LC-API/MS IN DRUG METABOLISM AND PHARMACOKINETIC STUDIES

GREGG ANDREW IMRIE

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Abstract

The use of API interfaces with quadrupole mass spectrometers has been shown to give rise to good sensitivity, selectivity, and robustness for the interfacing of LC to MS. Since their introduction in the 1990s the technique has rapidly become widespread, but at the outset of this research programme, there were still a number of problems associated with it, particularly when dealing with complex sample matrices. The aim of this research programme was to study illustrative examples of the kinds of problems associated with the analysis of biological samples using LC-API-MS in an attempt to arrive at strategies which could be employed to eliminate, or at least compensate for, the problems.

Commonly reported problems include the occurrence of matrix effects - a change in response of the target analyte(s) as a result of the presence in the samples of co-/late eluting interferences. An investigation which compared ESI with APCI ionisation illustrated a significant drawback in the accepted methodology for the elimination of matrix effects.

Optimal LC conditions for a number of assays may use non-MS-friendly mobile phases. A simple and convenient solution to this problem was found to be the post-column addition of organic modifier, which reproducibly and reliably enhanced sensitivity. This approach was initially used for a range of dihydropyridine calcium channel blockers and was subsequently applied to a range of chiral compounds from different therapeutic

groups to illustrate that this was applicable as a generic technique for increasing sensitivity (typically by around an order of magnitude) in low organic mobile phases.

Strategies to develop and validate methods for the determination of endogenous analytes in a biological fluid were investigated. This involved the use of a surrogate matrix, to develop a method for the determination of endogenous testosterone in human serum and the use of non-matrix calibration standards for the successful development and validation of a method for the analysis of indolyl-3-acryloylglycine (IAG) in human urine.

As a result of observations suggesting promotion of ionisation of deltamethrin in liver tissue sample extracts, it was postulated that this was due to the presence of high concentrations of surfactants. After confirming the effect, a series of systematic investigations were performed to attempt to understand the mechanism to be able to utilise this as a general method for the enhancement of signal with low sensitivity analytes. It was found that the type of surfactant and concentration used was directly associated with an increased (or decreased) response.

Although there remain a number of problems associated with the use of LC-API-MS, the work undertaken for this thesis has successfully demonstrated a number of techniques that can be applied to overcome these problems. Knowledge of the nature of the sample undergoing analysis, the required analytical conditions, and where required careful application of one of the techniques described will ensure that a robust method can be readily developed.

Acknowledgements

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Abbreviations

AGP	α1–acid glycoprotein
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
APPI	Atmospheric Pressure PhotoIonisation
CFAB	Continuous-Flow Fast Atom Bombardment
CI	Chemical Ionisation
CID	Collisionally Induced Dissociation
CMC	Critical Micellar Concentration
CSP	Chiral Stationary Phase
DC	Direct Current
DLI	Direct Liquid Introduction
EI	Electron Impact (Ionisation)
EM	Electron Multiplier
ESI	Electrospray Ionisation
eV	Electron Volts
FAB	Fast Atom Bombardment
GC	Gas Chromatography
GC-MS	Gas Chromatography - Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IAG	Indolyl-3-Acryloylglycine
LC	Liquid Chromatography

LC-API-MS	Liquid Chromatography – Atmospheric Pressure Ionisation - Mass Spectrometry
LC-MS	Liquid Chromatography – Mass Spectrometry
LLOQ	Lower Limit of Quantification
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Triple Quadrupole Mass Spectrometry
3-NBA	3-nitrobenzyl alcohol
Q1	First Set of Quadrupoles in a Triple Quadrupole Mass Spectrometer
Q2	Collision Cell in a Triple Quadrupole Mass Spectrometer
Q3	Third Set of Quadrupoles in a Triple Quadrupole Mass Spectrometer
QC	Quality Control (sample)
RF	Radio-Frequency
SDS	Sodium 1-dodecanesulphonate
SIM	Selected Ion Monitoring
SSI	Sonic Spray Ionisation
TFA	Trifluoroacetic Acid
UV-vis	Ultraviolet-Visible

Chapter 1

Introduction

1.1 Analysis of Drugs in Biological Fluids and Tissues

There are a number of reasons why it is necessary to analyse drugs in biological fluids, including in forensic toxicology, when looking for the presence of potential poisons, etc. [1] and in the monitoring of drugs of abuse [2]. However, the most common reason for this kind of analysis is to assist various stages of the drug development process [3]. These include pharmacology, toxicology, metabolism and clinical trial testing.

The programme of work described in this thesis was principally concerned with the analysis of pharmaceutical preparations where the concentrations in fluids are required for developmental purposes. However, while the issues discussed principally involved illustrative examples of the determination of plasma and urine concentrations of known drugs, the range of examples covered extended to the analysis of an insecticide in animal tissues where similar problems may also be encountered.

The drug development process is both complex and time-consuming with many different disciplines involved in creating the overall strategy towards developing a successful drug candidate. The process involves selection of a drug target and some (*in vitro*) efficacy testing, before selection of leading candidates based on a range of disciplines including toxicology, pharmacokinetics and pharmacodynamics. These are followed by more wide-ranging and rigorous toxicological evaluations and then extensive clinical trials before any submission of a drug candidate is permitted by the relevant regulatory authority. A brief summary of the drug discovery and development process is illustrated in Figure 1.1, with the elements most relevant to this thesis falling under the “Pharma” heading.

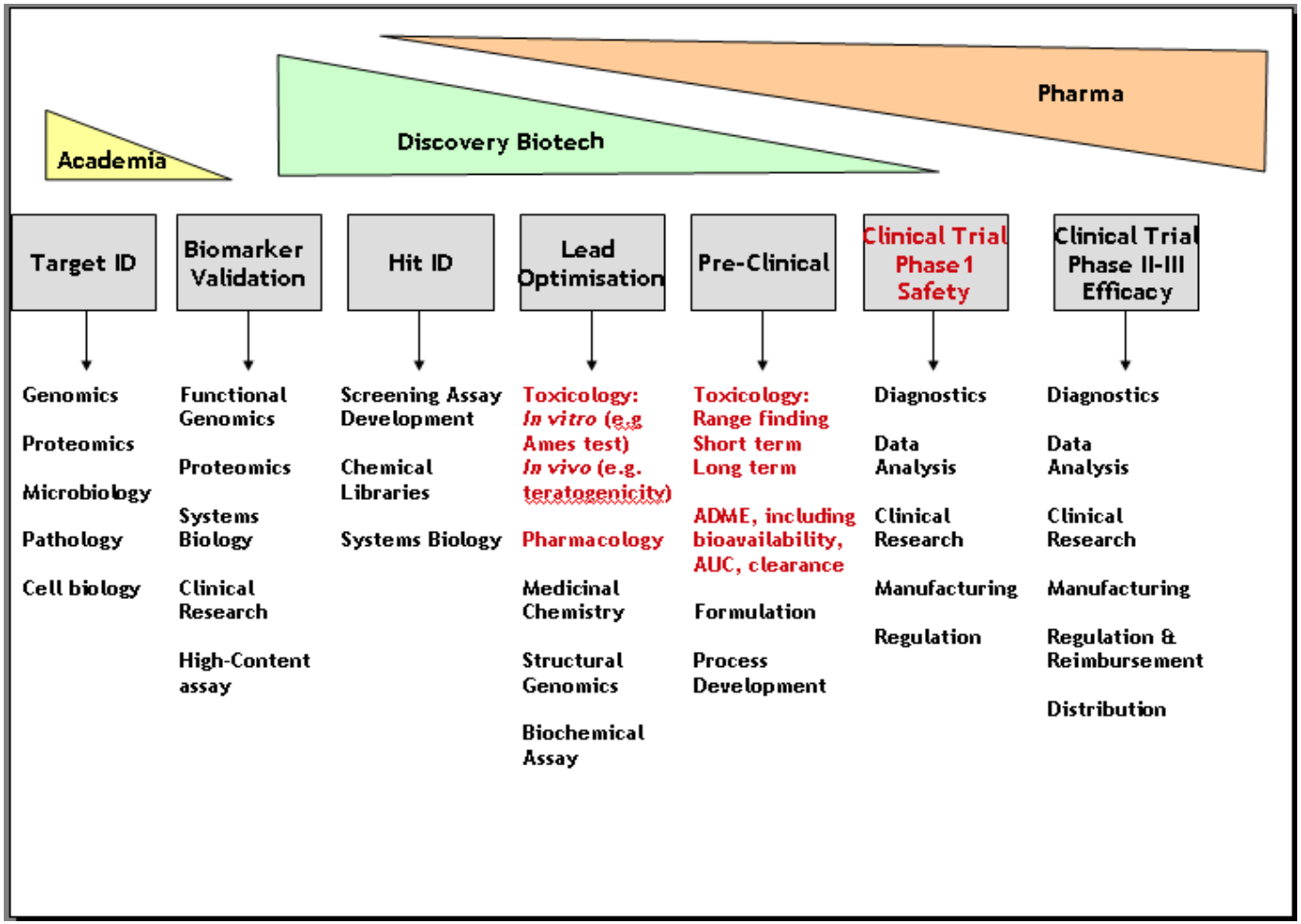


Figure 1.1 Schematic representation of the drug discovery and development process

As intimated already, drug bioanalysis is important in pharmacology studies. At an early stage of the drug development process pharmacological studies are performed in order to determine the plasma (or other relevant fluid or tissue) concentration that will produce the required pharmacological response. In many cases, at least as a simplified model for general use, the concentration of drug absorbed is directly proportional to the effect. Thus, at an early stage of development many drug analogues may be tested to determine their absorption. This will usually take the form of a single dose given to several animals with the plasma concentrations being mapped over a suitable time period. The analysis of these concentrations can provide information about the total absorption of the drug, which will generally be compared with other compounds that have similar chemical structures [4]. Thus the expected "active" part of the drug may be the same in all tests, with different side-groups attached to determine their effect in promoting or retarding absorption. Although low absorption may not initially appear to be a problem (as the dose could be increased for a poorly absorbed drug), in practice it is considered desirable to have as high a degree of absorption as possible, in order to provide a predictable behaviour model. For example, a drug with absorption of an average of 85% may, for example, vary from 80-90% between individuals, which represents a 12.5% variation in plasma concentration when comparing the lowest level to the highest. However a drug with absorption of an average of 8% may vary from 5-10% absorption, which represents a 100% variation in plasma concentration when comparing the lowest level to the highest. Thus, the more predictable behaviour of the highly absorbed drug is generally preferred to the poorly absorbed drug.

Once the pharmacological profile of the drug has been established (together with various *in vitro* safety tests) toxicology testing begins. This is essentially safety testing of the drug before it is allowed to be administered to man. In toxicology testing, the aim is to determine the level at which adverse effects occur, or to confirm that no adverse effects occur up to a certain concentration [5]. The main aim of the bioanalytical investigations performed as part of toxicology testing is to confirm the presence and concentration of the dosed drug (or a suitable product or marker compound).

Once a drug candidate is selected, based on a wide range of selection criteria from disciplines including molecular/cellular biology, chemistry, target specificity, cytological biomarker effects and drug metabolism [6], it then passes through the early stages of the toxicology testing process and moves on to clinical trials (i.e. testing in man). This initially involves Phase I Clinical trials [7]. These are safety tests only, i.e. using healthy volunteers to determine that the drug candidate does not cause any adverse effects at various concentrations, eventually rising to the intended therapeutic dose levels.

Metabolism studies to determine details of the absorption, distribution, metabolism and elimination of the drug are also carried out. These help to determine the metabolic pathways of the drug as well as providing information about the storage/accumulation or elimination of the drug, which help in the design of a therapeutic dosing regime. These are principally to help to justify the animal model chosen for the early toxicology work, by confirming the adsorption and distribution of the active part of the drug to the required therapeutic site [8]. Confirmation that the drug is not metabolised before having the

opportunity for efficacy is also provided by these studies. These studies will typically be performed around the same time as pharmacokinetic studies, where the absorption, distribution, transformation and elimination in man are investigated.

