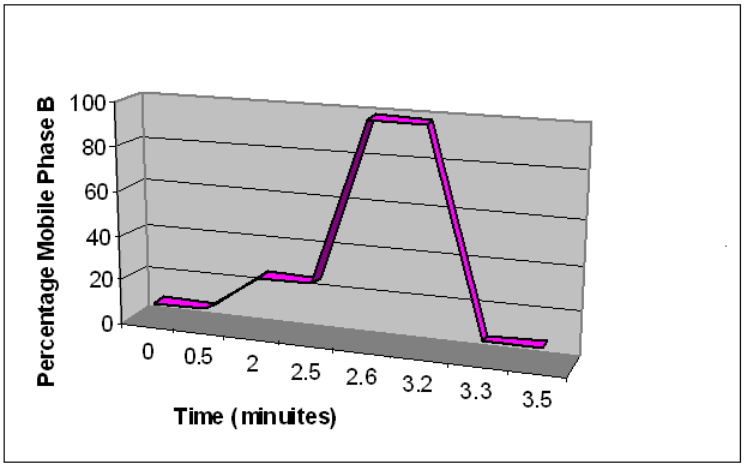
2.5.1 Sample preparation

Sample preparation for the assessment of the FAIMS interface comprised analyte isolation and concentration using Water's Oasis weak cation exchange (WCX) SPE (2.2.3).

2.5.2 LC conditions

LC was performed using an Accela© (ThermoFisher Scientific, Hemel Hempstead, UK) UHPLC system, using reversed-phase chromatography on a Water's Aquity BEH 130 Å C18 1.7 um, 2.1 x 100 mm column, and gradient elution as detailed in figure 4. The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The gradient profile can be seen in figure 26

Figure 26 - The percentage of mobile phase B (acetonitrile + 0.1% formic acid) used over time for gradient elution of AVP in FAIMS experiments



2.5.3 FAIMS-MS/MS

Instrumentation consisted of an Accela© (ThermoFisher Scientific, Hemel Hempstead, UK) UHPLC system, interfaced with the FAIMS device and a TSQ Ultra triple quadrupole mass spectrometer (figure 27). Instrumentation conditions are given in tables 3-5.

Figure 27 - FAIMS device with TSQ Ultra QQQ

The samples analysed on the instrument included calibration standards containing known amounts of AVP spiked freshly into plasma, plasma spiked with known amounts of AVP, then frozen and thawed for analysis, and unspiked plasma samples where no detectable AVP was present. The calibration standards were prepared at concentrations of 1, 3.125, 20, 47 and 160 ng/mL. They were extracted as described in section 2.6.4. The samples were injected in duplicate using 20 μL injections with a 50 μL loop, once with, and once bypassing the FAIMS device, to allow direct comparisons to be made.

Table 3 -MS conditions

Parameter	**Value**
Ion source and polarity	ESI, positive ion mode
Spray Voltage	3500 V
Vapouriser Temperature	350 ˚C
Sheath gas	40 units
Auxillary gas	60 units
Tube lens offset	100 V
Ion sweep gas pressure	60 psi
Helium: nitrogen	50:50
Q1 Resolution (where 0.7 is unit resolution)	0.7
Q3 Resolution (where 0.7 is unit resolution)	0.2

Table 4 –*m/z* values of ions monitored

Analyte	**Q1**	**Q3**
AVP	543.4	328.2
AVP-D5	545.5	328.2

Table 5- FAIMS conditions

Parameter	**Value**
Dispersion Voltage	-5000 V
Outer Bias Voltage	35 V
Compensation Voltage	-27 V
T_{inner} Electrode	70°C
T_{outer} Electrode	90°C
Total gas flow	4 mL/min

2.6 Quantitation Method for AVP in Human Plasma

2.6.1 Equipment

Oasis WCX 30 mg SPE extraction cartridges, UHPLC system, auto sampler, and columns (Acquity HILIC, 50 x 2.1 mm i.d, 1.7 µm particle size) were supplied by Waters (Manchester, UK), and the API 5000 triple quadrupole mass spectrometer by Applied Biosystems (Foster City, CA).

2.6.2 Preparation of Concentrated and dilute analyte stock solutions

Solutions of AVP and AVP-D5 were prepared in water: acetonitrile 70:30 v/v at a concentration of 1 mg/mL and stored at -20˚C. A diluted solution of the AVP-D5 internal standard was prepared at 5 ng/mL in acetonitrile:water:trifluoroacetic acid 10:90:0.1(v/v/v) and was used as the internal standard in the extraction.

2.6.3 Preparation of AVP spiked plasma standards

Quantitation standards were prepared fresh on the day of analysis -the highest concentration was prepared using the 1 mg/mL stock solution and this solution of AVP spiked into plasma was then serially diluted to generate the calibration standards. Quantitation standard levels were 10, 8, 6, 4, 2, 1, 0.5 and 0.25 ng/mL.

AVP spiked frozen plasma standards were prepared as per section 2.1.8 for the calibration standards and then frozen at -20ºC prior to analysis. AVP spiked frozen

plasma standards were prepared at 50 ng/ mL so that a dilution factor could be validated ensuring patient samples could be diluted accurately and back calculated to the true concentration (if the original concentration was above the highest quantitation standard concentration to avoid extrapolation of the calibration curve). The concentrations of these standards included the upper limit of quantification (ULOQ) at 10 ng/mL, 7.5 ng/mL, 5 ng/mL, 0.75 ng/mL, 0.5 ng/mL and the lower limit of quantification at 0.25 ng/mL.

2.6.4 Fractionation techniques for the quantitative analysis of AVP

0.5 mL plasma (AVP spiked plasma standards or study samples), 0.5 mL internal standard solution (AVP-D5 5 ng/mL in methanol) and 100 µL 10% phosphoric acid (aq) (used to eliminate protein binding to plasma proteins) were sequentially aliquotted into a 96 well plate [mixed and extracted using a Waters Oasis WCX 30 mg SPE 96-well plate. A wash step was performed using 0.5 mL H_2O: MeOH (90:10 v/v), a second wash was performed with 0.5 mL H_2O: MeOH: NH_4OH (80:20:2 v/v/v). A third wash of 0.5 mL acetone was then used. Two elutions each of 0.5 mL H_2O: MeCN:acetone: HCOOH (20:30:50:5 v/v/v/v) were performed which were collected into the same vessel to optimise recovery. The samples were then evaporated to dryness in a stream of nitrogen at 40˚C and reconstituted in 100 µL 20:80:0.5 (v/v/v) water: acetonitrile: formic acid, mixed, sonicated for 5 min at ambient temperature and centrifuged (5 minutes, 4ºC, 3000 rpm) prior to LC-MS/MS analysis.

2.6.5 UHPLC-MS/MS for the quantitative analysis of AVP

UHPLC-MS/MS analysis was performed using a Waters (Manchester, UK) Acquity UHPLC system with integrated auto sampler and column oven, and used an Acquity BEH HILIC, 1.7 µm, 100 mm x 2.1 mm (i.d) column. The mobile phases used were phase A = 10 mM ammonium formate with 0.5% (v:v) formic acid and B = acetonitrile (MeCN). The gradient consisted of 80% MeCN for 0.25 min, ramping from 80-65% MeCN over 2.75 min, lowering to 50% MeCN over 0.5 min and then holding at 50% MeCN for one min before returning to starting conditions over 0.5 min. A switching valve was used and diverted to waste between 0-2 min and 3.5-5 min.

Detection was performed using an API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex (Foster City, CA)). The TurboIonSpray source temperature was 650 ˚C, with gas settings of 50 psi (G1) and 70 psi (G2). The curtain gas was set to 20 psi and the collision gas setting was CAD=5. The transitions monitored for AVP were *m/z* 543.4 to 328.2. The transitions monitored for the internal standard were *m/z* 545.50 to 328.2. The dwell time used for each transition was 100 ms with a pause time of 5 ms.

The data were processed using the Analyst software (Applied Biosystems, Canada) version 1.4.2, to generate peak area ratios from the extracted ion chromatograms (XIC) for AVP and AVP-D5 transitions.

2.7 Validation of the quantitation method for AVP

2.7.1 Linearity

The concentration range assessed in this assay was 0.25 ng/mL to 10 ng/mL AVP in human plasma, with a sample aliquot volume of 0.5 mL. The calibration line consisted of ten quantitation standards prepared in human plasma, and the peak areas for AVP and AVP-D5 were determined from the extracted ion chromatograms. The AVP: internal standard peak area ratio was calculated by dividing the peak area of AVP by the peak area of AVP-D5. The peak area ratio values obtained were plotted against concentration of AVP, to generate a calibration line of best fit.

2.7.2 Linear range of the quantitative assay

The concentration range used in this assay was 0.25 – 10 ng/mL AVP with a dilute AVP spiked frozen plasma assessment which was prepared at 50 ng/mL as described in section 2.6.3 and frozen at -20 ◦C. Prior to analysis the 50 ng/mL AVP spiked frozen plasma standard was diluted 1:10 with human plasma to give an expected AVP concentration of 5 ng/mL. This was to replicate the dilution of patient samples which were above the upper quantification limit of the calibration curve to be analysed when diluted with human plasma.

An assessment of the linearity of the line of best fit for the quantitation standards was made for each experiment (on each day). There were five experiments each performed on different days The assessment reflected the a choice of a line of best

fit which had a regression curve which matched the calibration and quality control standards appropriately. The calibration lines were assessed using different statistical weightings to ensure there was less bias on the quantitation points at the top of the calibration line. The simplest regression for the calibration line was used as per FDA recommendations [9].

2.7.3 Matrix effects

The extraction procedure outlined in section 2.6.4 was performed using AVP or AVP-D5 spiked separately into either blank human plasma or HPLC grade water. At the end of the extraction, standard solutions of AVP and AVP-D5 were prepared separately in water: acetonitrile: formic acid (20:80:0.5 v:v:v). They were then spiked at 5 ng/mL individually into the extracted water and matrix samples, mixed, centrifuged and analysed by LC-MS/MS. The extent to which the matrix affects the response of the analyte and internal standard was then calculated by dividing the peak area of AVP as taken from the XIC for the MRM analysis of 543.4 to 328.2. sections in the presence of matrix by the peak area of AVP in the absence of matrix.

2.7.4 Selectivity

The selectivity of the quantitation method was assessed by using samples of unspiked human plasma from six different people [17-18] as well as a pool of plasma from ten individuals, and the impact of interferences on the chromatograms assessed visually at the retention times of AVP and AVP-D5. HILIC chromatography was chosen as it would retain analyte and internal standard most

effectively was chosen as this would ensure less interferences eluted with the analytes due to its polar nature.

2.7.5 Statistical outliers

The test used in this assessment was the Grubb's test G' [36] which was used to assess if a single outlier was present in the data set. The exclusion of a data value is then based on the fact that with 95% confidence, the value assessed does or does not fit within the normal Gaussian distribution of the other five values in the data set.

Accuracy and precision data were generated from the six replicates of each AVP spiked plasma standard level in each experiment and calculating the mean and standard deviation. The results were initially assessed by eye and any results that appeared to be anomalous were then assessed statistically.

2.7.6 Stability

The stability of AVP, frozen for 12 hours in plasma and then thawed and analysed was investigated using the AVP spiked frozen plasma standards prepared as described in section 2.6.3. Six replicates of these samples at the levels 0.25 ng/mL, 0.5 ng/mL, 0.75 ng/mL, 3 ng/mL, 5 ng/mL, 7.5 ng/mL and 10 ng/mL were extracted and analysed and their XIC peak area ratios for AVP: AVP-D5 Were determined and compared to the calibration curve.

Six replicates of AVP spiked frozen plasma standards as described in the paragraph above, at the levels of 0.75 and 10 ng/mL were used to assess the analytes stability in human plasma at room temperature. These samples consisted of AVP-spiked plasma which were frozen at -20˚C for 24 hours [17]. They were then removed from the freezer and left at room temperature for 24 hours before being extracted as in section 2.6.4. Recommendations are to store samples at room temperature for 4-24 hours [17-18] and since this bioanalytical assay required samples to be defrosted for more than 4 hours, it was appropriate to assess stability at 24 hours.