Formulation development is a key part of the drug development cycle which tends to take place at the same time as much of the Phase I and Phase II testing. Phase II clinical testing tends to involve confirmation of the kinetic profile of the drug in diseased subjects, (to confirm that the profiles seen in healthy volunteers are similar in patients).

There are a number of other trials performed over a similar time to some of the testing described above, including Proof of Concept and various other safety evaluations. Finally there are large scale (i.e. large numbers of subjects) clinical trials (some Phase II, but mainly Phase III) to provide substantive evidence for both safety and efficacy of the new drug candidate.

All of the above testing stages require the use of robust bioanalytical methods to analyse the samples from those tests.

1.2 Problems Associated with Biological Fluids

The biological fluids that are most commonly analysed for drug concentrations are plasma (or serum) and urine. In addition blood, saliva and many other fluids may be used depending on the chemical nature of the drug being analysed and the expected site of

action. All of these matrices are complex matrices, i.e. they contain not only the drug being analysed, but also many thousands of other compounds, which can impact on the analytical methodology employed [9].

The main problem associated with plasma or serum analysis is the presence of large quantities of protein [10]. Although the proteins will tend to have very different properties from the drugs being measured, there are frequently interactions between the proteins and drugs, which may impact on the analytical methodology. In addition there are large quantities of lipids in plasma, which can also impact on the analysis.

The main problems associated with urine are the presence of high concentrations of salts and the variation in concentration and content of urine which changes quite dramatically depending on diet, time of day, and various other factors. Urine has a wide range of pH values [11] which may affect the drug, for example by breaking it down into a degradation product [12].

1.3 Background

In order to appreciate the aims and objectives at the outset of the research programme described in this thesis, it is necessary not only to consider drug bioanalysis but also to address the fundamentals of triple quadrupole mass spectrometry: MS/MS created by connecting three quadrupole mass spectrometers in sequence. The first “instrument” is used for precursor ion selection, the second as a collision tube for CID (collisionally

induced dissociation), and the third for detection of fragment ions created by dissociation of precursor ions [13].

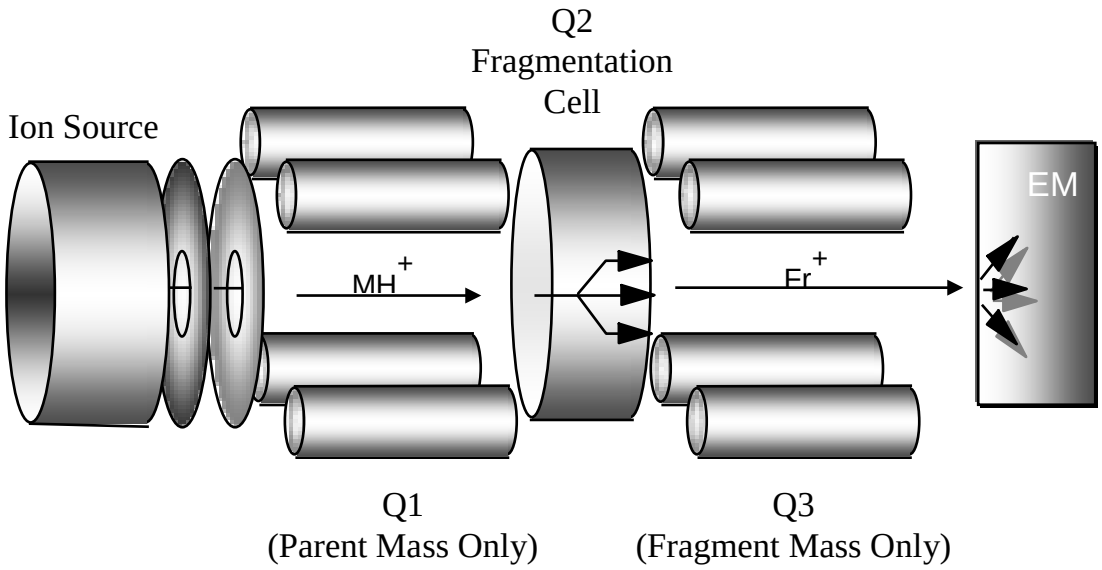


Figure 1.2 Schematic representation of a triple quadrupole mass spectrometer. Ions are generated at the ion source and drawn into the vacuum region of the mass spectrometer by a potential gradient. Initial separation of the parent ion occurs in the first set of quadrupoles (Q1), then this passes into a second set of quadrupoles which act as a fragmentation, or collision cell (Q2) to produce fragment ions which are separated in the third set of quadrupoles (Q3). These fragments then pass to an electron multiplier (EM) for signal enhancement prior to detection.

It is also necessary to be aware of the various existing approaches to overcoming typical analytical problems encountered in the use of high performance liquid chromatography (HPLC) with triple quadrupolar mass spectrometry for the analysis of analytes in biological samples and the challenges associated with these complex samples.

1.4 Hyphenated Techniques

The need to develop increasingly sensitive and specific analyses for organic compounds has grown significantly in recent years. Initially this was due to the advent of the "new" atmospheric pressure ionisation (API) techniques developed in the early 1990s [14] which enabled the analysis of compounds previously restricted to less specific, less sensitive or more complex techniques. Following the establishment of API LC-MS over the 1990s this requirement had increased due to a combination of the need to identify and isolate metabolites and the adoption of the technique for Drug Discovery work, where only low sample quantities are frequently available [5, 15, 16].

The techniques used for such analyses have generally been hyphenated techniques as the complex sample matrices require substantial separation of matrix components from the analyte to enable the development of robust and specific analytical methods [17, 18, 19]. For example, gas chromatography coupled with mass spectrometry (GC-MS) has been an excellent technique for the separation and quantitation of trace components in complex sample mixtures [20, 21], as well as in the confirmation of identity of unknown analytes [22].

A significant problem with GC-MS is that the analytes of interest must be volatile to be vapourised to allow separation in and elution from the gas chromatography column in the gas phase, prior to detection in the mass spectrometer. Accordingly the compounds of

interest must have a high degree of thermal stability to prevent degradation during separation and detection. Unfortunately, these requirements preclude the use of GC-MS for most polar and high molecular weight molecules. Critically, the majority of drugs in development over the last decade have fallen into one or both of these categories [23], with an increasing number of high molecular weight molecules being investigated due to advances in protein chemistry either as potential drug candidates [24, 25, 26] or, increasingly, as biomarkers that are used to determine the efficacy of drug candidates [27].

However, high performance liquid chromatography (HPLC) is usually performed at around ambient temperatures and is an effective technique for the separation of such polar molecules. Earlier detection techniques used in hyphenation with HPLC were based on absorbance of ultraviolet or visible radiation. This is a simple, reliable technique, but has the disadvantage of low sensitivity when the analytes have no significant chromophore (i.e. a group of atoms that absorbs light) present. In addition, there is low selectivity using this technique, in that analyte separation is based on retention characteristics which can be common among many completely different analytes. In the context of samples that come from complex matrices (such as plasma and urine) it is not possible to separate the desired analytes from all background interferences [17, 18, 28]; therefore additional resolving power needs to be added to the analytical technique being employed.

Fluorescence and electrochemical detectors can extend the sensitivity range of UV-vis detectors by two or three orders of magnitude [29, 30], if the compound exhibits (or can be made to exhibit) fluorescence or electroactivity. Such techniques also improve specificity, but are limited in range of application in that many compounds can not easily be made to exhibit these properties, without complex and potentially non-robust sample preparation methodologies.

Mass spectrometers provide both high sensitivity and selectivity. However, the problem with hyphenation of LC (a technique for the separation of complex mixtures that relies on the differential affinities of substances for a liquid mobile medium and for a stationary adsorbing medium through which they pass) and MS (an analytical technique used for separating ions or molecules that are dissolved in a solvent) was that mass spectrometers operate under high vacuum ($<10^{-5}$ Torr) and there is a requirement for the removal of the large volumes of mobile phase used in HPLC (liquid chromatography using tightly packed columns containing microspheres, where the mobile phase is pumped through the column with a high pressure pump) before MS analysis. In addition, in liquid chromatography the analytical species are generally not ionised in the liquid phase; whereas for mass spectrometric detection the analytes are required to be ionised in the gas phase. This results in a fundamental incompatibility between the two techniques.

Previous approaches for the combination of LC and MS have included use of the moving belt interface [31], direct liquid introduction (DLI) [32], particle-beam [33] and thermospray [32]. The main disadvantage with these techniques was that they required the removal of large amounts of solvent vapour from the vacuum system of the mass spectrometer. For example with the moving belt interface as illustrated in Figure 1.3 if evaporation is incomplete liquid passing into the mass spectrometer can cause significant pressure fluctuations leading to poor performance and reduced sensitivity.

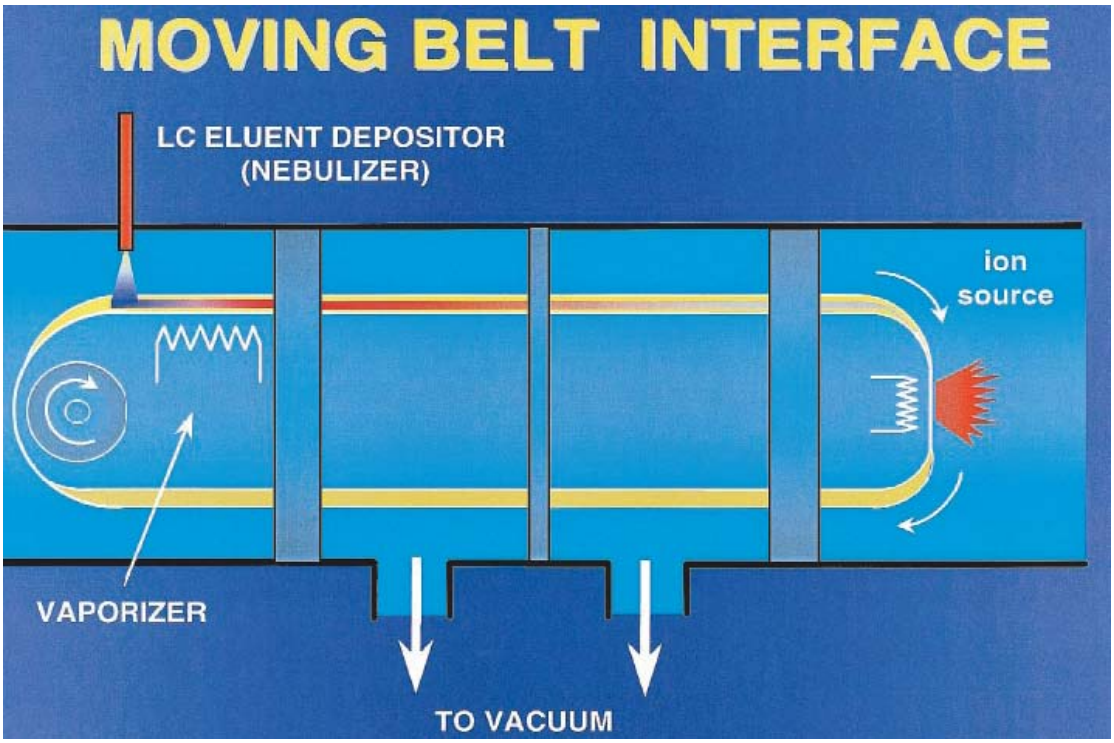


Figure 1.3 Schematic representation of moving belt interface [34**].**

1.5 Development of LC-MS Interfaces

The practical problems associated with the development of an LC-MS interface are much more severe than those associated with GC-MS interfacing. There are three particular difficulties:

a) high flow rates

b) involatile and labile samples

c) use of involatile buffers

The effective flow rates (i.e. in terms of vapour into the system) are much higher in LC than in GC and considerable energy input is required to separate the flow of solvent from the solute as vapour. A conventional source housing pumping system gives a pressure of 2.5×10^{-4} Torr when the flow rate of vapour into the system is of the order of 5-7 $mL.min^{-1}$.