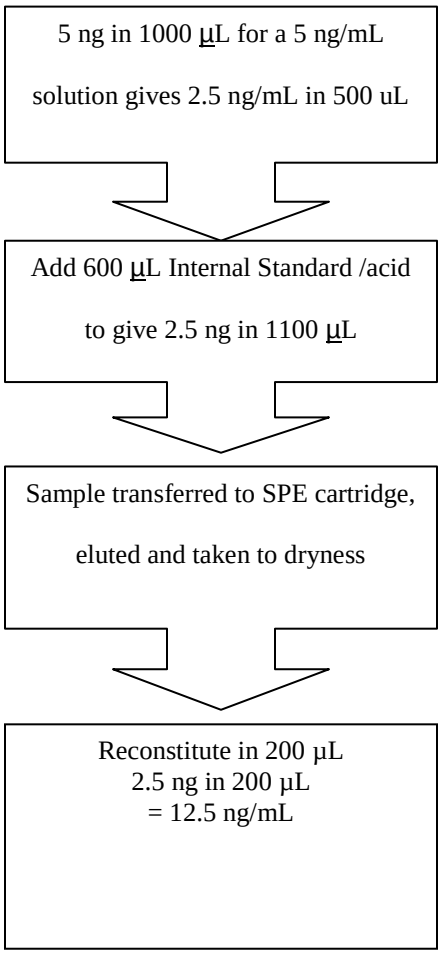
Six replicates of AVP spiked frozen plasma standards at the levels 0.75 and 10 ng/mL were also used to assess the analyte stability in human plasma on being frozen and defrosted. These standards were frozen for 24 hours after preparation, defrosted at room temperature for 1 hour and then returned to the freezer. This constituted one freeze-thaw cycle. Four of these cycles were completed and the standards thawed one final time and then assessed.

2.7.7 Maximum AVP recovery in a quanititative method

The assessment of how much AVP was recovered on extraction carried out as described in section 2.6.4 was performed using plasma spiked with AVP at concentrations of 0.75 ng/mL, 5 and 10 ng/mL. The maximum amount (100%) of AVP and AVP-D5 which could be recovered when using the fractionation techniques from the method detailed in section 2.6.4. Solutions of AVP in water:acetonitrile:formic acid (20:80:0.5 v:v:v) were prepared at the levels as if

100% of the analyte was recovered for the extracted analyte concentrations of 0.75 ng/mL, 5 ng/mL and 10 ng/mL. The calculation for 5 ng/mL is given in figure 27. Matrix blanks from pooled human plasma (ten individuals) was also extracted. The maximum recovery solutions as detailed above were added in place of reconstitution solution water:acetonitrile:formic acid (20:80:0.5 v:v:v) to the evaporated matrix blank extracts. These were then mixed, centrifuged and analysed by LC-MS/MS. The peak area of the XIC for AVP spiked frozen plasma standards was compared to the peak area of the extracted plasma blank which had a solution of AVP in it (at the 100% recovery concentration) which was spiked with AVP post extraction.

Figure 27- maximum recovery calculation (5 ng/mL AVP in human plasma)



3.0 Results and Discussion

Experimental design in bioanalysis permits many stages in the process. The first stage is method development of a quantitative bioanalytical assay and the second, the validation of the quantitative bioanalytical assay.

3.1 Method Development -Results and Discussion

Method development allows decisions to be made based on scientific experiments and the understanding of the chemistry behind them. The aims in all scientific investigation, including this method development are not to get the desired result every time, but to discover new ways to reach a goal. In this investigation, the aims were to identify suitable approaches to extract AVP from human plasma and quantify it by LC-MS/MS. There were many different approaches which could be taken and the testing of them can be referred to as method development.

In this thesis a range of different fractionation techniques, separation techniques and mass spectrometric optimisations were assessed, optimised and evaluated. The conclusions drawn from each of them were combined, to arrive at a quantitative method for the analysis of AVP in human plasma.

3.1.1 Fractionation techniques

The purpose of these experiments was to investigate the optimal conditions for SPE, including selection of the cartridges, and the washes and elutions to be used to fractionate AVP from the plasma matrix. The washes needed to elute the minimum amount of analyte whilst removing interferences, whereas for the elution solvent, it is important to elute as much of the analyte from the SPE cartridge as possible. To achieve this, SPE cartridges were primed and conditioned, and then diluted, acidified, AVP-spiked human plasma was loaded onto the extraction cartridge. HLB and WCX were used as they offer both reversed phase and mixed mode ion exchange mechanisms both of which are suitable for very basic compounds such as AVP (table 1).

0.5 mL Plasma containing 10 ng/mL AVP analyte to be extracted was loaded onto a HLB SPE plate (30 mg, Waters, Manchester) in 0.5 mL 0.1% formic acid (H_2O:MeOH, 90:10 v/v), to prevent AVP from adsorbing to the plastic surfaces. In order to assess the optimal % of methanol for eluting the AVP, a range of % of methanol in water (5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% v:v) acidified with 0.5 % formic acid were tested in duplicate[figure 28], and the intensity of the AVP response assessed using LC-SRM-MS, monitoring the 543.4-328.2 transition.

Figure 28- Elution profile for AVP when varying amounts of methanol were added to an elution solution containing 0.5% formic acid

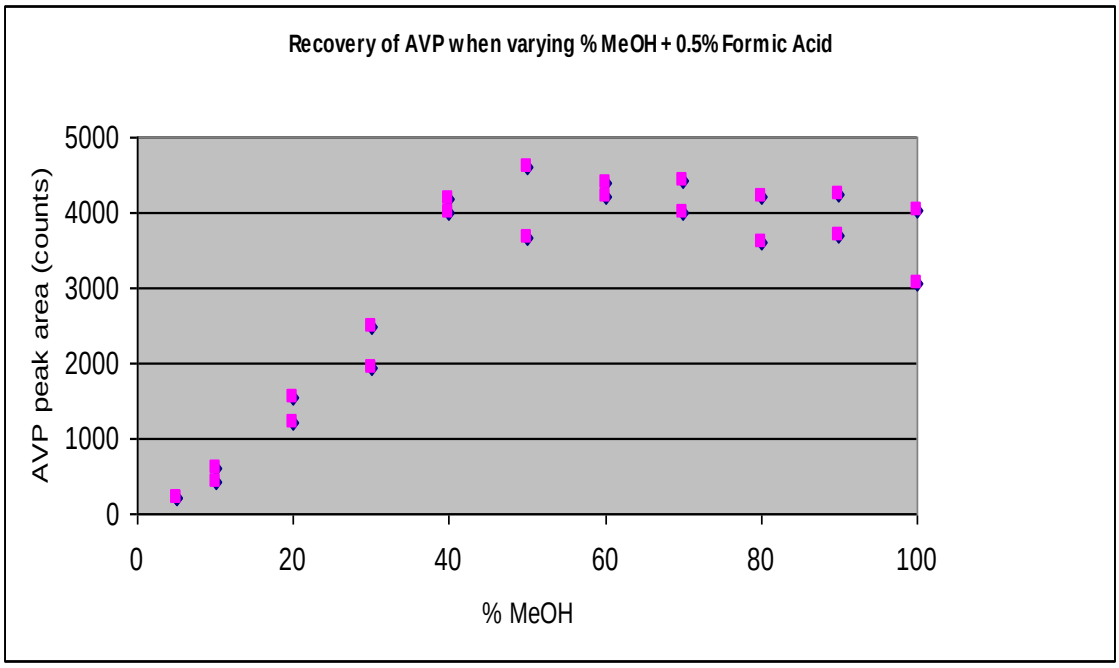
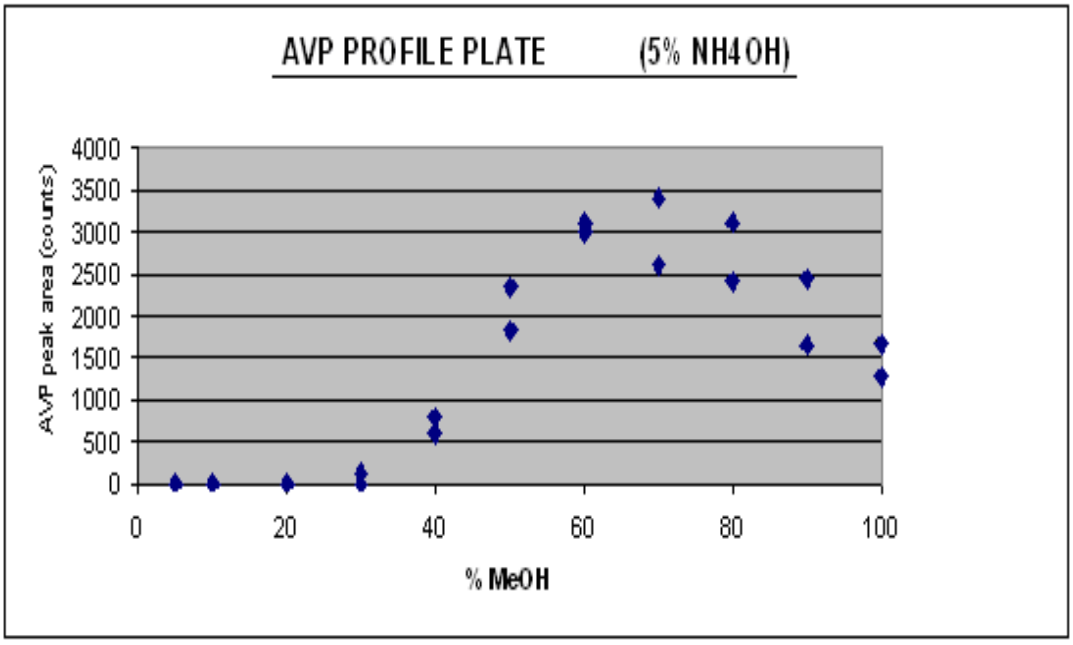


Figure 28 shows the area of the AVP peak obtained on UHPLC-SRM-MS analysis of the eluted AVP when the amount of methanol in the wash acidified with 0.5% formic acid is varied. At 5% methanol, there is very little analyte eluted. From 5% to 60% methanol, it is apparent that there is an increase in the amount of analyte eluted as the percentage methanol in the solution is increased. From 60%-100% methanol there is a plateau, with a slight decline in analyte recovery at 100%. From this data, it can be concluded that 5% methanol can be used to remove interferences but not analyte, as this solvent may be expected to remove water soluble matrix components without very much elution of the AVP analyte. It can also be determined that an appropriate percentage of methanol to elute AVP and to achieve maximum recovery would be 90%. This would also be suitable for rapid evaporation and would result in fast analysis times.

In order to improve the retention and further remove interferences, an additional mechanism can be used in the form of a WCX cartridge. This contains a carboxylic acid group in addition to the divinlbenzene polymer. 0.5 mL plasma containing 10 ng/mL AVP was loaded onto the SPE plate in 0.5 mL 0.1% formic acid (H_2O:MeOH, 90:10 v/v) . In order to assess the optimal percentage of methanol for eluting the AVP, a range of percentage of methanol in water (5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% v:v) in a basic solution of 2% ammonium hydroxide tested in duplicate [figure 29], and the intensity of the AVP response assessed using LC-SRM-MS, monitoring the 543.4-328.2 transition.

Figure 29- Elution profile for AVP when varying amounts of methanol were added to an elution solution containing ammonium hydroxide



In figure 29, the mass spectrometric AVP response versus percentage methanol in the wash solution is plotted. The data show that elution with between 5% and 30% methanol yields little or no analyte response. This is in contrast to the data shown in figure 28, and can be explained by the combined positions where the AVP can interact, these positions are on the carboxylic acid and also the benzene ring (structure shown figure 4) which are ionized by in the presence of 5% ammonium hydroxide. This retention can be seen by comparing figures 28 and 29. It is clear that from 0-25% Methanol that no elution of AVP occurs in the presence of ammonium hydroxide, whereas elution is seen from 5% Methanol in the presence of formic acid. This indicates that the ammonium hydroxide is increasing the interaction and retention of AVP to the SPE sorbent.

From 30% to 60% methanol there is a sharp increase in analyte response, which decreases again from 70% to 100% methanol. As the purpose of this experiment was to allow the N-vinylpyrrolidione part of the polymeric stationary phase to bind with the AVP, retention during the extraction was the aim.
These two profiles were used in combination to give the method bellow:

The stationary phase was primed with 0.5 mL methanol then conditioned with 0.5 mL water to equilibrate the stationary phase, followed by loading the sample, which contained 0.5 mL plasma containing 10 ng/mL AVP, 0.5 mL H_2O:MeOH:TFA (90:10:0.1 v/v/v) and 100 μL ortho-phosphoric acid (10% concentrated ortho-phosphoric acid in water). The methanol was to prevent the analyte adsorption to the

sample tube, TFA was used as an ion-pairing agent and ortho-phosphoric acid was included to prevent binding of the analyte to protein in plasma. As there are two elution profiles, they can both be used in recovery optimisation and sample purification. This can be done by using figure 28 which shows that 0.2% formic acid in 95:5 water:methanol will elute very little compound but will remove many water soluable components, this can also be used with the WCX as the sorbent structure is the same except for the addition of the carboxylic acid group. This should therefore behave in the same way for WCX as HLB in the presence of acid as the carboxylic acid will be protonated. The second wash, used for the removal of both some lipophilic plasma component removal in addition to any more polar components was consequently set at 20% methanol . This is based on the characteristics of the reagents and how they interact with polar and non-polar compounds.

The first wash used was H_2O: MeOH (90:5 v/v), used to remove water soluble plasma components and to keep the plate neutral and charged. Interactions take place between the stationary phase phenyl ring, which interact with AVP by binding to the phenyl ring on the phenlalanine, so on loading, the primary interactions were reversed phase. On washing with the 90:5 H_2O:MeOH the carboxylic acid group was deprotonated and the AVP is expected to bind via both RP and ion exchange mechanisms.