With the exception of the specially designed Thermospray ion source (where the majority of the liquid flow is volatilised as it emerges from a heated capillary, Figure 1.4), all of the flow that enters the ion source to be ionised is then directed into the source housing to be pumped away.

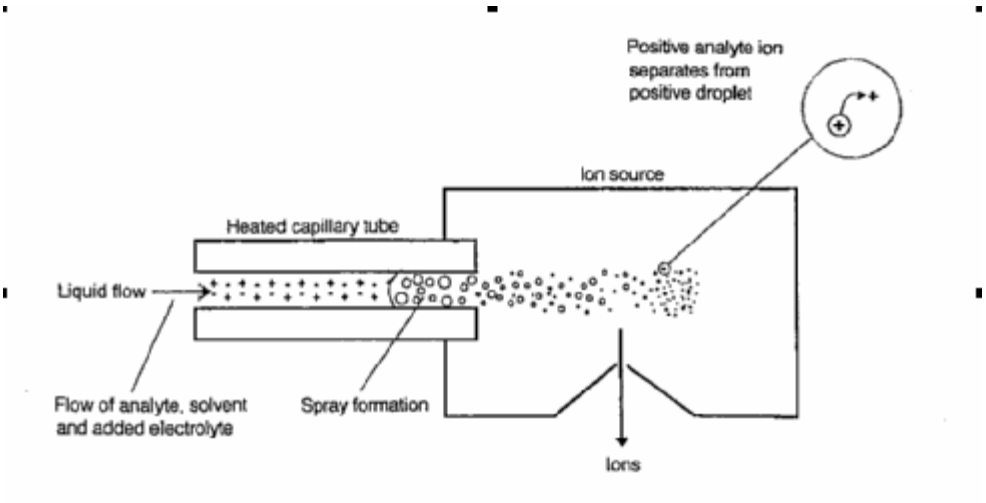


Figure 1.4 Schematic representation of thermospray ionisation process [35]. The analyte is ionised following ion-molecule reactions with reagent gas ions. The reagent gas ions are electrolytic ions generated when the sample solution is sprayed by heating from the end of a capillary tube under a low vacuum.

There were a number of possible solutions to the problem of restricted solvent flow rate that were available to the pioneers in the field of LC-MS as detailed below.

a) Splitting the flow from a conventional LC column and introducing only a fraction of this flow into the mass spectrometer. This course of action wastes a high proportion of the sample and is not acceptable if the sample quantity is limited [36].

b) Increasing the source housing pumping speed, for example (in the Sciex API III) by the addition of a cryopump. Under these circumstances, the maximum permissible flow rate may be increased.

c) Using low flow narrow-bore packed capillary (with a typical internal diameter of 1 mm [37]) or open tubular columns that eliminate the need for flow splitting [38].

d) Removal of the solvent prior to introduction of the sample into the mass spectrometer. This approach was used in the transport and particle beam interfaces, although the technique requires the sample to be at least partially volatile, and has limited tolerance to the kinds of buffers used in routine liquid chromatography [39].

e) Attaching additional pumping to the ion source itself, which can then accept much higher flow rates. This approach was used in the thermospray interfaces (Figure 1.4, [40]).

f) Ionisation at atmospheric pressure (Atmospheric Pressure Ionisation, API). This has the advantage that a curtain gas can be introduced to strip away solvent molecules prior to entry of the analyte(s) into the high vacuum mass spectrometer. Accordingly thermally labile compounds are more amenable to analysis using API techniques.

In many cases, the materials being analysed by LC-MS are relatively involatile and/or labile since they are generally compounds that are not amenable to GC-MS analysis. Conventional ionisation techniques such as electron impact* and chemical ionisation[#] are gas-phase ionisation techniques, which require thermal volatilisation of the sample. Although this limitation is acceptable for some compounds that can be analysed by LC, it is unsuitable for a larger number. For this reason, considerable use has been made of

liquid-phase ionisation techniques such as thermospray ionisation, continuous-flow fast atom bombardment** and electrospray ionisation [41].

*Electron Impact Ionization (EI): The sample is vaporised into the mass spectrometer ion source, where it is impacted by a beam of electrons with sufficient energy to ionise the molecule. This is a low energy process where the principal ions produced are protonated $[M+H]^+$ or deprotonated $[M-H]^-$ [42].

#Chemical ionization (CI) is a technique where the analyte is ionized by chemical ion-molecule reactions during collisions in the ion source. The reagent gas is ionised and this then ionises the analyte by charge transfer. As with EI, this is a low energy process where the principal ions produced are $[M+H]^+$ or $[M-H]^-$ [43].

**Fast atom bombardment (FAB) is a form of ionization where a fast moving beam of neutral atoms (typically Argon or Xenon at 8 keV) bombard a metal target coated with a liquid matrix (typically glycerol or 3-NBA) in which the sample has been dissolved. This is a soft ionization technique, i.e. does not result in significant fragmentation and produces mainly protonated or deprotonated molecules [44]. Continuous-flow FAB (CFAB) is a form of FAB in which the dissolved sample is introduced continuously to the sample probe [45].

Most LC-MS interfaces and/or ion sources do not accept the use of involatile buffer systems, as there is no simple way to prevent these from the entering the high vacuum

regions of the mass spectrometer, leading to contamination and loss of vacuum (and therefore sensitivity). Unless a transport interface is being used, volatile buffers based on materials such as ammonium acetate, ammonium formate, ammonium hydroxide, and acetic or trifluoroacetic acid should be used [46].

To sum up the situation, a number of different approaches have been used in interfacing LC and MS. The earliest attempts focused on methods of overcoming the incompatibility of the liquid flow rate and maintenance of the mass spectrometer high vacuum, while more recently (in particular since the development of the electrospray interface by Fenn and Yamashita in 1984 [47]), more attention has been focused on the practical use of ionisation techniques that do not require sample volatilisation.

1.6 Atmospheric Pressure Ionisation

The principal requirement for mass spectrometric analysis is to have the ability to ionise the analyte being investigated [48]. The degree to which the desired analyte ions are formed and the ability to discriminate the analyte ions from other, potentially interfering, species are the keys to providing the sensitivity and selectivity required to produce an acceptable analytical method using this technique. The design of the ion source is critical to the formation of the analyte ions and is therefore the key starting point for any mass spectrometric technique.

Development of atmospheric pressure ionisation (API) has resulted in a major improvement in the interfacing of LC and MS. In particular, ion-spray (pneumatically-assisted electrospray) [49] and atmospheric pressure chemical ionisation [50] (APCI) interfaces have been shown to be sensitive, selective, and most importantly, robust techniques for the interfacing of LC to MS. More recent developments include atmospheric pressure photoionisation (APPI) [51], which utilises a vacuum-ultraviolet lamp as a source of photons, and sonic spray ionisation (SSI) [52], which utilises a very high gas flow ("sonic", i.e. at the speed of sound) to deliver the analyte to the mass spectrometer. Both of these techniques have the advantage over APCI and ion-spray that high voltages or high temperatures are not required, which allows the analysis of thermally labile compounds.

Part of the success of API is due to the compatibility of the API interfaces with conventional LC conditions. For example, APCI can use the whole LC flow from conventional 4.6 mm i.d. columns (i.e. 1.0 $mL.min^{-1}$) for analysis. Meanwhile, ion-spray and the heated version of ion-spray, e.g. TurboIonSpray™ (PE Sciex), are compatible with the flows from microbore LC columns.

In API LC-MS, ions are formed from a liquid flow introduced into a source region maintained at atmospheric pressure; therefore the ion source contains sample ions together with solvent vapour and the ambient gas (usually nitrogen). The coupling of a liquid flow inlet to an API source offers some advantages over other approaches. For example, it avoids problems associated with the introduction of a liquid flow directly into

high vacuum. Again, it readily allows liquid chromatography systems, especially low flow systems such as capillary electrophoresis columns, to be operated under “normal” conditions, i.e. with the column exit at atmospheric pressure.

There are four forms of ionisation used in API sources for LC-MS:

a) Atmospheric pressure plasma ionisation

b) Electrospray

c) Ion spray

d) Sonic spray

Atmospheric pressure plasma ionisation consists primarily of atmospheric pressure chemical ionisation (APCI) and the relatively recent atmospheric pressure photo ionisation (APPI), introduced by Robb, Covey and Bruins in 2000 [53].

The APCI interface (Figure 1.5) uses pneumatic nebulisation to convert the liquid flow into droplets which are then swept by means of a sheath gas through a quartz tube heater to vapourise the solvent and analyte. The mixture of vapourised solvent and analyte flows towards the ion formation region where a corona discharge initiates chemical ionisation at atmospheric pressure (APCI) using the vapourised solvent as the reagent gas. These CI processes take place within a wall-less reaction region, which is defined by the gas flow in the source volume. Flow rates from 0.5 to 2 $mL.min^{-1}$ of solvent and the use of volatile or involatile buffers are permitted. Memory effects are minimised by the large-scale, open construction of the source volume.

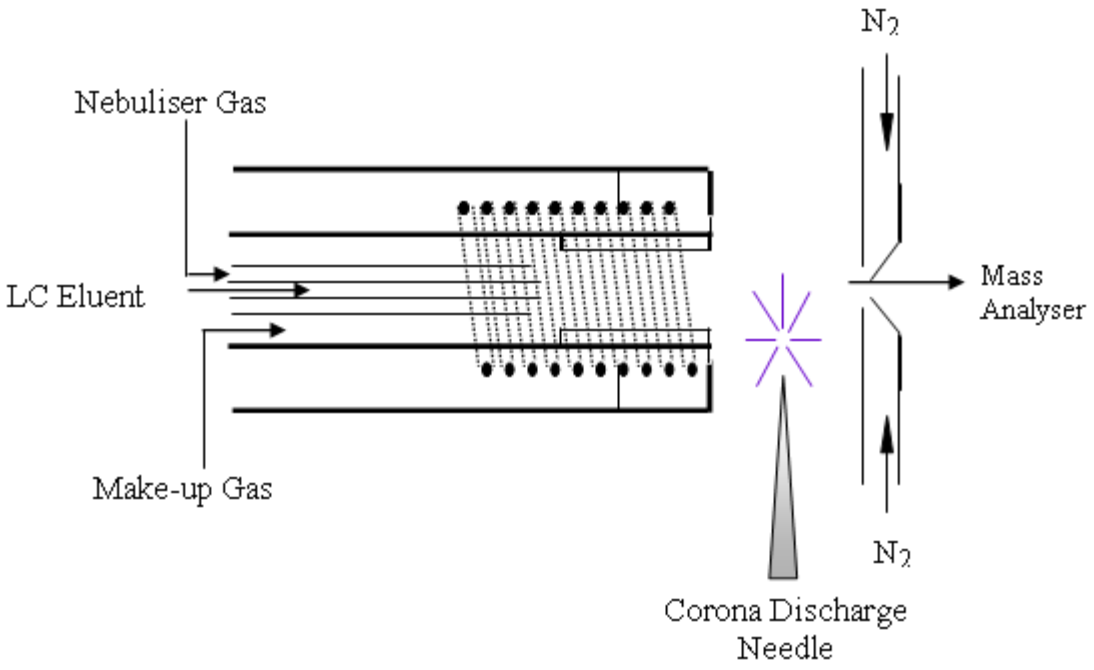


Figure 1.5 Schematic representation of Sciex APCI interface.