A third step was trialled and this was a wash with 0.5 mL acetone. This was to ensure lipid removal from the stationary phase prior to analyte elution. The elution solution comprised H_2O: MeCN: HCOOH (20:80:5) with the acid included to elute the cationic AVP from the carboxylic acid groups in the ion-exchange resin; ultimately the sorbent-reagent interaction is greater than the sorbent-analyte interactions and therefore the analyte is eluted.

3.1.2 Separation techniques

The purpose of this experiment was to determine if there was a difference in the limit of detection which could be reached when comparing AVP spiked into solvent suitable for injection onto the chromatographic column it was trailed on, using RP and HILIC chromatography.

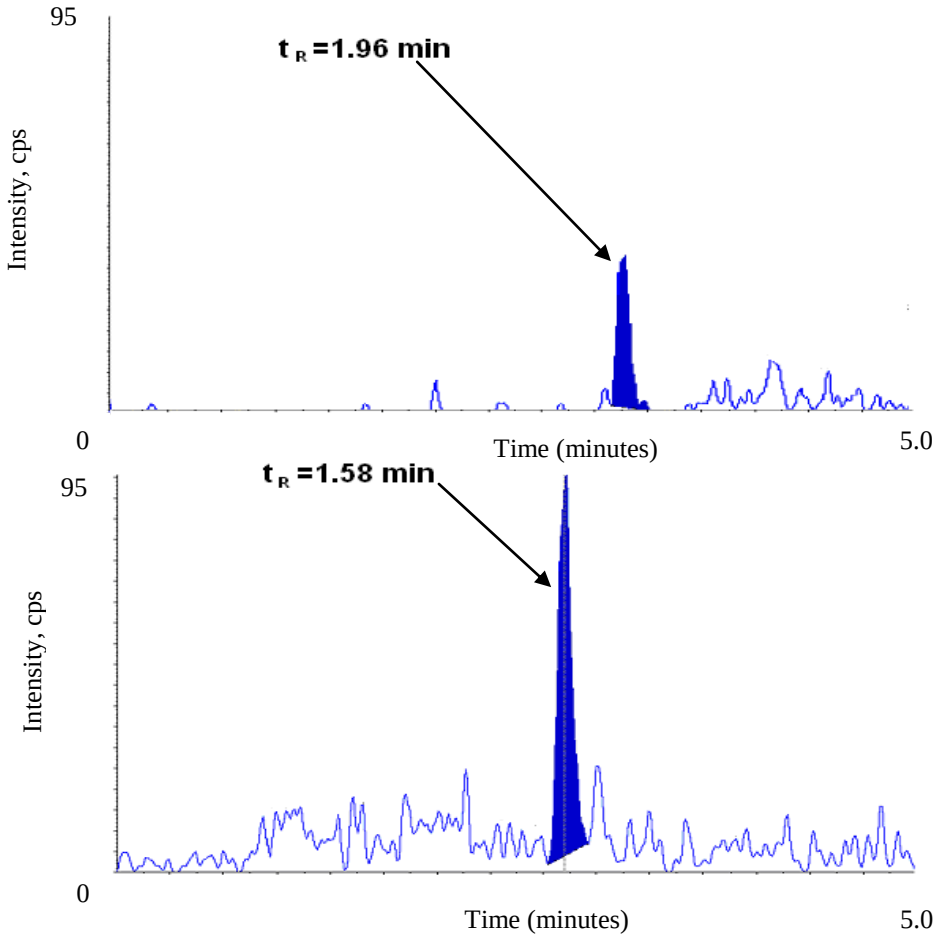
Various concentrations of AVP were analysed by both RP and HILIC UHPLC-SRM-MS/MS and the XIC peak area for AVP measured in each case. The results are shown in table 6.

Table 6 - Peak Areas of for AVP analysed using RP-UHPLC-SRM-MS/MS and HILIC-UHPLC-SRM-MS/MS

Concentration AVP (ng/mL)	AVP peak area reversed-phase (cps)	Reversed phase AVP t_R (min)	AVP peak area HILIC (cps)	HILIC t_R (min)
1	104	1.97	128	1.67
10	1202	1.96	1381	1.68
100	13332	1.96	14121	1.68
1000	123487	1.95	142329	1.68

The results in table 6 show that the peak areas for HILIC analysis are greater than for those of reversed-phase analysis for the same amounts of AVP. This is probably due to improved ionization efficiencies when eluting the analyte at higher levels of organic solvent, which is the case in HILIC chromatography. The extracted ion chromatograms for 1 ng/mL AVP are given in figure 30, which also show a larger sharper peak at this level for HILIC analysis when compared to RP.

Figure 30 – Extracted ion chromatograms obtained from injection of 1 ng/mL AVP, RP (t_R =1.96 min) and HILIC (t_R=1.58 min)



These results show that HILIC chromatography is more suitable than RP chromatography for the analysis of AVP in human plasma and so was used in the quantitative method (section 2.2).

3.1.3 Internal Standards in quantitative analysis

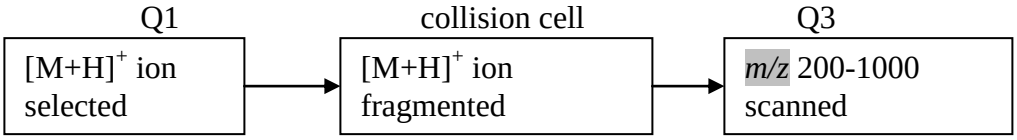
AVP is the analyte in this validation and the purpose is to quantify it. AVP is extracted in the presence of a deuterated internal standard AVP-D5, where the deuteriums are on the phenylalanine ring. This is of particular significance to the MRM transitions selected. The internal standard showed co-elution (or very slight separation) with the analyte in the majority of trials, due to the deuterium atoms being smaller than hydrogen.

3.1.4 Optimisation techniques in quantitative mass spectrometry

Precursor and product ion Identification

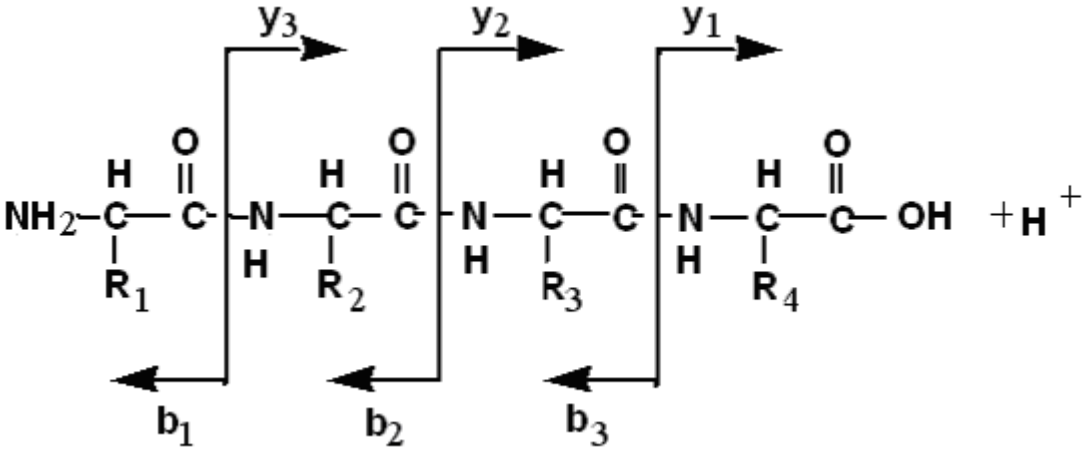
In order to record peptide product ion spectra, infusions were carried out using solutions of **AVP** and AVP-D5 (in MeOH:H_2O:HCOOH 50:50:0.1 v/v/v) prepared at 1 ng/mL and infused at 10 µL/min. They were ionised using TurboIonSpray and their product ion spectra recorded in positive ion mode, where the analyte precursor ion was selected in Q1 and then fragmented in the collision cell. In Q3 all the ions in the range *m/z* 200-1000 were scanned (figure 31); this then gives a detailed picture of how the analyte breaks down and this can be rationalized in terms of the peptide's primary structural. Figures 33-34 give the product ion spectra of AVP and AVP-D5.

Figure 31 – product ion scan



A proposed mechanism for peptide fragmentation is given in figure 21. This produces b and y ions as shown in figure 32.

Figure 32– peptide sequence ions of the b and y series



Determination of the ion to be monitored in Q1 was decided based on ion intensity where $[M+ 2H]^{2+}$ was the most abundant ion.. The product ion to be selected for MRM analysis is also shown in figures 33-34. A common fragment for these two peptides is the y3 ion. This fragmentation is favorable as the AVP has a disulfide link between the two cysine residues in the peptide (table 1) and thus y type fragmentation occurs to give the y3 ion. Subsequently the y3 ions are chosen for analysis in Q3 for MRM analysis.

Figure 33- prominent ions in the product ion spectrum of AVP

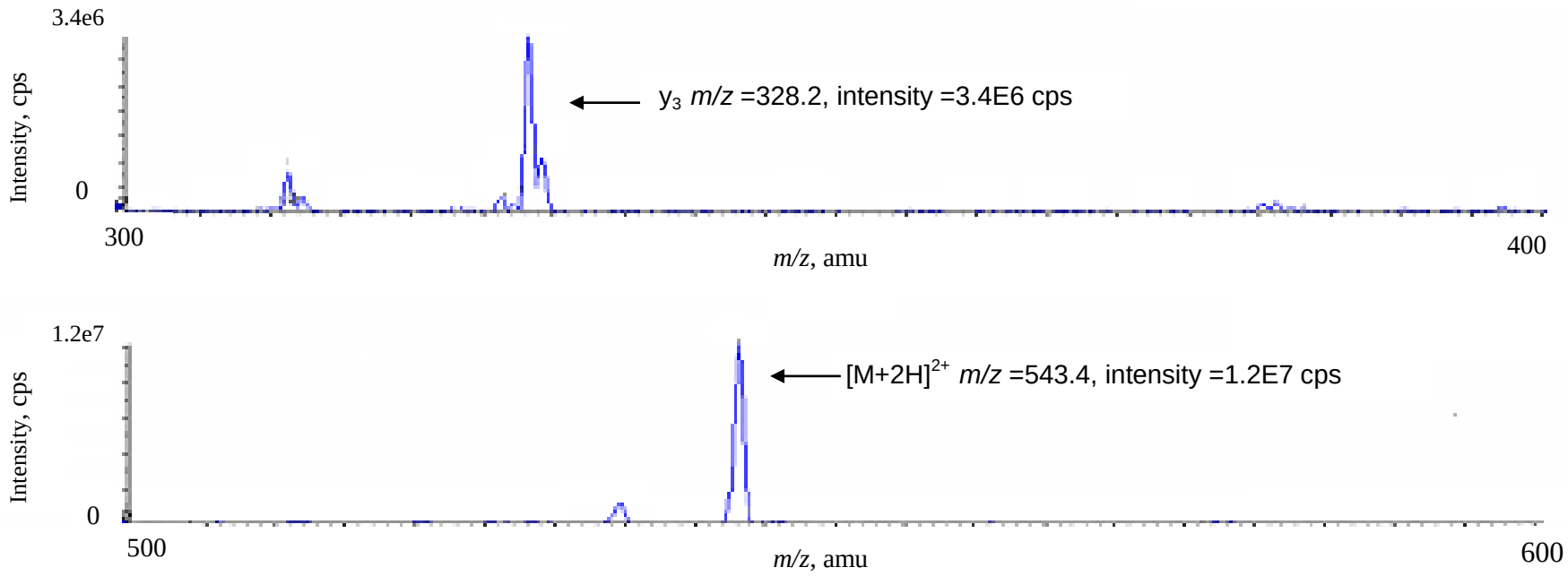
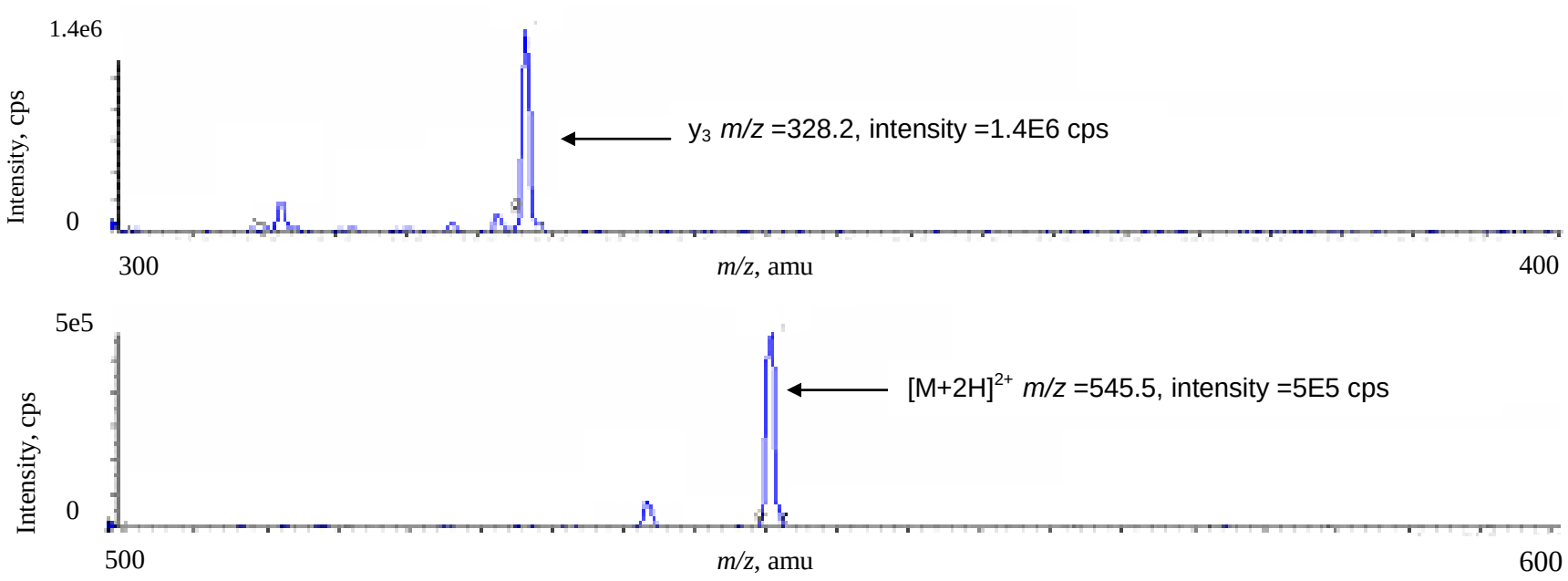


Figure 34- prominent ions in the product ion spectrum of AVP-D5



3.1.5 Comparison of API 3000 and API 5000 instruments for the quantitation of AVP

In order to determine the limits of detection for AVP using the API3000 and API5000 triple quadrupole mass spectrometers, equal-sized aliquots of standard solutions of AVP containing 0.5, 5, 50, 500 and 5000 pg/mL AVP were injected onto the UHPLC system using the HILIC separation developed (section 2). The API3000 and API5000 instruments are comparable in design, with the API3000 being an earlier model and the API5000 incorporating more recent design developments; the specifications of the API5000 include the advantages of an additional RF only quadrupole (Qjet) on the front of the analyser, between Q1 and the ionization source. For increased ion focusing, as well as a redesigned ionization source, which maximizes ionisation efficiency and analyte transfer into the mass analyzer.

In order to compare the performance of the two instruments for the detection of AVP, the peak obtained for AVP on selected reaction monitoring of the transition *m/z* 543.4-328.20 was plotted for each of the five analyte dilutions (figures 35-39). Figure 35, shows the XIC for AVP on both an API 3000 (a) and an API 5000 (b) with 0.5 pg AVP on the column. This clearly shows that no AVP can be detected when using an API 3000 instrument whereas analyte is detected when using an API 5000. Figure 36 shows the XIC for AVP on both an API 3000 (a) and an API 5000 (b) with 5 pg AVP on the column. The chromatograms show that the intensity is much greater when detection is performed using an API 5000. This is also the case

for figure 37 where 50 pg AVP was analysed, for figure 38 where 500 pg AVP was analysed on column and figure 39 where 5000 pg was analysed on column. From these chromatograms it can also be seen that AVP elutes with a retention time of 1.80 min (API3000 system) or 1.82-1.83 min (API5000), and an intense and sharp peak was observed for AVP in all the chromatograms obtained on the API5000. For the API3000, intense, sharp peaks were obtained at this retention time from the 5, 50, 500 and 5000 pg injections, but no peak was discernible at this retention time from the 0.5 pg injection.