One method used in transferring ions from an APCI source into a mass analyser is illustrated in Fig. 1.5. Ions are driven towards a sampling orifice (approximately 125µm in diameter), that leads directly from atmospheric into the mass spectrometer vacuum system, by a combination of gas flow and electric fields. The flow of an inert barrier gas (nitrogen in the illustration) across the sampling orifice can be used to minimise the number of neutral solvent molecules that enter the vacuum system. The same barrier gas will also quench any ion-molecule reactions initiated by the corona discharge. With this sampling method, a very high pumping speed, provided in earlier generations of instruments by cryopumping and cryotrapping, and more recently using ultra-high speed Turbo pumps, is used to maintain a suitable operating pressure.

An alternative interfacing method for API sources uses a larger sampling orifice (approximately 200-300 μm in diameter), which leads into an intermediate vacuum stage. Successive vacuum stages may be used to improve the ion-to-neutral ratio in the stream which eventually enters the mass analyser by preferentially pumping away neutral molecules while ions are directed towards the next orifice by means of electric fields. As in all LC-MS interfaces, the neutral flow has to be reduced to a level at which the mass spectrometer pumping system can maintain a good working pressure.

In both electrospray and ionspray an electric field is generated at the tip of the sprayer by the application of a high voltage range directly on to the sprayer, with a counter electrode a few millimetres away.

IonSpray is pneumatically assisted electrospray, where the liquid carrying the analytical sample (i.e. the LC column effluent) is passed through a sprayer which is maintained at high voltage, in order to ensure that a mist of highly charged droplets is formed. As the droplets evaporate, ions are ejected into the gas phase by a low energy process that does not induce fragmentation [54].

A comparison of the three main atmospheric pressure techniques reveals differences in the type of compounds that can be analysed and the liquid flow rates that can be accommodated. The electrospray and ion-spray techniques are based on the desorption of ions from small droplets with virtually no heat input and are therefore very suitable for highly polar molecules. The heated nebuliser, on the other hand, depends on a gas-phase

ionisation process and is therefore only suitable for moderately involatile or labile molecules. The APPI interface is suitable for extremely non-polar compounds, and is ideal for the analysis of biological molecules such as steroids [55, 56].

Atmospheric pressure photoionisation utilises a very high gas flow ("sonic", i.e. at the speed of sound) to deliver the analyte to the mass spectrometer. The PhotoSpray^{TM} source utilises a vacuum-ultraviolet lamp as a source of 10 eV photons. A specially selected mobile phase additive, the dopant (e.g toluene [57]), is used to enhance photoionisation, by creating large numbers of ionised dopant molecules. This excess of charged dopant molecules created a cascade of ion-molecule reactions involving protonated solvent clusters as intermediate species leading to the final protonation of the analytes. This is the principal form of ionisation in the PhotoSpray^{TM} source and is typically more intense than in APCI for non-polar compounds. In addition, there is a secondary mode of ionisation within this source, which involves charge transfer directly from the ionised additive to the analyte.

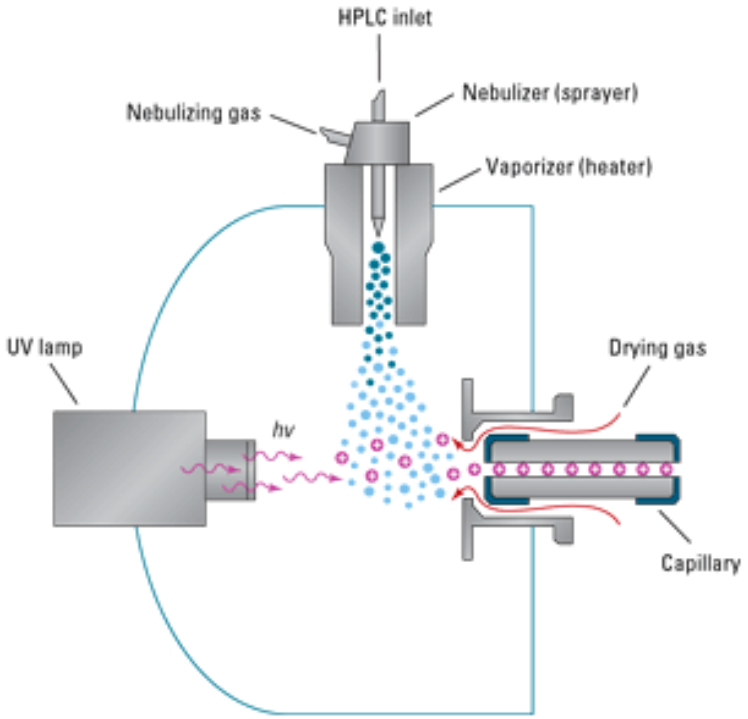


Figure 1.6 Schematic representation of atmospheric pressure photoionization. PhotoMate source, Agilent Technologies, www.agilent.com

An alternative interfacing method for API sources used a larger sampling orifice (approximately 200-300 µm in diameter [58]) which leads into an intermediate vacuum stage. Successive vacuum stages are then used to preferentially pump away neutral solvent molecules while analyte ions are directed towards the next orifice by means of electric fields. As in all LC-MS interfaces, the neutral flow has to be reduced to a level at which the mass spectrometer pumping system can maintain a good working pressure.

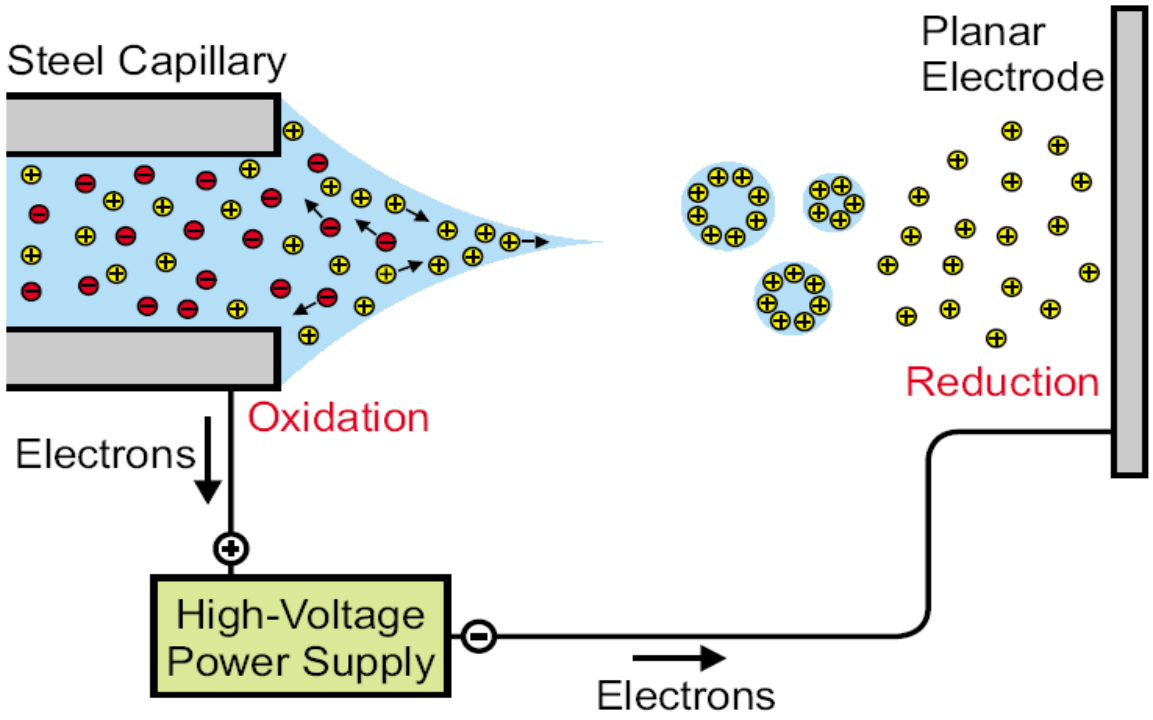


Figure 1.7 Schematic representation of electrospray ionization.

IonSpray is pneumatically assisted electrospray, where the liquid carrying the analytical sample (i.e. the LC column effluent) is passed through a sprayer which is maintained at high voltage, in order to ensure that a mist of highly charged droplets is formed. As the droplets evaporate, ions are ejected into the gas phase by a low energy process that does not induce fragmentation [59]. The liquid effluent is either infused at low flow rates, or more typically, split so that approximately 20 $\mu L.min^{-1}$ reaches the interface. Preformed ions in solution are desorbed from highly charged droplets by a process known as ion evaporation. The ion-spray interface is operated at ambient temperature, which results in a mild ionisation process, leading to molecular ion formation. Once formed, the ions are again passed through a curtain gas into the vacuum region of the mass spectrometer.

A commonly used modification of ion-spray is the TurboIonSpray^{TM}, which uses a heated drying gas to aid ion evaporation, thereby allowing higher solvent flows (up to 1 mL.min^{-1}) to the mass spectrometer.

Sonic spray ionisation generates molecular ions using a neutral mobile phase. Charge separation occurs around the surface of the solution, then vaporisation is enabled by passing a fast gas flow over the surface to produce electrically charged droplets. Although developed in 1994 [60], this technique has not seen widespread use to date.

Differences in the nature of the main atmospheric pressure techniques make them more or less suitable for both the ideal types of compounds that can be analysed by a particular technique and the liquid flow rates that can be accommodated by the technique. As electrospray and ion-spray are based on the desorption of ions from small droplets with minimal heat input, they are therefore ideal for highly polar molecules. The heated nebuliser, however, utilises a gas-phase ionisation process and is therefore only suitable for moderately involatile or labile molecules. The APPI interface is suitable for extremely non-polar compounds, and is ideal for the analysis of biological molecules such as steroids [55, 61].

Part of the success of API is due to the compatibility of the API interfaces with conventional LC conditions. For example, APCI can use the whole LC flow from conventional 4.6 mm i.d. columns (i.e. 1.0 mL.min^{-1}) for analysis. Meanwhile ion-spray and the heated version of ion-spray, e.g. TurboIonSpray^{TM} (PE Sciex) are compatible

with the flows from microbore LC columns. In API LC-MS, ions are formed from a liquid flow introduced into a source region maintained at atmospheric pressure. Therefore the ion source contains sample ions together with solvent vapour and the ambient gas (usually nitrogen). The coupling of a liquid flow inlet to an API source offers some advantages over other approaches. For example, it avoids problems associated with the introduction of a liquid flow directly into high vacuum. Again, it readily allows liquid chromatography systems, especially low flow systems, such as the output from a capillary electrophoresis interface, to be operated under "normal" conditions, i.e. with the sample exit from the initial separation technique at atmospheric pressure.

Current developments include a combined electrospray and APCI interface [62], which allows alternate electrospray and APCI scans with switching polarity possible within a single sample run. The ability to use both types of ionisation combined with both polarities of ionisation is expected to enable a sharp reduction in instrumentation time required for development of future methods, or when utilised for the analysis of complex compound mixtures. The requirement to optimise tuning parameters in either mode still exists, and whether there is a reduction in sensitivity in each mode remains to be established.

1.7 Quadrupole Mass Analyser

The principal type of mass spectrometers used for the quantification of drugs in biological fluids is the triple quadrupole mass spectrometer [63].

Ions from the source (interface) enter the mass analyser region under an accelerating potential of around 5-15 V. Pairs of rods have a two component voltage applied to them – one set have a standard DC potential, with the other set having an alternating radiofrequency (RF) component.

Ions follow an oscillating trajectory between the rods, with only ions of a specific kinetic energy passing between the rods and out the other end. All other ions collide with the rods. The selected ions enter a collision cell, where collisions with an inert gas lead to fragmentation. These collision cells are typically a second quadrupole, with only alternating RF present, to focus the ions. The ions enter the collision cell with a low energy (in the region of 100 eV). This low ion velocity and the relatively long ion path of a quadrupole collision cell increase the possibility of multiple collisions. The fragments (product ions) are then passed through a second set of mass selection quadrupoles so that only fragments of a particular kinetic energy emerge from the mass analyser to the detector.