Figure 35–XIC for 0.5 pg of AVP using A) API 3000 and B) API 5000

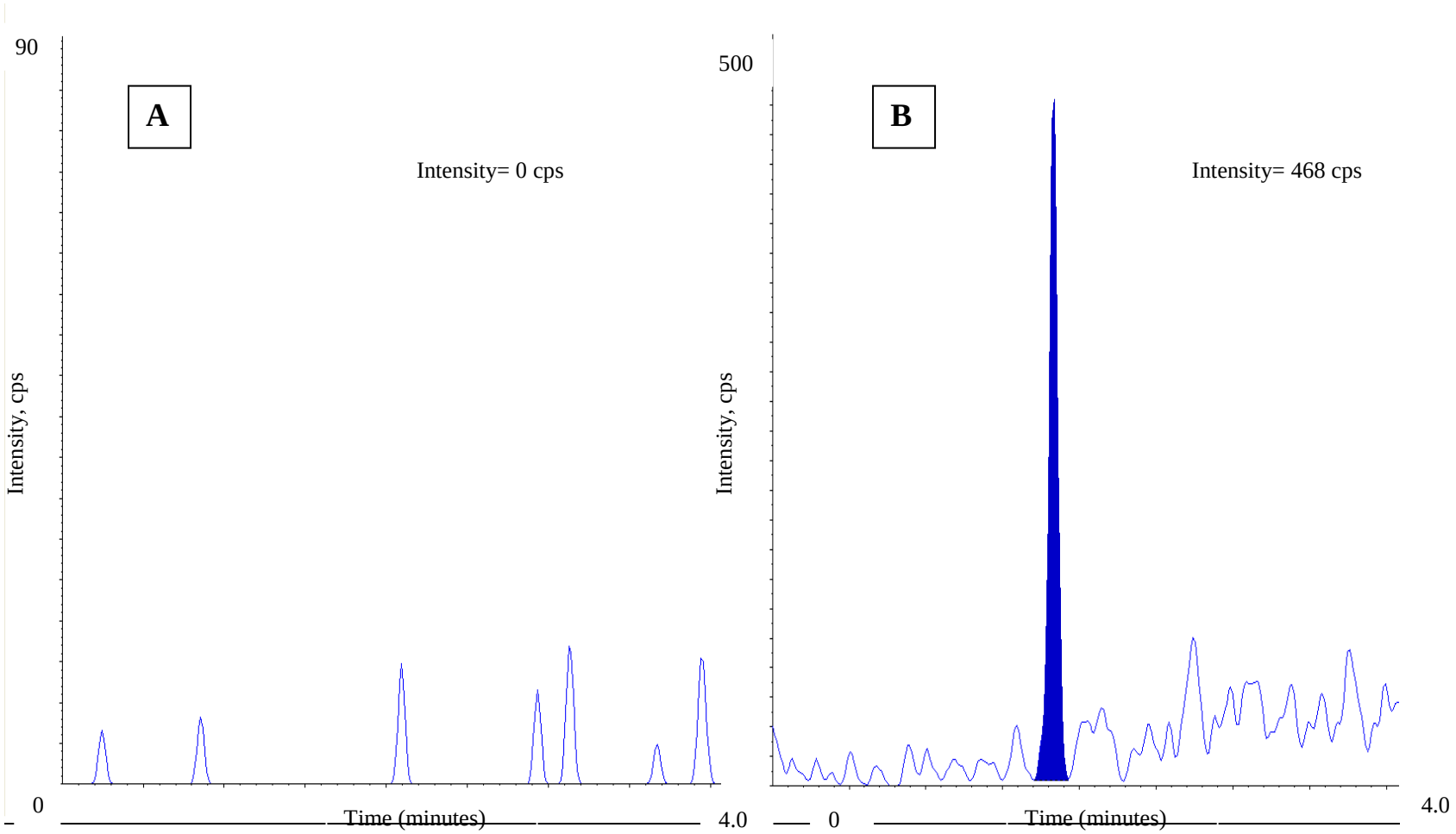


Figure 36 – XIC for 5 pg of AVP using A) API 3000 and B) API 5000

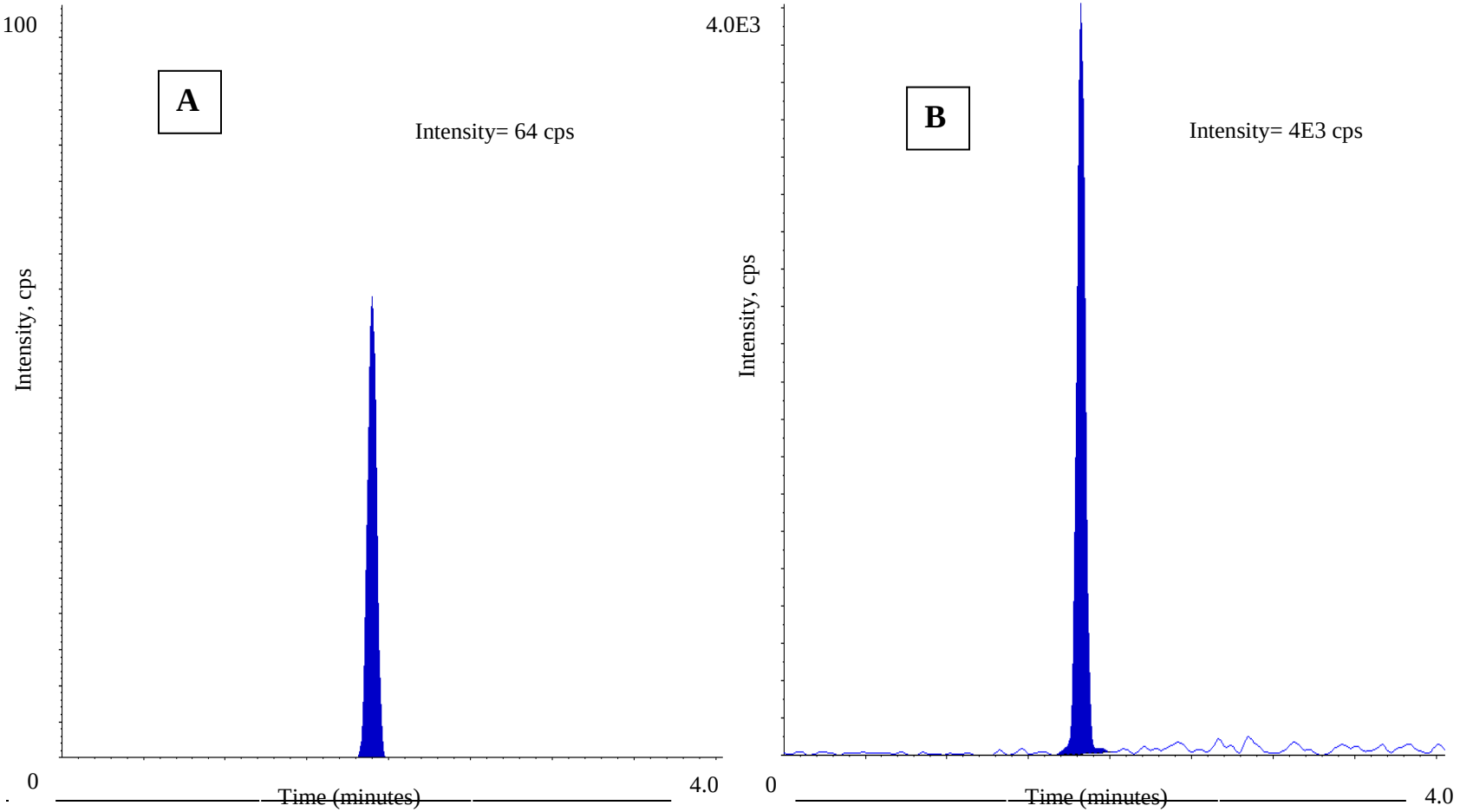


Figure 37- XIC for 50 pg of AVP using A) API 3000 and B) API 5000

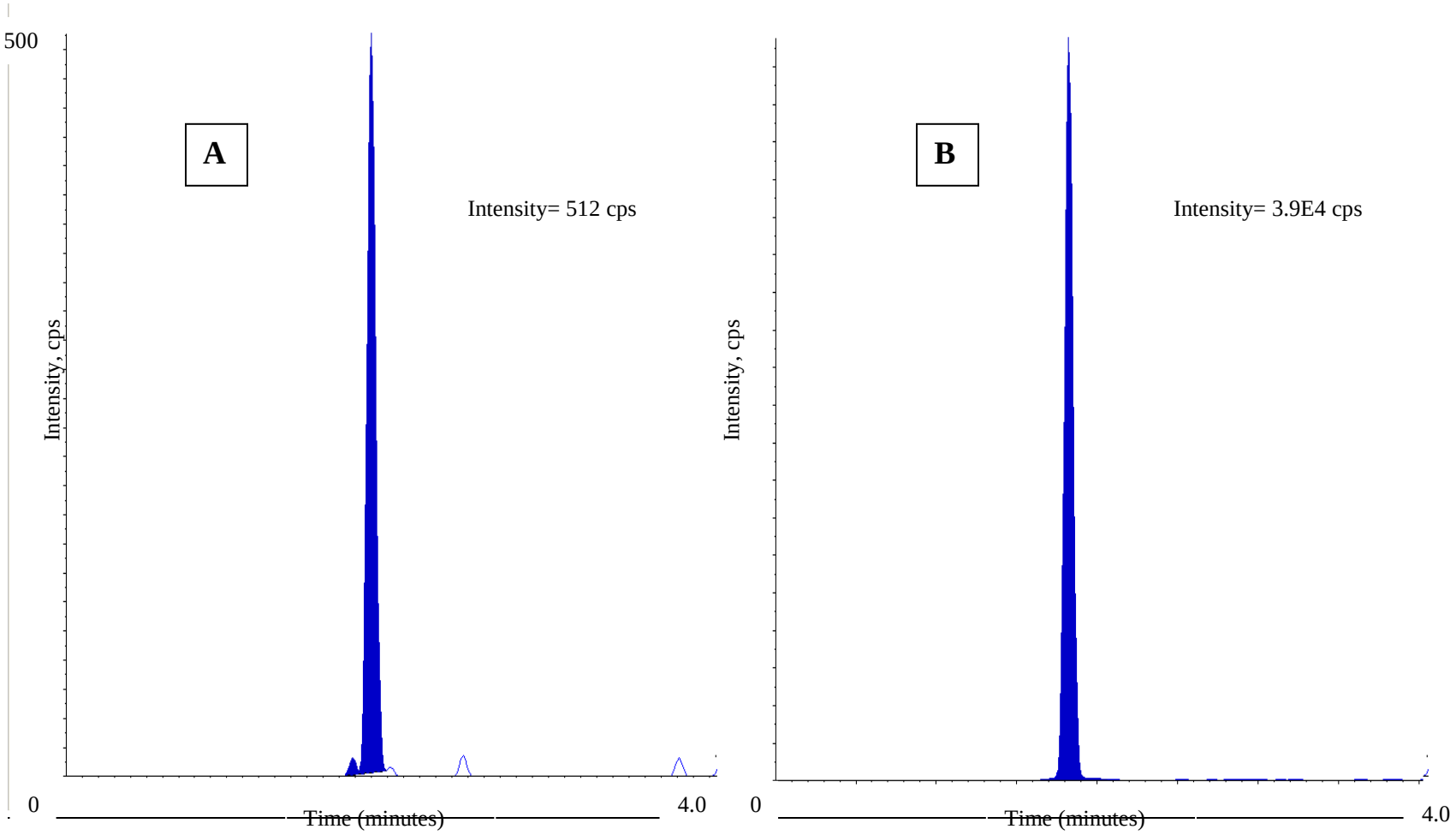


Figure 38- XIC for 500 pg of AVP using A) API 3000 and B) API 5000

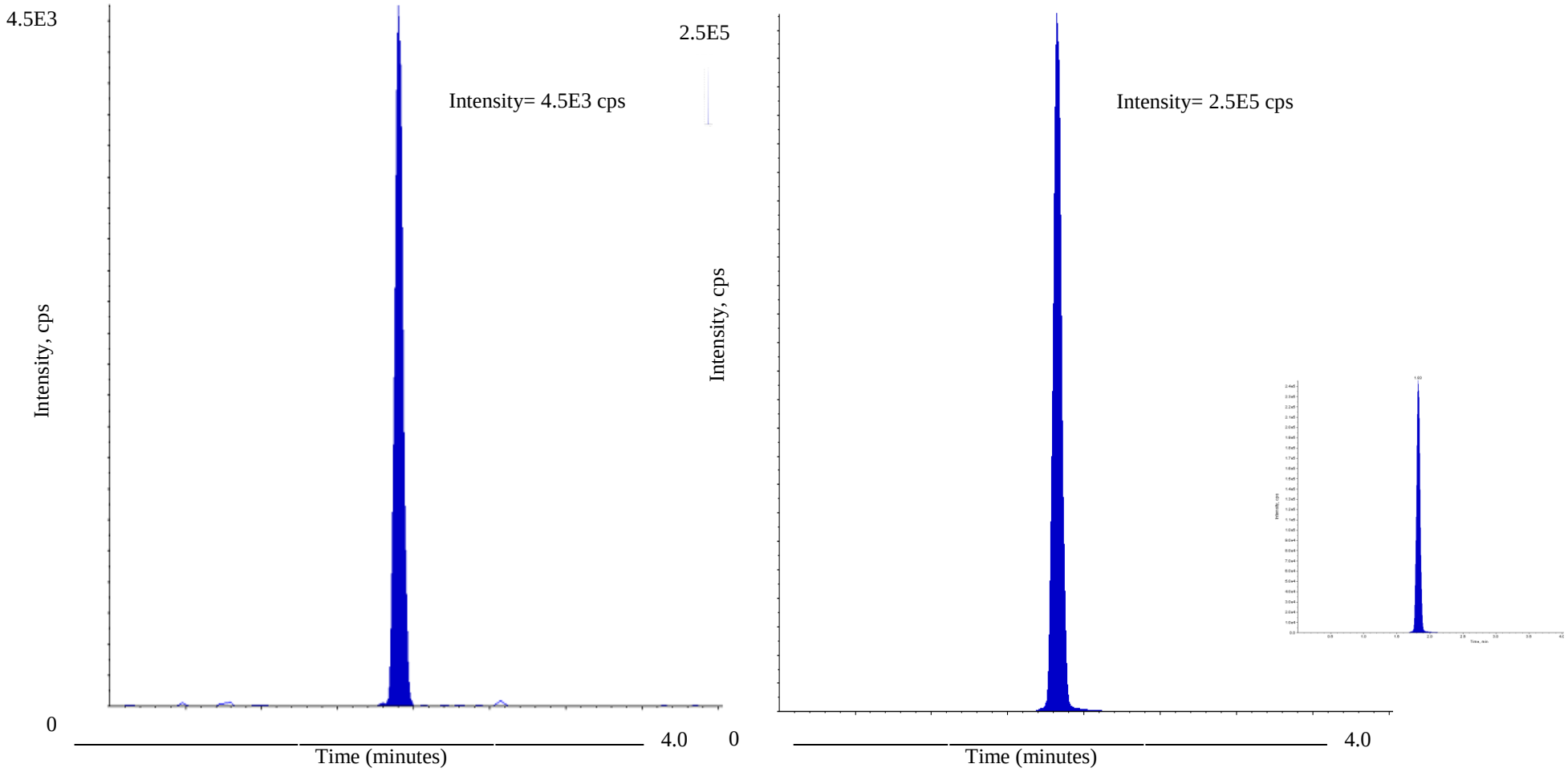
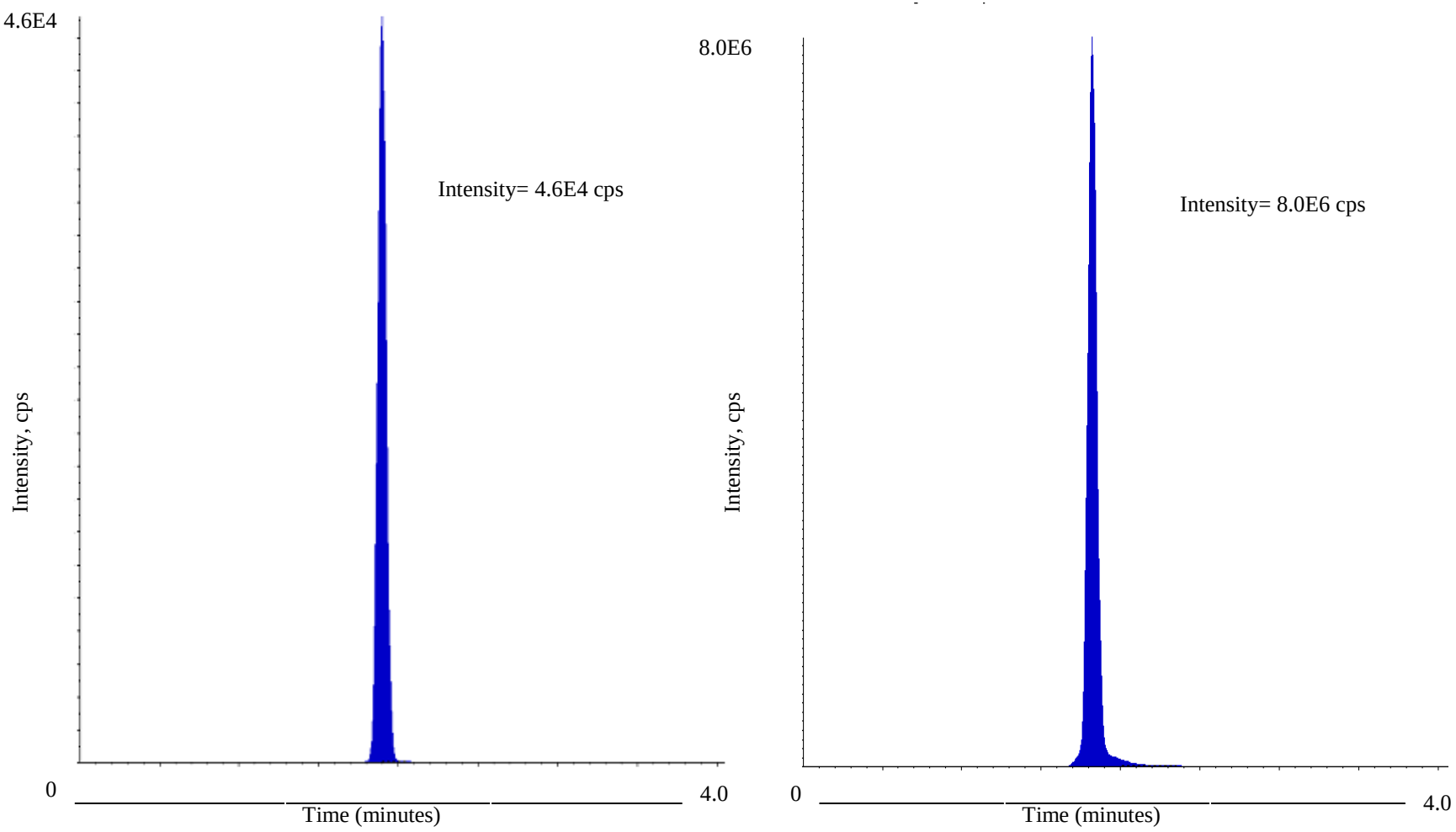


Figure 39 XIC for 5000 pg of AVP using A) API 3000 and B) API 5000



From these results, it is clear that using an API 5000, 0.5 pg on column AVP can be detected from standard solution, whereas the lowest amount detectable using an API 3000 is 5 pg. The API 5000 is therefore a more suitable instrument for use in the quantitative analysis of AVP if amounts less than 5 pg need to be detected. Applied Biosystems, the manufacturer of the two instruments, suggest this is due to improvements in the ionisation efficiency of the source on the API5000.

3.1.6 FAIMS

In the assessment of the potential of the FAIMS device for improving the detection of AVP in human plasma by lowering background chemical and endogenous noise, samples of AVP spiked freshly into human plasma, plasma spiked with AVP then frozen and rethawed for analysis, and unspiked plasma samples were extracted using weak cation exchange SPE cartridges. A calibration curve was constructed using the peak areas obtained on LC-MRM analysis, both with and without the FAIMS device, of the plasma spiked with known amounts of AVP (six replicates were analysed for each of the 1, 3.125, 20, 47 and 160 ng/mL levels)). Six replicates of the AVP spiked frozen samples were prepared and analysed using LC-MRM both with and without the FAIMS device; the amounts of AVP in these samples were determined from the peak areas of the AVP peak using the calibration curve. The accuracy values at the

LLQQ were 7.0% from the nominal value and with FAIMS 3.3% from the nominal value. The precision values at the LLOQ were 4.9% without FAIMS and 7.2% with FAIMS. The accuracy and precision values for both sets of data met the precision and accuracy criteria for validations laid down by the FDA [9] and industry guidelines [60-61] as described in chapter 1.

table 4 shows that there is improved precision when not using FAIMS, which can be explained as FAIMS reduces the number of ions reaching the detector; it would therefore be expected to have lower precision as there are lower numbers of ions to be detected. There was very little difference in the accuracies when FAIMS was utilised or bypassed.

As both the data obtained with and without the FAIMS device met the criteria for validation, the limits of detection were then compared.