The signal is usually then amplified prior to entering the detector region, e.g. using an electron multiplier, before being sent for detection and interpretation.

The specificity obtained by the use of the triple quadrupole mass spectrometer is particularly important when the original sample is in a complex matrix, containing numerous, chemically similar, compounds which would lead to high background noise, without the specificity generated by MRM. All samples from biological matrices fall into this category, whether urine, blood, plasma, or some other biological fluid or tissue. As these are the kinds of samples required for analysis in the development of pharmaceutical products, this covers a wide range of sample analysis types of relevance to the research programme described in this thesis.

Although these techniques can allow the analysis of a number of compounds that were previously unsuitable for mass spectrometric methods, there are large differences in the suitability of each technique to various compound classes.

1.8 Aims and Objectives

Although liquid chromatography with triple quadrupole mass spectrometry has become the pre-eminent technique used for the analysis of drugs in biological fluids, there remain a number of problems to be resolved, or reduced. The aim of the research programme was to investigate a number of the more common problems and determine ways to eliminate the problems, or reduce them to an acceptable level. The focus of the investigations was on the ionisation process itself.

Initially, the project focused on developing a range of novel approaches to some of the most scientifically challenging of the bioanalytical applications to more effectively exploit the significant advantages of LC-API/MS. The effects of co-eluting and late eluting compounds in samples produced from pharmacokinetic studies was investigated, to determine efficient and robust methodologies for reducing or eliminating the matrix effects that these compounds were responsible for. As part of this investigation, the mode of ionisation used was investigated, to determine whether some matrix effects may be alleviated using a simple change in ionisation mode.

Other studies included an investigation into the application of the technique of post-column modification of mobile phase, where the optimum chromatographic separation required a non-MS-friendly mobile phase to be used. The aim was to enable the development of a robust method, without changes to the initial chromatographic separation, thereby enabling an established separation methodology to be used whilst making the analytical instrumentation capable of operating at the required limits of detection.

The problem of developing robust and specific methods for the analysis of endogenous analytes is often encountered in the analysis of biological samples. Investigations were made using working examples to illustrate the suitability of several methods to overcome the problems created by this issue, including utilising a surrogate matrix and getting truly representative blank matrix to develop an assay.

Finally, as a result of observations made during previous work and with the knowledge gained of the mechanism of ionisation and associated factors which combine to provide a suitable method for analysis, an investigation into the addition of surfactants to samples in order to affect the response of target analytes was made, with the aim of increasing sensitivity for a specific analyte.

In each of the separate application studies, in order to properly investigate the issues discussed there was a thorough investigation into factors that affected the sensitivity, specificity, accuracy and precision of LC-API/MS in bioanalysis. This included an examination of the influence of factors such as matrix effects, sample preparation modalities, ionisation technique and mobile phase design. These issues of optimisation of experimental variables were embodied in the individual application studies.

The focus has always been on relatively easy to apply modifications of standard techniques in order to increase the applicability of these modified techniques for a wider range of analyte types.

Chapter 2

Modification of Analyte Response Due to Matrix Effects

As intimated previously, one of the most common problems encountered in the analysis of samples in biological matrices is as a result of so called “matrix effects”, i.e. modification of analyte response as a result of the presence of chemical components of the sample matrix.

There are two main causes of matrix effects in bioanalytical analysis using HPLC as the principal separation technique – those caused by co-eluting compounds and those caused by late-eluting compounds:

Co-eluting compounds: These are compounds with similar retention characteristics to the analyte(s) of interest, under the chromatographic conditions employed, which therefore elute from the sample separation stage at approximately the same time as the target analyte(s).

Late-eluting compounds: These are compounds that are more strongly retained on the retention medium under the chromatographic conditions employed (in HPLC - the stationary phase on the analytical column) than the target analyte(s). For example if the target analyte has a retention time of 3 min, with a total sample run time of 5 min, then a late-eluting compound that elutes after 8 min will co-elute with the target analyte after the first sample.

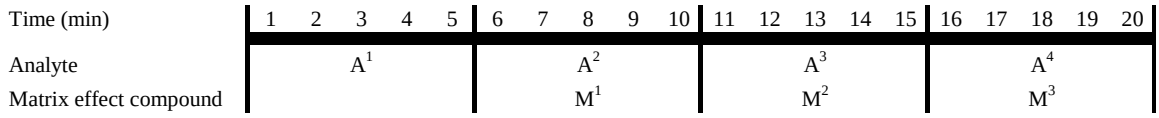


Figure 2.1 Comparison of elution time of target analyte (A) and potential late-eluting interferent (M). Assumes a run time of 5 min, with no pause between samples. The superscript number indicates the sample injection number, i.e. M^1 is the matrix effect compound elution time from injection 1, which co-elutes with the target analyte elution time from injection 2 (A^2).

In this example (Figure 2.1) the co-elution occurs from the second sample onwards. With the relative retention times selected for this example co-elution occurs for all samples after the first, but it could just as likely be from the 3rd, 4th, 5th, etc. In addition, the co-elution need not necessarily occur on all samples after a particular time point. This is one of the main reasons that some methods can suffer from matrix effects in early development as it is not always apparent that there is a problem with only a few samples

injected. A further problem with late-eluting interferents is potential cumulative effects (Figure 2.2).

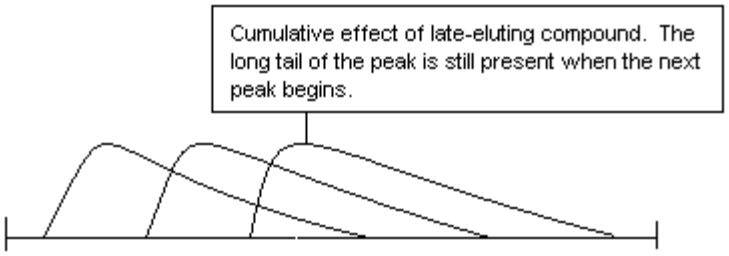


Figure 2.2 Cumulative effect of late-eluting compound. The matrix effect-inducing compound illustrated has a long chromatographic tail, which in successive injections falls under the "main peak" of the compound. This will lead to an increase in the matrix effect observed through the course of a chromatographic run. In the example shown the cumulative effect is not highlighted.

Essentially both types of interferences to an assay are similar in both cause and effect – the presence of interfering compounds causes a change in response of the target analytes in the mass spectrometer, which makes the development and validation of robust and accurate analytical methods difficult. The majority of matrix effects result in suppression of the analyte signal, e.g. as discussed by Annesley [64], although much less usually in some cases the presence of these interfering species can cause enhancement of the analyte response [65]. The critical problem associated with matrix effects is the lack of

robustness of the method, as the degree of suppression (or enhancement) would be expected to vary considerably from sample to sample.

In addition the same effect can be the result of the "dosing vehicle effect" [66], the dosing vehicle being the agent that serves as a carrier used to disperse or solubilise the test item.

This is an increasingly common problem where the vehicle used for (particularly) intravenous dosing causes modification of ionisation of the target analytes [67]. The main problem with this is that it is an effect that is not typically considered during the early stages of the development and validation of a method, which typically occurs in isolation to the vehicle selection process. This type of effect is most often only detected when using a stable isotopically-labelled internal standard within the analysis. In full method development it is important to consider this as a possible effect and carry out a small amount of additional development prior to the analysis of valuable (both ethically and financially) samples.

In addition, during the Discovery stage of drug development it is usual to perform only limited method development. At this stage significant matrix effects caused by the dosing vehicle can even result in the rejection of perfectly viable drug candidates at an early stage as their bioavailablity may appear to be low due to poor "recovery" compared with other candidate drugs [68]. In Discovery investigations care should be taken to minimise any vehicle effects that may lead to rejection of viable drug candidates [69].

Strategies for the elimination, or at least control, of these effects are discussed in the following sections, for different types of problems encountered.

As illustrated in Figures 2.1 and 2.2 late-eluting interferences occur as a result of compounds which are retained on the HPLC stationary phase for a longer period than the target analyte(s).

There are four main approaches to dealing with compounds associated with late-eluting interferences that may normally be adopted:

a) Greater sample clean-up to remove the interfering compounds from the samples [70]. Overall, this is always preferable as the "cleaner" the sample the more robust the final method is likely to be. There are a number of reasons to not adopt this strategy including limitations on the chemical nature of the target analyte (which preclude very specific sample preparation methodologies), time constraints on developing the method and the requirement to analyse multiple target analytes of differing chemical natures. All of these may make greater sample clean-up impracticable without significant material loss, which may be a significant factor, particularly when the available sample volumes are limited.
b) Modification of the mass spectrometric conditions [71] (including, if necessary, the mode of ionisation being used) to reduce the effects that the late-eluting interferents have on the analytes of interest. The mass spectrometric conditions will routinely be optimised for the target analyte(s), but sometimes the conditions that produce the

greatest response in solutions of the analyte(s) are less optimal when using extracted samples. Mass spectrometric conditions are routinely optimised using a "pure standard solution" (i.e. a solution containing only a suitable solvent and the target analyte). Re-optimising the mass spectrometer conditions with pooled extracted samples is an additional step that, although time-consuming, can potentially remove or reduce any problems associated with the sample matrix.

c) Modification of the chromatography to ensure that the interferents no longer elute at the same time as the analytes of interest [72]. The effectiveness of this strategy is dependent on the differences in chemical properties between the target analyte(s) and the potentially interfering species. This can be a particularly difficult strategy to employ if the analysis is for multiple analytes, which require chromatographic separation (e.g. structural or optical isomers), as any attempt to remove the potential interferents will impact on the required chromatographic resolution.

d) Use of a post-sample gradient to ensure that the late-eluters are removed from the analytical column before the introduction of the next sample [73]. This is perhaps the simplest strategy to employ, as the only significant negative factors are an increased run time and increased use of solvents, etc.

In practice, the methodology employed to eliminate, or control matrix effects, is usually a compromise between the four available methods, to reduce the interferences as comprehensively as possible, whilst minimising method development time and maintaining relatively short sample analysis times (compared to traditional techniques such as HPLC).

The simplest method to try is modifying the mass spectrometric conditions; in particular, this might often involve changing the mode of ionisation to APCI.

Previous work involved the validation of a method for the analysis of a new drug candidate undergoing investigation in dog plasma in accordance with recognised international standards [74]. This validation exercise involved confirmation that the assay being described was precise, accurate and robust over the range investigated.

In the course of developing a method for the analysis of the same compound in dog urine, the intention was to use the same methodology, where possible, for analyst convenience and, it was assumed, in order to minimise the time required to develop a robust analytical method. Accordingly, with the exception of the sample preparation method, all steps of the method applied were initially identical, i.e. the chromatographic and mass spectrometric conditions were identical. Initial results showed poor precision and varying accuracy in all quality control samples. Quality control samples consisted of six replicates at each of five concentrations across the planned linear concentration range for the assay.

The presence of late-eluting interferents had not initially been detected using the now common method of post-column infusion of the compound whilst injecting matrix blanks. This was because the isocratic mobile phase conditions, which were optimised for the new drug candidate, only resulted in allowing these late-eluting interferents to

enter the mass spectrometer, and therefore be detected, approximately one hour after injection.

Included in the earlier investigation (producing poor precision) were “matrix effect samples”, which consisted of six separate individual sources of matrix (i.e. urine taken from six different dogs) which was spiked to three separate concentrations at the low end, middle and high end of the planned concentration range. Normally a matrix effect is illustrated by poor precision for these samples (as they consist of different matrices, therefore potentially different quantities of interfering species); however with late-eluting interferences which elute so long after the target analyte, these samples may not (and in this case did not) highlight the problem (i.e. they were as inconsistent as the other quality control sample sets). Often, poor precision for quality control samples may be as a result of system effects such as partial blockage of the LC or mass spectrometric system. However in this case the internal standard (which was similar in structure to the target analyte) produced a consistent response throughout the run; therefore system effects could be discounted. It was thus suspected that a matrix effect caused by late-eluting interferences was the cause.

In order to confirm that the differences in response of the matrices was a real effect and not as a result of inconsistent spiking or sample preparation, a solution of the target analyte was infused through a tee-piece at the same time as a blank injection of extracted matrix was injected onto the chromatographic system, with the sample run time increased to 2 hours. The response of the analyte was seen to be acceptable (i.e. no significant

change in response) at the analyte retention time; however after approximately one hour under the conditions used, there was a significant drop in analyte response for a period of approximately 5 minutes. This confirmed that the matrix effects observed were as a result of late-eluting interferences, which were only detected after a large number of samples were injected (run time for plasma method using the same isocratic conditions was 6 min).

Of the main strategies for the elimination of such interferences previously described it was decided to try to eliminate the matrix effects by the simplest means possible, i.e. by changing the mode of ionisation from pneumatically assisted electrospray to atmospheric pressure chemical ionisation (APCI). (The sample preparation involved relatively specific sample clean-up using mixed-mode solid phase extraction cartridges; therefore it would have been expected to take a long time to eliminate the interferences by modifying the sample preparation methodology). Although in some cases APCI may be susceptible to significant matrix effects [75] it is generally considered to have reduced and in many cases no matrix effects [68]. The change from pneumatically assisted electrospray to APCI often reduces or eliminates matrix effects as the mode of ionisation is completely different; therefore the chemical properties that are responsible for causing the matrix effect may not be relevant. In general, ion suppression in electrospray ionisation can be considered to be caused by the presence of non-volatile solutes in the mass spectrometer source spray, which alter the droplet solution properties, thereby altering the response that would be expected for the target analyte [76].

This simple change, with no effects on the sample preparation methodology or chromatographic separation reduced the effects that had previously been observed to negligible proportions. Although change of mode of ionisation was sufficient to eliminate this suppression of ionisation as a result of late-eluting interferents, an additional chromatographic step was added, consisting of the introduction of a rapid gradient after the time of the elution of the analytes of interest. Although at first glance this may appear a superfluous step, it was included to ensure that as many of the interferents as possible could be removed without significantly affecting analysis time. If these interferents were not removed it was considered a possibility that, over the course of multiple analytical batches, a build-up of late-eluters could occur, which might result in matrix effects even after the change in mode of ionisation to APCI.

As the isocratic conditions for the sample analysis utilised acetonitrile - ammonium acetate (40:60, v/v); introducing a rapid gradient to 100% acetonitrile over 1 min, then leaving at 100% acetonitrile for 1 min resulted in an increased run time of 4 min (including time to re-equilibrate to the original conditions). This increase from a sample run time of 6 min to 10 min although undesirable from an efficiency standpoint was considered to be easily acceptable as an additional safeguard to the validation of a robust analytical method for the required analysis.

The use of these two small modifications to the validated (in plasma) method allowed the new method (in urine) to be validated to the same rigorous internationally recognised standards.

This simple approach meant that only one or two additional days of development time was required to solve the problems encountered with the method, rather than several weeks, which would be more likely if the sample preparation methodology and overall chromatographic conditions had to be radically altered, by effectively restarting the development as a completely new method (rather than as a modification of an existing method).

2.1 An Approach to Dealing with Matrix-Related Suppression of Ionisation caused by Co-Eluting Interferences: The Influence of Ionisation Mode on Ion Suppression in LC-MS/MS – A Comparison Between Electrospray and Atmospheric Pressure Chemical Ionisation

2.1.1 Introduction

Liquid chromatography-tandem mass spectrometry has become the current principal analytical technique for the quantitative analysis of drugs and drug metabolites in biological matrices [16, 35]. Use of tandem mass spectrometry provides a highly selective methodology; therefore theoretically sample clean-up and chromatographic separation can be reduced compared to other less selective methodologies. However there are still a number of potential problems with the techniques, with one of the most significant being ion suppression [64, 77]. As discussed earlier, the principal causes of ion suppression are the presence of coeluting, or less-commonly, late-eluting compounds in the prepared sample. The presence of these compounds results in competition with the desired analyte ions for the surface of the droplet undergoing ionisation in electrospray [78], or direct competition for charge in APCI [75].

The presence of ion suppression can be evaluated in several ways, but is most commonly investigated by infusion of a solution of the test compound(s), which is added into the eluent stream post-column *via* a tee-piece [77]. Samples of blank matrix are injected

under normal chromatographic conditions and the effect on the response of the infused compound(s) monitored. Theoretically the infused compound should produce a straight line of response over time, but typically in practice there is a significant drop in response which corresponds to the void volume of the analytical column and there may be other changes in response (usually reductions, though less typically the signal may be enhanced in certain regions). The number and degree of these changes in response tend to be strongly related to the degree of sample clean-up used in the sample preparation method [79]. Thus “dilute and shoot” methods (i.e. methods where the sample matrix undergoes simple dilution with a suitable solvent, usually a buffer, before direct injection into the LC/MS system) will produce the largest number of response changes, followed by protein precipitation, with more specific clean up strategies such as solid phase extraction producing the fewest response changes.

Use of the above methodology enables the determination of the presence, absence or (semi-quantitatively) the degree of suppression or enhancement of ionisation of the target compound(s). This allows the decision to be made as to whether this is a significant enough issue to require further method development. In order to eliminate or at least control such matrix effects the principal choices for method adjustment are enhanced sample clean up [80]; alteration to chromatography, to separate the interfering species in the matrix from the analytes of interest [81]; and the use of an effective, ideally radiolabelled, internal standard.

Although it has been shown that there are significant differences in matrix effects between the two most common methods of ionisation in LC-MS/MS, i.e. electrospray and atmospheric pressure chemical ionisation [78, 82], changing mode of ionisation in order to reduce or eliminate matrix effects does not seem to be as widespread as would be expected [83, 84].

An investigation to establish a robust method for the determination of a new drug candidate in human urine was required. During the course of the method validation matrix effects were initially evaluated as required by regulatory guidelines [74]; however some limitations of the most common method for evaluating matrix effects were observed and further work was required to eliminate the problems identified. Building on previous experience it was decided to investigate whether changing the mode of ionisation would reduce the level of ion suppression encountered to an acceptable level (i.e. to have a low enough effect to enable the "matrix effect" samples to pass the acceptance criteria as required by the regulations being followed).

2.1.2 Experimental

2.1.2.1 Materials

The new drug candidate (target compound) and its primary metabolite were supplied by the Sponsor, the internal standard was supplied by Sigma-Aldrich (Poole, Dorset, UK). All reagents were of analytical grade and were supplied by Fisher Scientific UK

(Loughborough, Leicestershire, UK). A Hypersil BDS Cyano column (50 x 4.6 mm, 5 µm) was supplied by Thermo Electron Corporation (Basingstoke, Hampshire, UK). Plasma was supplied by B&K Universal (Aldbrough, Hull, UK). Urine was supplied in-house and 96-well mixed-mode solid phase extraction plates containing HCX-3 were supplied by Argonaut (Hengoed, Glamorgam, UK).

2.1.2.2 Instrumentation

The system used for this work consisted of a PE Series 200 Micro-pump (Perkin Elmer, Thornhill, Ontario, Canada), an online DGU-14A degasser (Shimadzu, Kyoto, Japan), and a PE Series 200 Autosampler (Perkin Elmer, Thornhill, Ontario, Canada) connected to a Perkin Elmer API 4000 triple quadrupole mass spectrometer. Chromatographic system control, data acquisition and analysis were performed by means of Analyst software version 1.2 (Applied Biosystems – MDS Pharma, UK).

2.1.2.3 Chromatographic Conditions

The target analyte, which was a basic and relatively non-polar drug undergoing investigation for a novel (and currently confidential) use, its principal metabolite (which was a demethylated version of the parent and is therefore more polar than the parent) and the internal standard, were separated from matrix components using a Hypersil Cyano 50 x 4.6 mm, 5 µm analytical column. The mobile phase was acetonitrile - ammonium acetate (10 mM; pH 4) (35:65, v/v) (pH adjusted using acetic acid), delivered at a rate of

1.0 mL.min^{-1} through the analytical column. The retention times were approximately 1.5 min for the target analyte, 1.3 min for the principal metabolite and 3 min for the internal standard.

2.1.2.4 Sample Preparation

A 250 µL sample was added to a polypropylene tube. Twenty microlitres of working internal standard solution were added to each sample except the blanks. The samples were then buffered with 1.0 mL of ammonium acetate (50 mM; pH 6), vortex mixed and then centrifuged.

An Isolute HCX-3 solid phase extraction plate was conditioned using 1 mL methanol, and then equilibrated using 1.0 mL of ammonium acetate (50 mM; pH 6). The samples were then added to the plate and a very low vacuum applied to slowly load the sorbent material. The plate was then washed sequentially with 1 mL of ammonium acetate (50 mM; pH 6), 1 mL of acetic acid (1 M) and 1 mL of methanol and then dried. The samples were eluted into a polypropylene collection plate using 400 µL of ammonia - methanol (5:95, v/v) and then evaporated to dryness under nitrogen at 40°C. The residue was re-dissolved in 200 µL of ammonium acetate (10 mM; pH 4) – acetonitrile (50:50, v/v). The plate was then sealed, vortex mixed gently and centrifuged, then submitted for LC-MS/MS analysis.

2.1.2.5 Mass Spectrometric Detection – Starting Conditions

A Perkin Elmer API 4000 triple quadrupole mass spectrometer was used in positive ion, multiple reaction monitoring mode using a pneumatically and thermally assisted electrospray interface (TurboIonSpray) with the drying gas at 450°C. The mass spectrometer voltage settings i.e. the source voltage and the voltage at the interface and at all stages of the ion optics, were optimised for the target analyte, its principal metabolite and the internal standard.

2.1.2.6 Post-Column Infusion for Assessment of Matrix-Related Modification of Ionisation

A solution containing the target analyte, its principal metabolite and the internal standard was added to the eluent from the analytical column *via* an infusion pump, which was connected to a flow splitter (Figure 2.3), while the mass spectrometer was scanning the selected ion transitions for the three analytes.

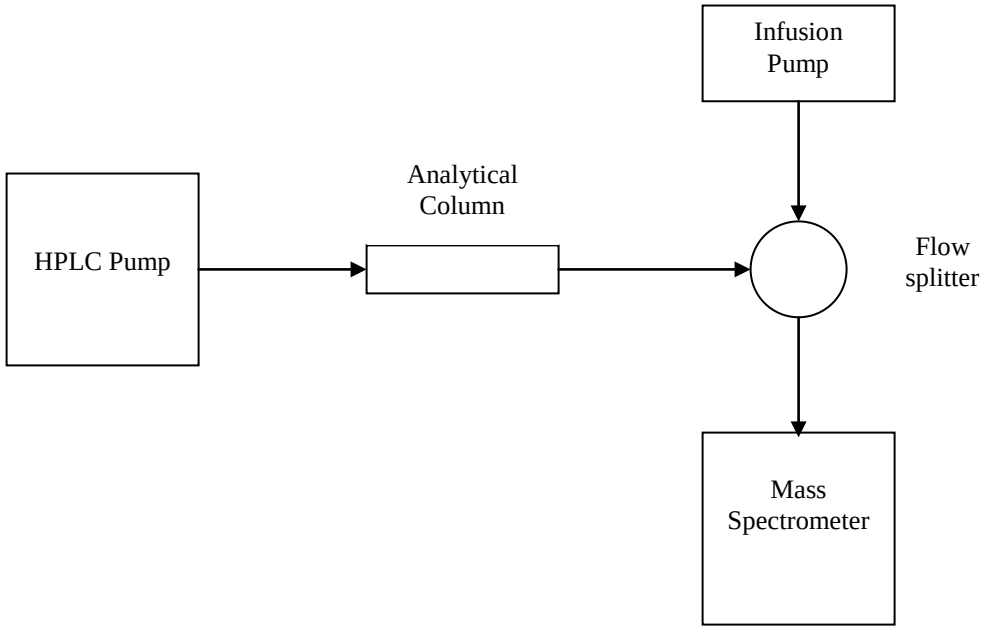


Figure 2.3 System configuration for post column infusion system. HPLC system PE Sciex API 4000 and PE Series 200 pumps. Column Hypersil Cyano 50 x 4.6 mm, 5 µm particles. Mobile phase acetonitrile - ammonium acetate (10 mM; pH 4) (35:63, v/v), flow rate 1 $mL.min^{-1}$. The analyte or metabolite was added to the system by infusion of a 1 $\mu g\ mL^{-1}$ solution at approximately 10 $\mu L.min^{-1}$ (i.e. approximately 100 $ng\ min^{-1}$).