Table 7- LC-MS/MS

precision and accuracy of AVP in spiked frozen plasma samples

	concentration AVP spike introduced (ng/mL)				
	1	3.125	20	47	160
	observed concentration (ng/mL)				
mean (ng/mL)	1.07	3.14	19.5	46.7	161
standard deviation (n-1)	0.0526	0.141	0.811	1.63	3.83
precision (%)	4.9	4.5	4.2	3.5	2.4
accuracy (%)	107.0	100.5	97.5	99.4	100.6

Table 8- LC-FAIMS-MS/MS

precision and accuracy of AVP in spiked frozen plasma samples

	concentration AVP spike introduced (ng/mL)				
	1	3.125	20	47	160
	observed concentration (ng/mL)				
mean (ng/mL)	0.967	3.06	20.0	48.8	165
standard deviation (n-1)	0.0693	0.181	1.66	2.45	7.50
precision (%)	7.2	5.9	8.3	5.0	4.5
accuracy (%)	96.7	97.9	100	103.8	103.1

To evaluate whether there are changes in the limits of detection with and without FAIMS, the signal-to-noise ratios (S:N) for the extracted ion peak for AVP for spiked frozen plasma samples were compared (table 9). The results show the analyte peak height divided by the height of the background noise for different AVP concentrations in plasma. The data in table 9 shows that when using FAIMS, the S:N ranges from 4.6-1550 whereas without FAIMS it ranges from 3.2-995. For validation, the overall aim of the work described in this thesis, it is desirable to have S:N values in excess of 5:1 [9]. Although neither of the methods meets this criterion for AVP at 1 ng/mL, using FAIMS brings improved S:N when analysing AVP at this level, and at all other concentrations tested. However there is not an improvement in the limit of detection, as table 9 shows that the 1 ng/mL level does not meet the S:N requirements (5:1) as using FAIMS gives a S:N of (4.6:1) whereas without FAIMS (3.2:1). This criterion has been met under both conditions for the concentration 3.125 ng/mL.

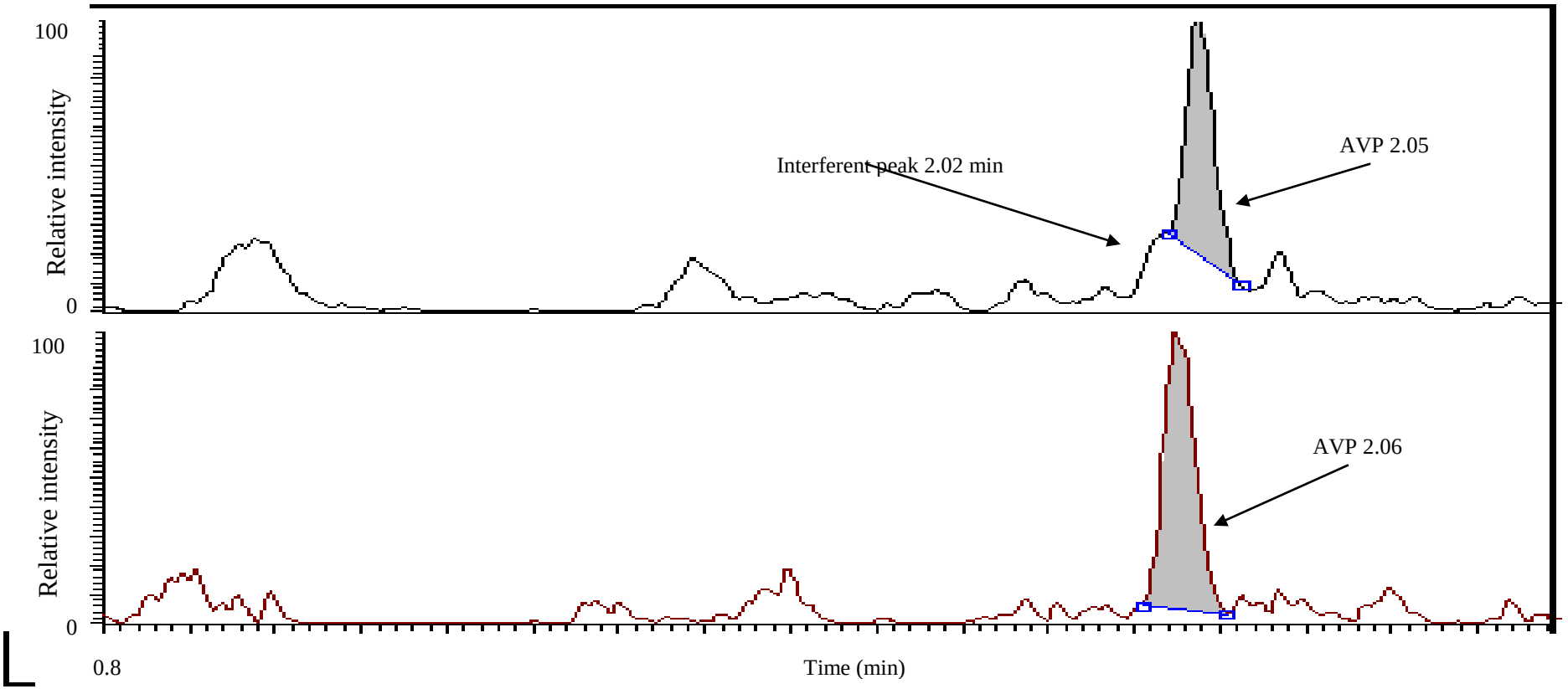
Table 9 –S:N for AVP extracted ion chromatograms from spiked frozen plasma sample with and without FAIMS

	Spiked AVP Concentration in human plasma				
	1 ng/mL	3.125 ng/mL	20 ng/mL	47 ng/mL	160 ng/mL
Mean S:N without FAIMS	3.2	15.8	116	773	995
Mean S:N with FAIMS	4.6	23.7	229	956	1550

As well as lowering baseline noise and subsequently increasing the S:N, FAIMS acts to separate and remove co-eluting interferences from the analyte peak. To determine whether differences are observed in the XIC for AVP (*m/z* 543.4 -328.2) two aliquots of 3.25 ng/mL AVP spiked into plasma, one analysed utilising and the other bypassing the FAIMS interface (figure 40), were compared.

It can be seen in the top chromatogram that there is a shoulder on the main AVP peak at 2.0 minutes. This is believed to be an interfering peak and is not apparent when FAIMS is used (bottom chromatogram). The S:N is also noticeably improved when using FAIMS; the peak for AVP at t_R 2.1 min in the top chromatogram gives S:N of 13:1, whereas the peak for AVP in the bottom chromatogram gives S:N of 22:1. This clearly shows that although a lower detection limit (the lowest concentration where the S:N $\geq$5:1) could not be reached by using FAIMS, the analysis is more selective when used in conjunction with FAIMS.

Figure 40- XIC for 3.25 ng/mL AVP spiked into plasma, analysed without (upper panel) and with (lower panel) FAIMS



3.2 Validation of the quantitative bioanalytical method for AVP

The second stage in development of a bioanalytical method is its validation. This is crucial prior to sample quantitation using the method as it establishes the robustness of a method, ensuring that sample analysis will result in precise, accurate results for the amount of analyte in the analytical samples.

The purpose of this bioanalytical method validation was to determine whether the method developed (section 2.0) would satisfy the requirements of industry guidelines as set out by the FDA, MHRA and EMEA. If the method failed to comply with these guidelines, it may be deemed unsuitable and subsequent sample analysis not appropriate for regulatory submission for NCEs.

In order for concentration determination of AVP in human plasma to be possible, there needs to be proof that the method is appropriate for this purpose. This begins with the construction of the calibration line. A calibration line formed from of the line of best fit for the quantitation standards in the range 0.25 ng/mL to 10 ng/mL AVP prepared in human plasma. These standards were extracted in the presence of AVP-D5 internal standard as described in section 2.2 and analysed by LC-MS/MS using MRM for the transitions 543.4 and 545.5 to 328.2 The peak areas at the retention time for AVP and AVP-D5 from the extracted ion chromatograms (XIC) were determined and the ratio of AVP: AVP-D5 for the peak areas calculated. This

area ratio was plotted in a graph against the known concentration of AVP in the original plasma samples. The relationship between the peak area ratio and AVP concentration is defined by a line of best fit between the quantitation standard points. Experiments were performed and the area ratio of the sample compared to those of the line. Other standards quantified by the line included AVP spiked, frozen plasma samples which were stored for 24 hrs at room temperature; AVP spiked frozen plasma samples which were frozen and defrosted twice prior to analysis. Human plasma without AVP, and solutions containing AVP without human plasma were also analysed. The purpose of these analyses was to test if AVP was stable at room temperature for 24 hours in plasma, if AVP was stable in plasma that had been frozen and defrosted twice, also other experiments were required to assess recovery and ensure that there were no endogenous interferences.

3.2.1 Construction of Calibration Curve

In order to determine the linearity of response, five replicate sets of quantitation standards, each set prepared on a different day but using the same plasma and dilute stock solution were prepared, each containing 10 different concentration levels of 0.25, 0.5, 1, 2, 3, 4, 6, 8, 9 and 10 ng/mL in human plasma. This will be referred to as inter and intra-day data, and will show that the assay is robust and reproducible on different days.

The aim of the experiment was to assess the lowest level which could be quantified by LC-MS/MS. This was because I wanted to have a calibration range appropriate

to assess the levels of AVP in human plasma samples, where elevated levels of AVP may act as a biomarker for disease states, a suitable LLOQ for this kind of assessment would be below 1 ng/mL (chapter 1). The lowest levels assessed were at 0.1 ng/mL and 0.25 ng/mL. The level 0.1 ng/mL was also included however the XIC for 543.4>328.2 did not give a peak for AVP and subsequently no data could be reported. The next level which was assessed was 0.25 ng/mL and therefore the range of the quantitation standards, and ultimately the range for which plasma samples of unknown AVP concentrations could be assessed, was therefore 0.25 ng/mL to 10 ng/mL. Therefore the range was limited to 0.25 ng/mL to 10 ng/mL.

The quantitation standards were extracted using the method described in section 2.2 and analysed using HILIC UHPLC-MS/MS using multiple reaction monitoring for the ions 543.4>328.2. The peak area for AVP was determined from the extracted ion chromatogram and divided by the peak area of the internal standard obtained in the same way. This was plotted against the nominal concentration of AVP. The simplest appropriate form of regression was chosen as required by the FDA [17] In this case it was linear 1/x where x was the concentration. This gave the calibration curve a stronger weighting towards the lower end of the calibration curve.

3.2.2 Accuracy, precision and statistical analysis

Prior to the assessment of the accuracy and precision of the AVP spiked frozen plasma sample outliers were removed using the Grubbs test. The single outlier test was used and this is based on a normal Gaussian distribution of points, which

calculates the probability of the suspected outlier of being part of the normal distribution generated by the other five values.

In order to determine how close a value is to the nominal concentration, it is important to know what it is being measured against. In this validation there were five experiments performed, each of which consisted of measuring six replicates of different levels of AVP spiked into plasma at the levels 0.75, 5 and 10 ng/mL. All samples were measured using the peak area ratios for the XICs of AVP and AVP-D5.

Precision was determined by assessing the distribution of AVP values obtained on analysing six replicate extracted AVP spiked, frozen plasma samples each at concentrations 0.25, 0.5, 0.75, 5, 7.5 and 10 ng/mL AVP spiked into human plasma. These values were measured against the calibration line, and compared to the known amount of AVP in the AVP spiked frozen plasma standards.

The AVP: AVP-D5 XIC area ratios of the AVP spiked frozen plasma standards are compared to the calibration line to determine the concentration of AVP in unknown plasma samples. It is therefore important that the line be a true reflection of AVP concentrations at the levels spiked into plasma. The AVP spiked plasma samples confirm whether or not the samples are stable when frozen.

The quantitation standard data are reported in table 11 and this shows all values to be consistent with guidelines including the FDA and MHRA for bioanalytical methods [18].

Table 11 shows the data collected from the AVP spiked plasma quantitation standards at the levels 0.25, 0.5, 1, 2, 3, 4, 6, 8, 9, 10 ng/mL from the five different days; this is referred to as inter-day data and gives the accuracy and precision values comparing the standards on different days. The results show that the accuracy was within ±4.8% of the nominal spiked concentration and the precision (given as relative standard deviation) below 8.6%. The requirements for this validation were to meet the FDA guidelines, was to be within ±15% of the nominal spiked concentration. These results are within that criterion.

Accuracy and precision was also assessed for the spiked, frozen plasma samples which were analysed using six replicate AVP spiked samples at each level on the same day. This was performed on five days and the results collated and compared to show that the assay was repeatable.

The results in table 12 show the accuracy and precision data for the spiked frozen stability samples for the levels 0.25, 0.5, 0.75, 5 and 10 ng/mL which were extracted and analysed on day 1. The requirement for the data was for the mean concentration to be within ±15% of the nominal concentration and also within ±15% for precision.

The data shown in table 12 meets these requirements as the accuracy is within ± 13.6 % of nominal spiked concentration and the precision within 9.2 %.

The results in table 13 show the accuracy and precision data for the spiked frozen stability samples for the levels 0.25, 0.5, 0.75, 5 and 10 ng/mL which were extracted and analysed on day 2. The requirement for the data was for the mean concentration to be within ±15% of the nominal concentration and also within ±15% for precision. The data shown in table 13 meets these requirements as the accuracy is within ± 13.2 % of nominal spiked concentration and the precision within 8.1 %.