2.1.3 Results and Discussion

The method described had been validated (by the author) for the same analytes in human plasma, using TurboIonSpray ionisation. The same method was applied to human urine and during development it was initially shown that there were no significant matrix effects present. However although this was performed using the almost ubiquitous

method of post-column infusion of the analytes of interest with injected matrix blanks, there were subsequently major problems within the validation process.

During method development prior to formal validation, the modification of ionisation experiment had taken the standard form of post-column infusion of the analytes of interest, with several injected matrix blanks. These injections suggested that the regions of suppression of ionisation were separated from the analyte retention times and that matrix-related modification of ionisation was therefore not a problem with this method (Figures 2.4 and 2.5).

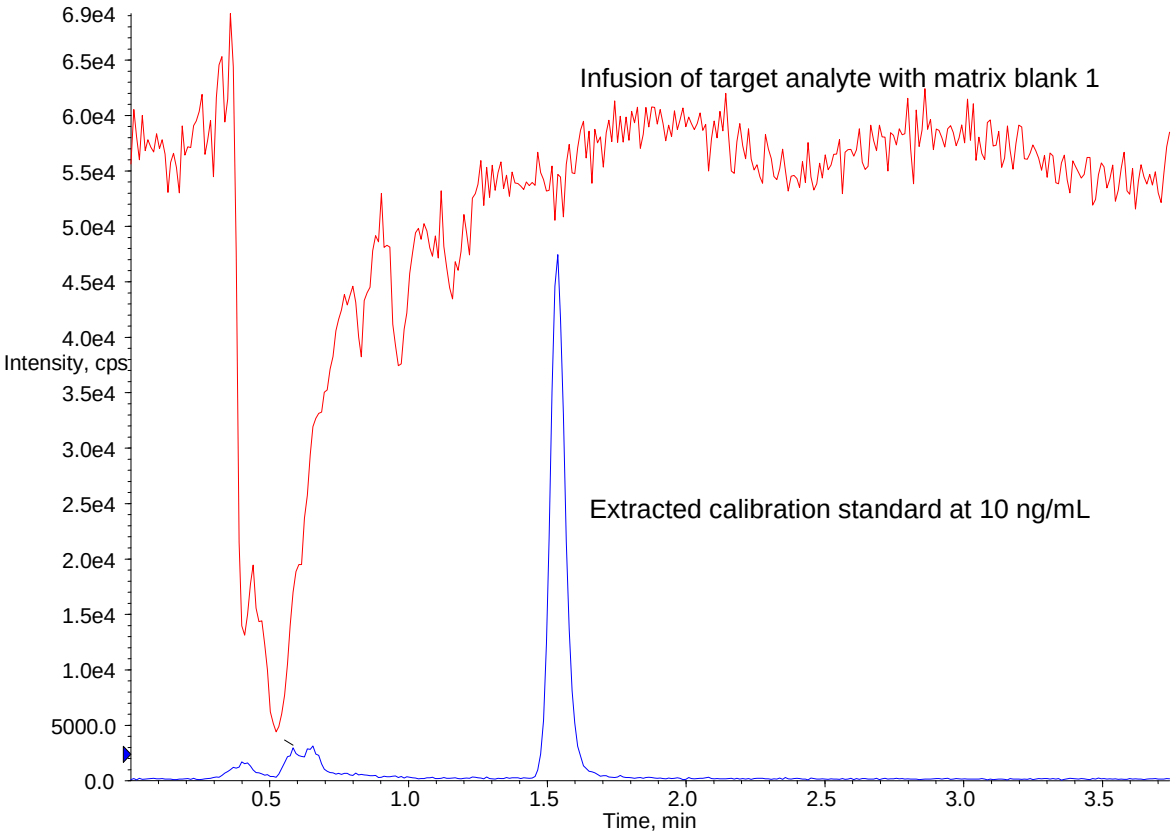


Figure 2.4 Ion suppression profile for matrix blank 1 (target analyte) using TurboIonSpray. HPLC system: PE Sciex API 4000 and PE Series 200 pumps, SIM for the target analyte. Column Hypersil Cyano 50 x 4.6 mm, 5 µm particles. Mobile phase acetonitrile - ammonium acetate (10 mM; pH 4) (35:65, v/v), flow rate 1 mL.min^{-1}. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 1 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time.

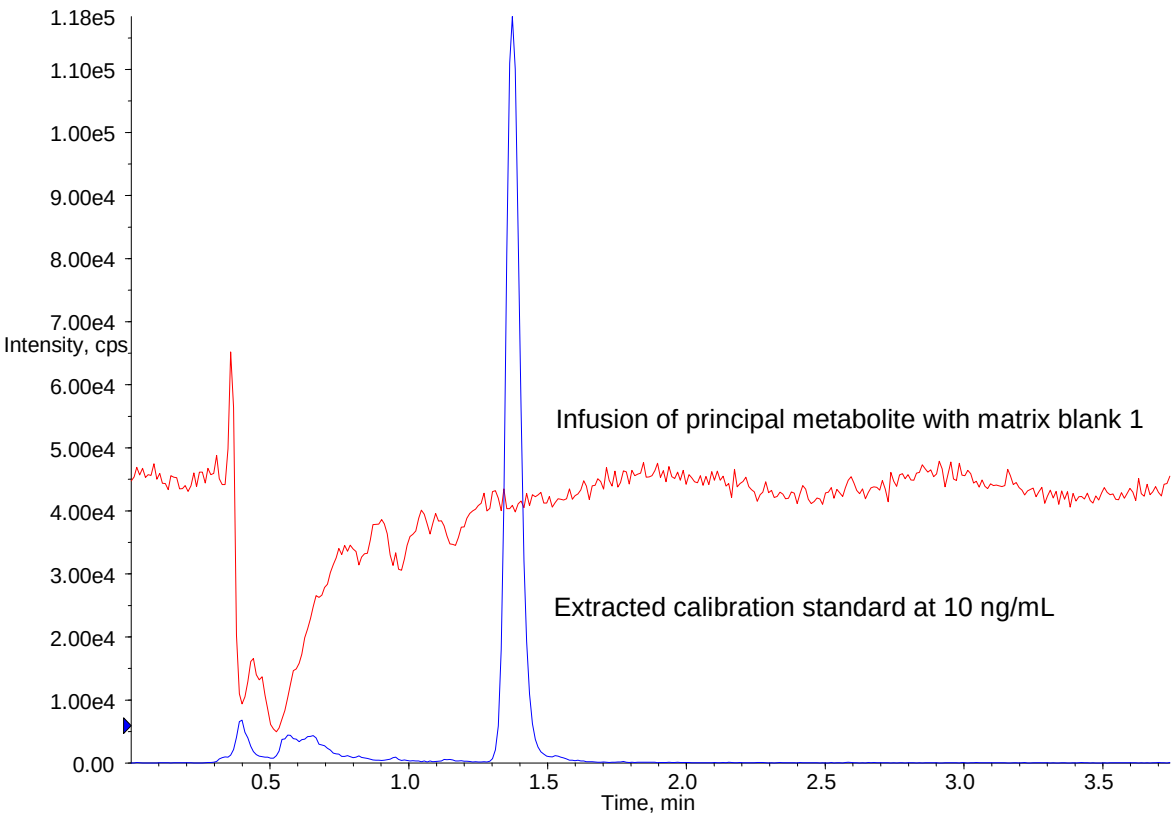


Figure 2.5 Ion suppression profile for matrix blank 1 (principal metabolite) using TurboIonSpray. HPLC system: PE Sciex API 4000 and PE Series 200 pumps, SIM for the target metabolite. Column Hypersil Cyano 50 x 4.6 mm, 5 µm particles. Mobile phase acetonitrile - ammonium acetate (10 mM; pH 4) (35:65, v/v), flow rate 1 mL.min^{-1}. The metabolite was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 1 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the metabolite retention time.

However, as part of the formal validation six individual matrices were spiked at concentrations at the LLOQ and at a mid-calibration level and prepared according to the method. It was found that two of these matrices (here numbered 4 and 6 in Table 2.1) provided results that were outside the required acceptance criteria for the principal metabolite, in that due to ion suppression these two matrices produced extremely low accuracies, which resulted in the overall accuracy and precision (measured by coefficient of variation) being outside the required range at each concentration of 85-115% for accuracy and ≤15% for precision.

Table 2.1 Accuracy and precision for primary metabolite of individual matrix effect samples analysed using TurboIonSpray ionisation.

Nominal concentration (ng/mL)	Control matrix ID	Observed concentration (ng/mL)	Accuracy (%)	Mean accuracy (%)	Coefficient of variation (%)	n
0.100	1	0.106	106.0	80.1	38.6	6
	2	0.0899	89.9			
	3	0.0875	87.5			
	4	0.0236	23.6			
	5	0.105	105.0			
	6	0.0683	68.3			
25.0	1	26.6	106.4	91.4	24.2	6
	2	26.7	106.8			
	3	26.0	104.0			
	4	13.2	52.8			
	5	25.5	102.0			
	6	19.1	76.4			

Although the target analyte had provided acceptable results which suggested that the sample preparation was performed correctly, in order to rule out any analytical errors in the preparation of the matrix effect samples, a further run was performed, using the same six matrices, but with three replicates of each matrix at each concentration. Each of the three replicates was distributed through the run, i.e. one near the start, one near the middle and one near the end of the run.

These results confirmed the original results (Table 2.2), i.e. there were differences in response between several of the individuals investigated, the agreement of the replicates for each individual being satisfactory.

Table 2.2 Accuracy and precision for primary metabolite of multiple replicates of individual matrix effect samples analysed using TurboIonSpray ionisation.

Nominal concentration (ng/mL)	Control matrix ID	Observed concentration (ng/mL)	Accuracy (%)	Mean accuracy (%)	Coefficient of variation (%)	n
0.100	1	0.107	107.0			
		0.0893	89.3	95.2	10.8	3
		0.0892	89.2			
	2	0.0903	90.3			
		0.0918	91.8	89.9	2.3	3
		0.0877	87.7			
	3	0.0850	85.0			
		0.0868	86.8	87.4	3.1	3
		0.0903	90.3			
	4	0.0275	27.5			
		0.0321	32.1	28.0	14.0	3
		0.0243	24.3			
	5	0.100	100.0			
		0.0989	98.9	97.7	3.2	3
		0.0941	94.1			
	6	0.0488	48.8			
		0.0588	58.8	51.1	13.4	3
		0.0457	45.7			
25.0	1	24.8	99.2			
		26.5	106.0	102.3	3.4	3
		25.4	101.6			
	2	26.0	104.0			
		27.1	108.4	107.1	2.5	3
		27.2	108.8			
	3	26.7	106.8			
		26.3	105.2	103.9	3.6	3
		24.9	99.6			
	4	17.0	68.0			
		16.4	65.6	66.5	1.9	3
		16.5	66.0			
	5	26.8	107.2			
		25.4	101.6	103.1	3.5	3
		25.1	100.4			
	6	20.0	80.0			
		20.1	80.4	81.2	2.1	3
		20.8	83.2			

Accordingly it was determined that the original post-column infusion experiment should be revisited.