The results in table 14 show the accuracy and precision data for the spiked frozen stability samples for the levels 0.25, 0.5, 0.75, 5, 10 and 50 ng/mL which were extracted and analysed on day 3. The requirement for the data was for the mean concentration to be within ±15% of the nominal concentration and also within ±15% for precision. The data shown in table 14 meets these requirements as the accuracy is within ± 6.6 % of nominal spiked concentration and the precision within 13.7 %.

The results in table 15 show the accuracy and precision data for the spiked frozen stability samples for the levels 0.25, 0.5, 0.75, 5 and 10 ng/mL which were extracted and analysed on day 4. The requirement for the data was for the mean concentration to be within ±15% of the nominal concentration and also within ±15% for precision. The data shown in table 15 meets these requirements as the accuracy is within ± 5.8 % of nominal spiked concentration and the precision within 10.6 %.

The results in table 16 show the accuracy and precision data for the spiked frozen stability samples for the levels 0.25, 0.5, 0.75, 5 and 10 ng/mL which were extracted and analysed on day 5. The requirement for the data was for the mean concentration to be within ±15% of the nominal concentration and also within ±15% for precision. The data shown in table 16 meets these requirements as the accuracy is within ± 13.6 % of nominal spiked concentration and the precision within 8.8 %.

Data were also collated from the experiments performed on different days. This is referred to as the inter-day results as is given in table 17. The accuracy and precision of AVP spiked frozen plasma standard (AVP spiked frozen plasma standards) AVP concentrations to the mean was compared for experiments performed on different days to ensure repeatability. These experiments all used different batches of SPE cartridge for sample preparation and mass spectrometers for quantification and detection. This was to ensure the experiments were robust and repeatable.

The results in table 17 show the mean accuracy and precision data for the spiked frozen stability samples for the levels 0.25, 0.5, 0.75, 5 and 10 ng/mL assessed over five days, where the data from tables 12-16 has been collated. The requirement for the data was for the mean concentration to be within ±15% of the nominal concentration and also within ±15% for precision. The data shown in table 17 meets these requirements as the accuracy is within ± 7.6 % of nominal spiked concentration and the precision within 11.1%.

All intra-day and inter-day statistics in tables 12- 17 shows that the acceptance criteria as defined by the regulatory agencies has been met [15-18].

Table 11

inter-day precision and accuracy of quantitation standard data

	quantitation standard level (ng/mL)									
	0.25	0.5	1	2	3	4	6	8	9	10
mean (ng/mL)	0.238	0.504	1.04	2.07	2.99	4.09	6.04	7.83	8.79	10.1
standard deviation (n-1)	0.0204	0.0271	0.0425	0.0480	0.127	0.136	0.227	0.358	0.277	0.284
RSD (%)	8.6	5.4	4.1	2.3	4.2	3.3	3.8	4.6	3.2	2.8
accuracy (%)	95.2	100.8	104.0	103.5	99.7	102.3	100.7	97.9	97.7	101.0

Table 12

intra-day precision and accuracy data of AVP spiked frozen plasma samples

experiment 1 – day 1

AVP concentration	0.25 ng/mL	0.5 ng/mL	0.75 ng/mL	5 ng/mL	7.5 ng/mL
	observed concentration (ng/mL)				
mean (ng/mL)	0.284	0.526	0.791	4.99	7.24
standard deviation (n-1)	0.0261	0.0391	0.0630	0.171	0.213
RSD (%)	9.2	7.4	8.0	3.4	2.9
accuracy (%)	113.6	105.2	105.5	99.8	96.5

Table 13

intra-day precision and accuracy data of AVP spiked frozen plasma samples

experiment 2 – day 2

AVP concentration	0.25 ng/mL	0.5 ng/mL	0.75 ng/mL	5 ng/mL	10 ng/mL
	observed concentration (ng/mL)				
mean (ng/mL)	0.283	0.521	0.818	4.82	9.82
standard deviation (n-1)	0.0228	0.0403	0.0409	0.246	0.536
RSD (%)	8.1	7.7	5.0	5.1	5.5
accuracy (%)	113.2	104.2	109.1	96.4	98.2

Table 14

intra-day precision and accuracy data of AVP spiked frozen plasma samples

experiment 3 –day 3

AVP concentration	0.25 ng/mL	0.5 ng/mL	0.75 ng/mL	5 ng/mL	10 ng/mL	50 ng/mL*
	observed concentration (ng/mL)					
mean (ng/mL)	0.237	0.480	0.739	4.67	9.49	50.5
standard deviation (n-1)	0.0325	0.0131	0.0407	0.199	0.229	2.16
RSD (%)	13.7	2.7	5.5	4.3	2.4	4.3
accuracy (%)	94.8	96.0	98.5	93.4	94.9	101.0

* analysed after ten-fold dilution with blank human plasma to validate samples above range being diluted into range with blank plasma.

Table 15

intra-day precision and accuracy data of AVP spiked frozen plasma samples

experiment 4 – day 4

AVP concentration	0.25 ng/mL	0.5 ng/mL	0.75 ng/mL	5 ng/mL	10 ng/mL
	observed concentration (ng/mL)				
mean (ng/mL)	0.257	0.500	0.713	4.71	9.47
standard deviation (n-1)	0.0215	0.0429	0.0753	0.134	0.349
RSD (%)	8.4	8.6	10.6	2.8	3.7
accuracy (%)	102.8	100.0	95.1	94.2	94.7

Table 16

intra-day precision and accuracy data of AVP spiked frozen plasma samples

experiment 5 –day 5

AVP concentration	0.25 ng/mL	0.5 ng/mL	0.75 ng/mL	5 ng/mL
	observed concentration (ng/mL)			
mean (ng/mL)	0.284	0.527	0.797	4.92
standard deviation (n-1)	0.0127	0.0466	0.0446	0.0818
RSD (%)	4.5	8.8	5.6	1.7
accuracy (%)	113.6	105.4	106.3	98.4

Table 17

inter-day precision and accuracy data of AVP spiked frozen plasma samples

	0.25 ng/mL	0.5 ng/mL	0.75 ng/mL	5 ng/mL	7.5 ng/mL	10 ng/mL	50 ng/mL
	observed concentration (ng/mL)						
mean (ng/mL)	0.269	0.512	0.770	4.82	7.24	9.59	50.5
standard deviation (n-1)	0.0299	0.0403	0.0646	0.204	0.213	0.403	2.16
RSD (%)	11.1	7.9	8.4	4.2	2.9	4.2	4.3
accuracy (%)	107.6	102.4	102.7	96.4	96.5	95.9	101.0

3.2.3 Stability

In the assessment of stability, three experiments were performed. The first assessed the short term stability of AVP in plasma over five days when stored at -20°C. Other experiments assessed the room temperature stability and freezing / defrost stability of AVP in human plasma. The aim of these experiments was to show whether or not AVP was stable in human plasma at -20◦C for up to five days, if it could be stored at room temperature for 24 hours and still be stable and also if it could be frozen and defrosted up to four times.

Spiked frozen plasma samples at AVP concentrations 0.75 ng/mL and 10 ng/mL were used to assess the frozen stability of AVP in human plasma, the room temperature stability of AVP in plasma and the freezing and thawing stability of AVP in human plasma. They were prepared as per section 2.2. These were then analysed using six replicate samples at each concentration, and the concentration of AVP determined by comparison to the calibration curve . The data in tables 12-17 show that the accuracy and precision meet the acceptance criteria initially set out in the introduction and taken from the FDA and MHRA guidelines [17]. It can be concluded that AVP is stable in plasma according to these guidelines [17] over 5 days (which was the longest amount of time the AVP spiked frozen plasma samples were stored for and then compared to unfrozen quantitation standards.

The stability of AVP in human plasma was trialed for 24 hrs at room temperature by preparing spiked, frozen plasma samples and freezing these for 24 hours, then

storing at room temperature for 24 hours before analysis. AVP spiked plasma samples were also used in the assessment of freeze-thaw stability. This involved freezing the standards for 24 hours then defrosting them for 1 hour at room temperature; the samples were then returned to the freezer for 12 hours then defrosted for 1 hour at room temperature. This process was repeated until the standards had been frozen and defrosted in total four times. This was to test whether patient samples could be frozen and defrosted in this way without affecting the quantity of AVP detected.

The data in table 18 shows the concentrations of AVP spiked into human plasma compared to the concentrations of AVP determined experimentally in the samples frozen for 24 hours then extracted. The data in table 18 shows that there is good accuracy and precision at both the 0.75 and the 10 ng/mL levels, which is indicated by the accuracy being within 8% of nominal spiked concentration. The precision of the six replicate extracts is also very close at bellow 5% for RSD at both levels. This data shows that samples would be stable if defrosted and left at room temperature for up to 24 hours and that they could be analysed (and re-frozen between analyses) up to four times.

Table 18

Stability of AVP in human plasma stored for 24 hours at room temperature

Replicate	0.75 ng/mL	10 ng/mL
	observed concentration (ng/mL)	
1	0.782	10.5
2	0.820	10.3
3	0.764	10.9
4	0.820	11.4
5	0.851	10.7
6	0.773	11.1
mean (ng/mL)	0.802	10.8
standard deviation (n-1)	0.0339	0.402
RSD (%)	4.2	3.7
accuracy (%)	106.9	108.0

The data in table 19 shows the concentrations of AVP spiked into human plasma compared to the concentrations of AVP determined experimentally in the samples frozen for 24 hours then extracted. The data in table 18 shows that there is good accuracy and precision at both the 0.75 and the 10 ng/mL levels, which is indicated by the accuracy being within 8% of nominal spiked concentration. The precision of the six replicate extracts is also very close at bellow 5% for RSD at both levels. This data shows that samples would be stable if defrosted and left at room temperature for up to 24 hours and that they could be analysed (and re-frozen between analyses) up to four times.

Table 19
stability of AVP in human plasma following four freeze/thaw cycles

Replicate	0.75 ng/mL observed concentration (ng/mL)	10 ng/mL observed concentration (ng/mL)
1	0.786	9.01
2	0.758	10.1
3	0.794	9.53
4	0.801	10.5
5	0.806	11.0
6	0.802	10.9
mean (ng/mL)	0.791	10.2
standard deviation (n-1)	0.0177	0.786
RSD (%)	2.2	7.7
accuracy (%)	105.5	102.0

3.2.4 Matrix effects

The effects human plasma had on AVP and AVP-D5 detection were assessed by analysing plasma which was extracted as per section 2.2 then spiked with a solution of AVP and AVP-D5 at 5 ng/mL prior to HILIC-UPLC-MS/MS analysis, and comparing the results to those obtained from a sample of water extracted as per section 2.2 which was spiked with the same AVP : AVP-D5 solution prior to analysis.

The results in table 20 show the extent of modification of ionisation response of the analyte in the presence of matrix varies very widely, ranging from 85.1 % to 177%

enhancement. This may be caused by the AVP in the absence of matrix adhering to the plastic container and therefore giving a lower result than in the presence of matrix or may be a genuine reflection of how AVP behaves in human plasma, meaning that there may be components in the matrix that alter the detected level of AVP by either suppressing or enhancing the signal. On analysis of unspiked plasma, no AVP was detectable. As these matrix effects are significant, they could seriously impact the performance of the study.

Table 20

Matrix effects – comparison of AVP levels in extracted blank plasma and water samples spiked with AVP and AVP-D5 post extraction at 5 ng/mL

blank matrix sample number[5]	AVP mean peak area for post spiked water extract (RSD %)	AVP mean peak area for post spiked blank plasma extract	percentage difference
1	46757	127203	172.0
2	(2.4)	86534	85.1
3		128228	174.2
4		122224	161.4
5		129703	177.4
6		98142	109.9

[5] There were six blank matrix samples, these are samples of blank matrix from one human plasma sample and the six samples are reflective of a population sample.

To compensate for any potential analyte extraction variability, an internal standard was used as previously described in chapter 1. The purpose of including an internal standard is to compensate for any variability in the assay including how different matrix samples – in this case from different people can affect the AVP levels quantified.

When selecting an internal standard, it was important to consider the impact matrix effects can have on the experiment. Analogues of the analyte are often used but do not always behave in the same way as the analyte. One way to account for matrix effects is to use a stable isotope labeled-internal standard [18].

The matrix effects for the internal standard were assessed and the results presented in table 21. The results shown in table 21 are consistent with those in table 20 as the percentage modification in the presence of unspiked human plasma ranges from 82.8-170.7% enhancement

Table 21

Matrix effects – comparison of AVP-D5 levels in extracted blank plasma and water samples spiked with AVP and AVP-D5 post extraction at 5 ng/mL

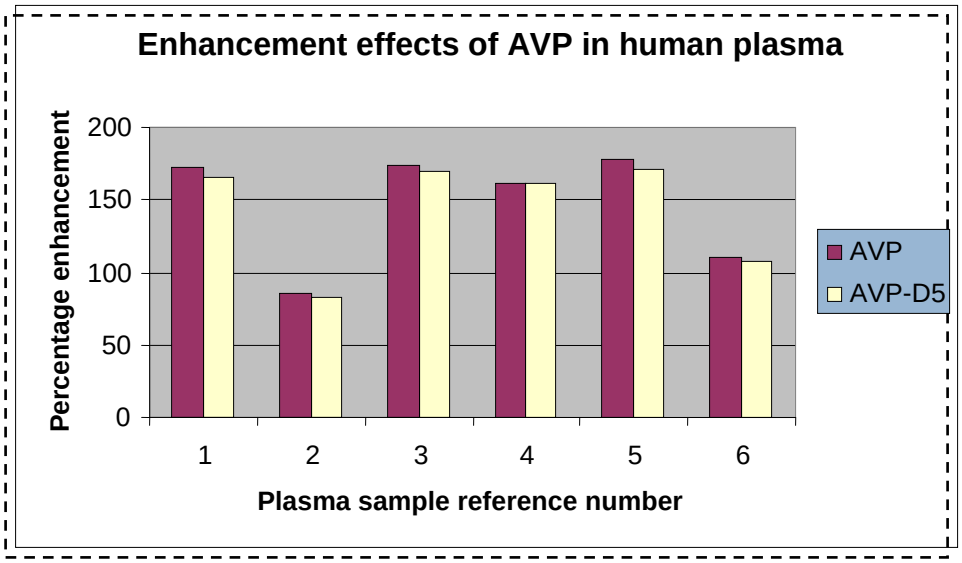
blank matrix sample number	AVP mean peak area for post spiked water extract (RSD %)	AVP mean peak area for post spiked blank plasma extract	percentage difference
1	3795131	10075356	165.5
2	(1.5)	6935912	82.8
3		10209229	169.0
4		9915660	161.3
5		10271622	170.7
6		7857274	107.0

Figure 41 shows a histogram comparing the percentage modification of AVP and AVP-D5 in each of the six plasma samples (from different donors) where close correlation of the percentage enhancement experienced by AVP and AVP-D5 in human plasma can be seen.

Ionisation suppression or enhancement experienced by the analyte in the matrix will also affect the internal standard and therefore be compensated for. It is also important to note that AVP and AVP-D5 co-elute and therefore experience the same conditions as one another when they enter the mass spectrometer.

The matrix effects in this study have been assessed and with the use of a stable isotope-labelled internal standard do not impact on the integrity of the study as the internal standard compensates for the enhancement effects that can be seen for AVP in human plasma.

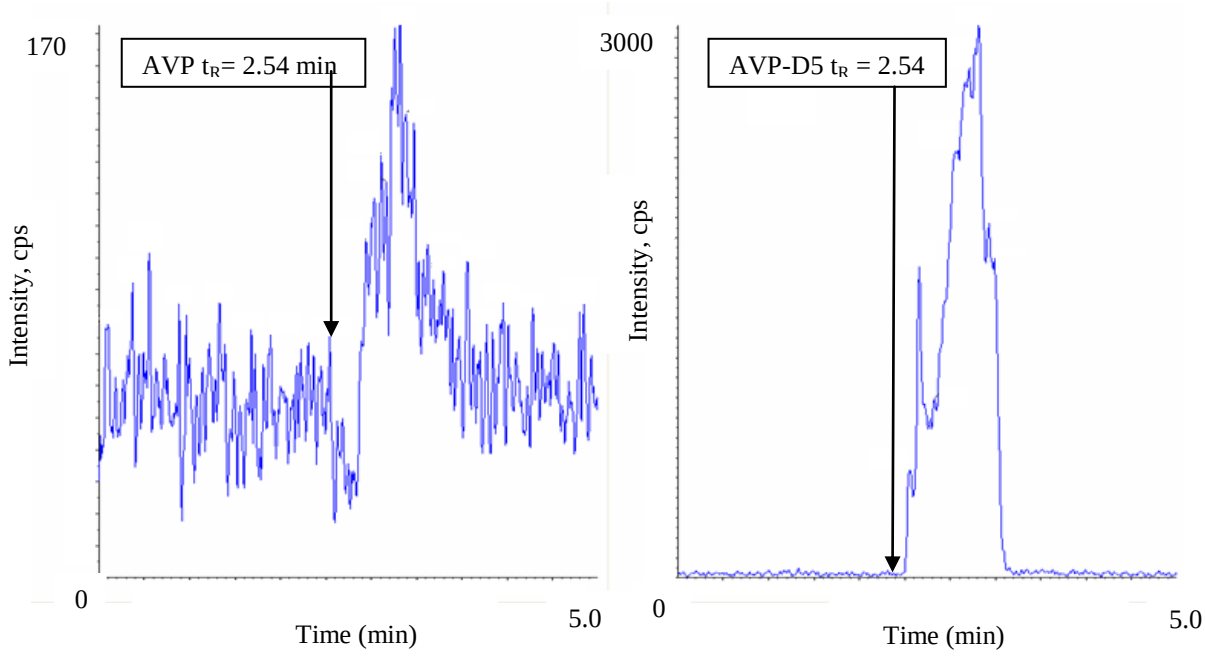
Figure 41 – Graph to show the normalisation of matrix enhancement and suppression of six samples of human plasma from different donors when AVP and AVP-D5 are added post extraction.



3.2.5 Selectivity

In this assay it was important to ensure there were no interferences with the analyte peak which is being quantified. This was achieved by screening six samples of blank human plasma from different individuals. There were no peaks in these samples at the retention time of 2.54 minutes for AVP or AVP-D5 which complied with the requirement of no blanks exceeding 20% of the LLOQ peak area ratio [16-18]. A sample chromatogram of an extracted unspiked human plasma sample is illustrated in figure 42

Figure 42- XIC for unspiked plasma sample monitoring AVP and AVP-D5 (for the transitions 543.4>328.2 and 545.5>328.2)



3.2.6 Recovery

In order to detect the smallest amount of AVP possible, the recovery of AVP was calculated. This was to determine how much was being lost in the extraction. To do this, maximum recovery, assuming no loss of analyte through the extraction was calculated for the concentrations 0.75, 5 and 10 ng/mL AVP and solutions of AVP and AVP-D5 prepared at these concentrations. This was added to unspiked plasma at the end of the extraction. The peak area of both AVP and AVP-D5 in these samples was compared to the peak area of the extracted AVP spiked frozen plasma samples at the levels 0.75, 5 and 10 ng/mL AVP and 5 ng/mL AVP-D5, these are shown in tables 22 and 23.

Table 22 shows recovery levels of between 55.1 and 67.6%. This level of recovery is not optimal and could be improved which would have a direct effect on the lowest limit of AVP which could be detected. The mean recovery over the levels is 60%. This shows consistency in the extraction as all three levels are within 7.5% of the mean.

Table 22

Percentage of AVP recovered from spiked frozen human plasma extracted as

AVP concentration (ng/mL)	mean peak area AVP (red %)	mean peak area (RSD %)	recovery (%)
0.75	6613.9 (24.4)	11500.1 (11.0)	57.5
5	35595.0 (12.6)	64626.0 (7.4)	55.1
10	74431.2 (11.8)	110086.6 (21.2)	67.6
Mean recovery			60.1

The results in table 23 shows that the recovery of the internal standard AVP-D5 is 55.9%. This is very close to that of the mean percentage recovery of AVP of 60.1% which is shown in table 22. This result is to be expected as the internal standard is a deutertaed form of the analyte, so should behave chemically in the same way throughout the extraction as the analyte. As recovery is consistent throughout the concentration range, a recovery value of less than 100% does not impact on the study, but will be later suggested as an area of further work.

Table 23

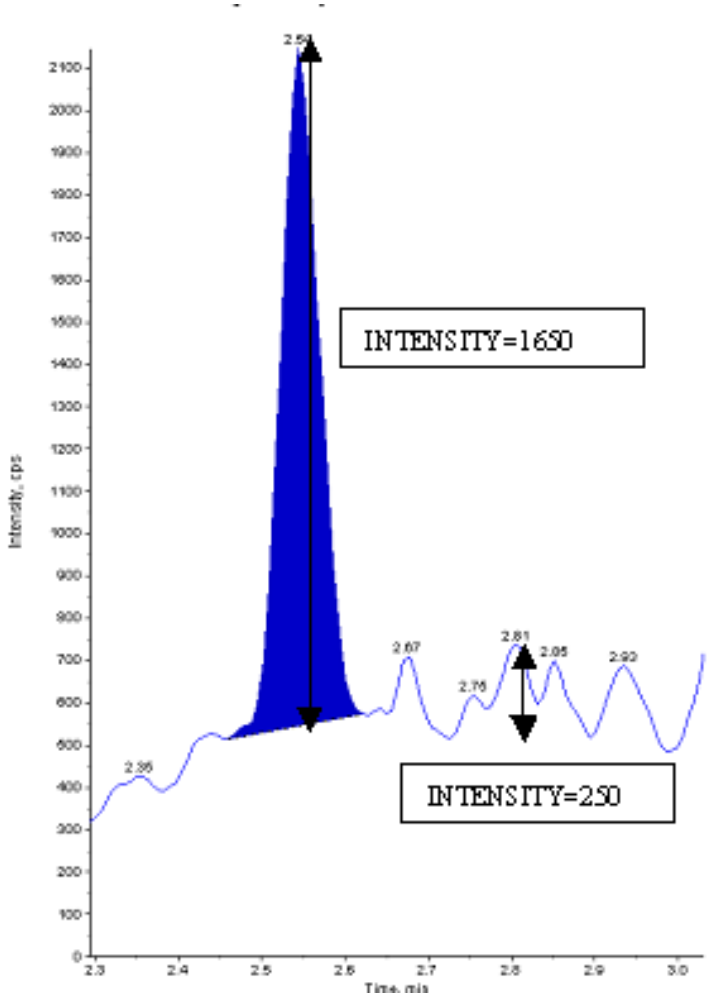
recovery of AVP-D5 from human plasma

AVP-D5 concentration (ng/mL)	mean peak area AVP-D5 (red %)	mean peak area post extraction AVP-D5 spike (RSD %)	recovery (%)
5	52514.3 (15.9)	94027.0 (14.9)	55.9

3.2.7 Lower limit of quantification

To assess the lowest amount of AVP which can be quantified using the assay, the signal-to-noise ratio of the lowest quantitation standard at the concentration 0.25 ng/mL was assessed. A chromatogram for the standard at 0.25 ng/mL is shown in figure 43. The AVP peak (t_R = 2.54 min) has an intensity of 1650 (cps) whereas the noise has an intensity of 250 (cps), so that the criterion of requiring the signal to have better than 5:1 signal: noise ratio [3,4] is fulfilled as this gives the ratio of 6.6:1.

Figure 43 – XIC (543.4>328.2) for AVP concentration 0.25 ng/mL



Chapter 4- Conclusion and further work

The aim of this thesis was to develop a bioanalytical assay for the development and quantification of the nona-peptide AVP in human plasma at the lowest quantifiable limit which can be validated.

In order to achieve these aims a method was developed which explored fractionation techniques using both reversed phase and mixed mode ion exchange solid phase extraction. The process of evaluating these included profiling to optimise analyte retention and recovery. The best results in terms of limit detection were using weak cation exchange chromatography techniques.

Separation techniques were also investigated in the form of ultra high performance liquid chromatography in two different modes – reversed phase and HILIC. In this assessment the lowest detection limits were obtained when using HILIC.

The mass spectrometric techniques used in this method included TurboIonSpray© source for optimal ionisation combined with MRM which utilizes the optimal duty cycle. The ions monitored were $[M+2H]^{2+}$ as the precursor and the y3 for the product ion. Tandem mass spectrometry gave this method selectivity over Immunoassays such as ELISA, this is due to the MRM being able to differentiate between the analyte and closely related metabolites which is not always possible in Immunoassay.

A technique interfaced with MS which was trialled was FAIMS. The aim was to investigate if the S:N was improved for the quantitative assay so that a lower LOD could be utilised. The results in chapter 3 show improved S:N at the LLOQ, however this was not sufficient enough to lower the LLOQ. Subsequently FAIMS was not used in the final quantitative bioanalytical assay for vasopressin.

The ultimate aim for a bioanalytical method is to ensure it is fit for use in sample analysis. To do this it must be validated. The validation which took place aimed to assess linearity, range, precision, accuracy, matrix effects freeze-thaw stability, room temperature stability, short term frozen matrix stability and recovery. The method complied with industry guidelines set out by the FDA, MHRA and EMEA [9-11]. This method would therefore be suitable for a regulatory bioanalytical study for the assessment of AVP in human plasma.

The levels quantified in this method were 0.25 -10 ng/mL. They do not compete with the 1 pg/mL levels that immunoassay offers. However as a more selective technique than immunoassay, with faster set up time, quantitative bioanalysis does still have its advantages. This method meets the requirement of an LLOQ of less than 0.5 ng/mL and is subsequently could be used as a screening method for elevated biomarker vasopressin levels. This could be used as a complementary technique to immunoassay which can achieve greater sensitivity but less selectivity.

Further work to improve this assay should focus on lowering the limit of detection. A proposed method for doing this is to improve the fractionation techniques and maximise recovery above 60%.

Chapter 5 - Bibliography

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