The six individual matrices were extracted according to the method detailed above and were then injected onto the chromatographic system with the analytes infused post-column, as illustrated in Figure 2.3. The first matrix was the one that had been used for the calibration standards and quality control samples – all of which had originally provided extremely accurate results during the validation. This again confirmed that there was no matrix effect present for this individual matrix. However as is indicated by the specimen suppression profiles illustrated in Figures 2.6 - 2.11, there was a huge amount of inter-individual variation in the suppression profile seen using this method. In particular, the region of suppression in matrix 4 is highlighted, thereby confirming the reason for the low accuracies detected for this individual matrix. For this matrix it is illustrated that the target analyte was eluting just after a significant suppression region (Figure 2.10), but the principal metabolite was eluting on top of a region with significant ion suppression (Figure 2.11).

These results illustrated the need to investigate a number of individual sources of matrix to determine the presence/degree of matrix related modification of ionisation.

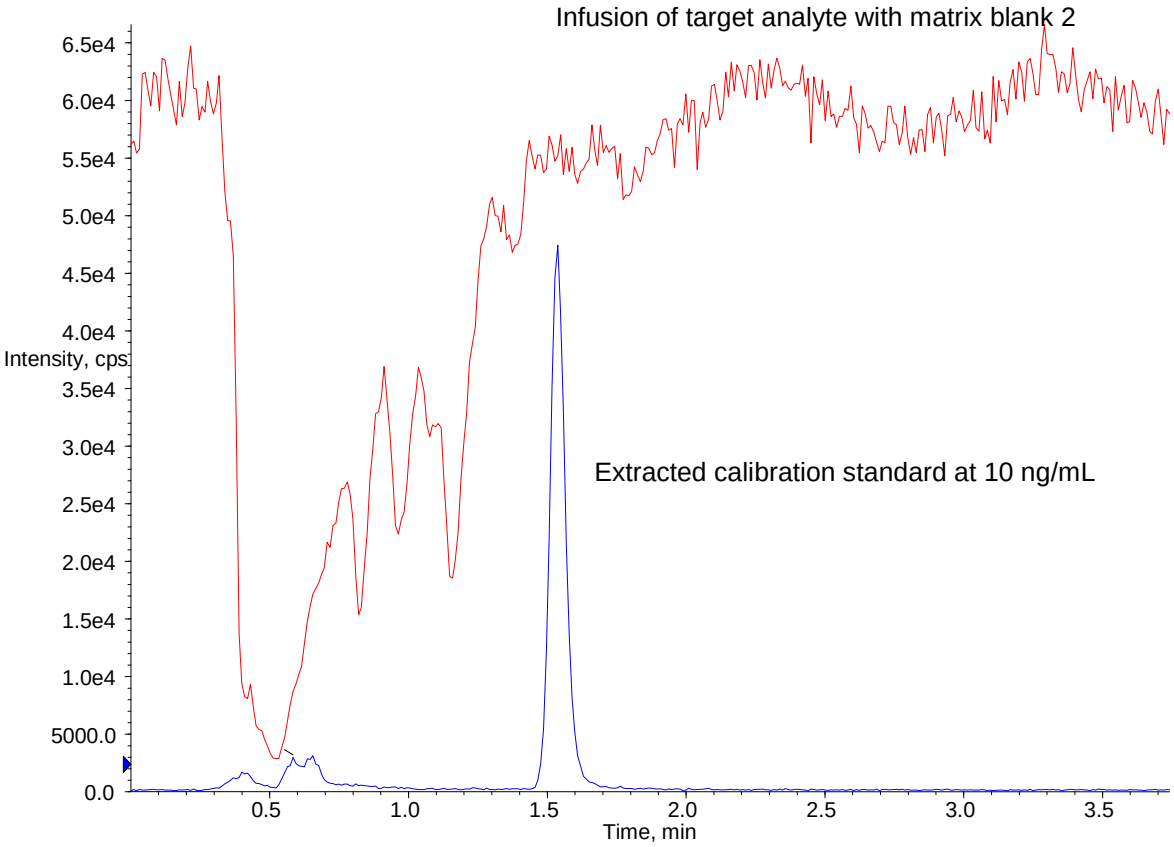


Figure 2.6 Ion suppression profile for matrix blank 2 (target analyte) using TurboIonSpray. HPLC system: PE Sciex API 4000 and PE Series 200 pumps, SIM for the target analyte. Column Hypersil Cyano 50 x 4.6 mm, 5 µm particles. Mobile phase acetonitrile - ammonium acetate (10 mM; pH 4) (35:65, v/v), flow rate 1 mL.min^{-1}. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 2 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time.

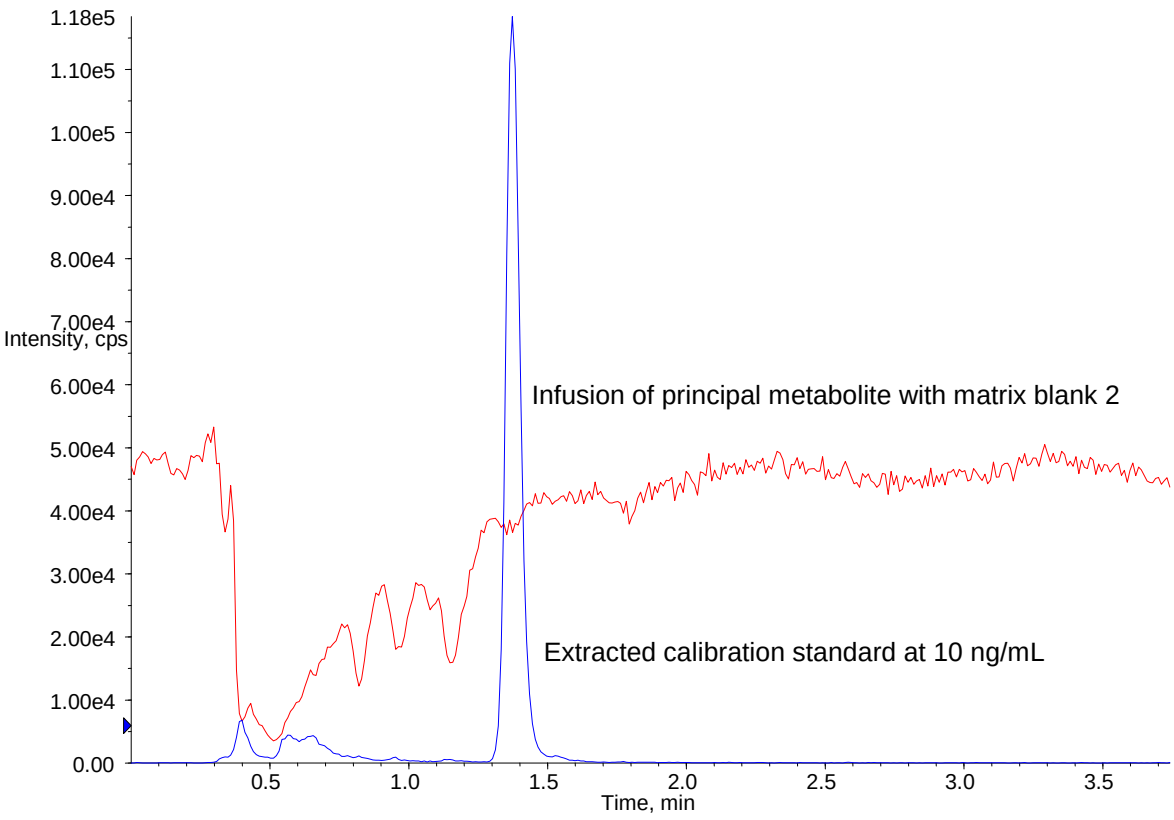


Figure 2.7 Ion suppression profile for matrix blank 2 (principal metabolite) using TurboIonSpray. HPLC system: PE Sciex API 4000 and PE Series 200 pumps, SIM for the principal metabolite. Column Hypersil Cyano 50 x 4.6 mm, 5 um particles. Mobile phase acetonitrile - ammonium acetate (10 mM; pH 4) (35:65, v/v), flow rate 1 mL.min^{-1}. The principal metabolite was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 2 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (just) before the metabolite retention time (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore little or no ion suppression would be expected at the metabolite retention time.

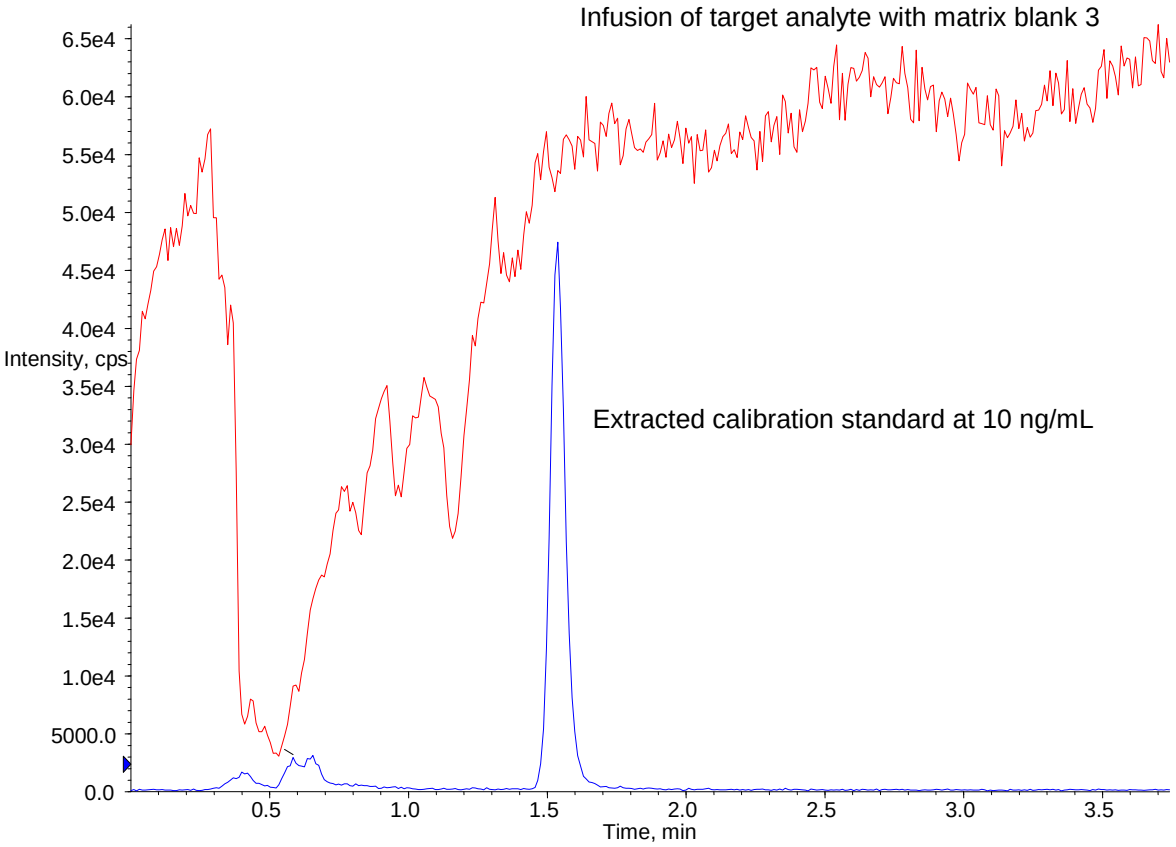


Figure 2.8 Ion suppression profile for matrix blank 3 (target analyte) using TurboIonSpray. HPLC system: PE Sciex API 4000 and PE Series 200 pumps, SIM for the target analyte. Column Hypersil Cyano 50 x 4.6 mm, 5 µm particles. Mobile phase acetonitrile - ammonium acetate (10 mM; pH 4) (35:65, v/v), flow rate 1 mL.min^{-1}. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 3 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time.

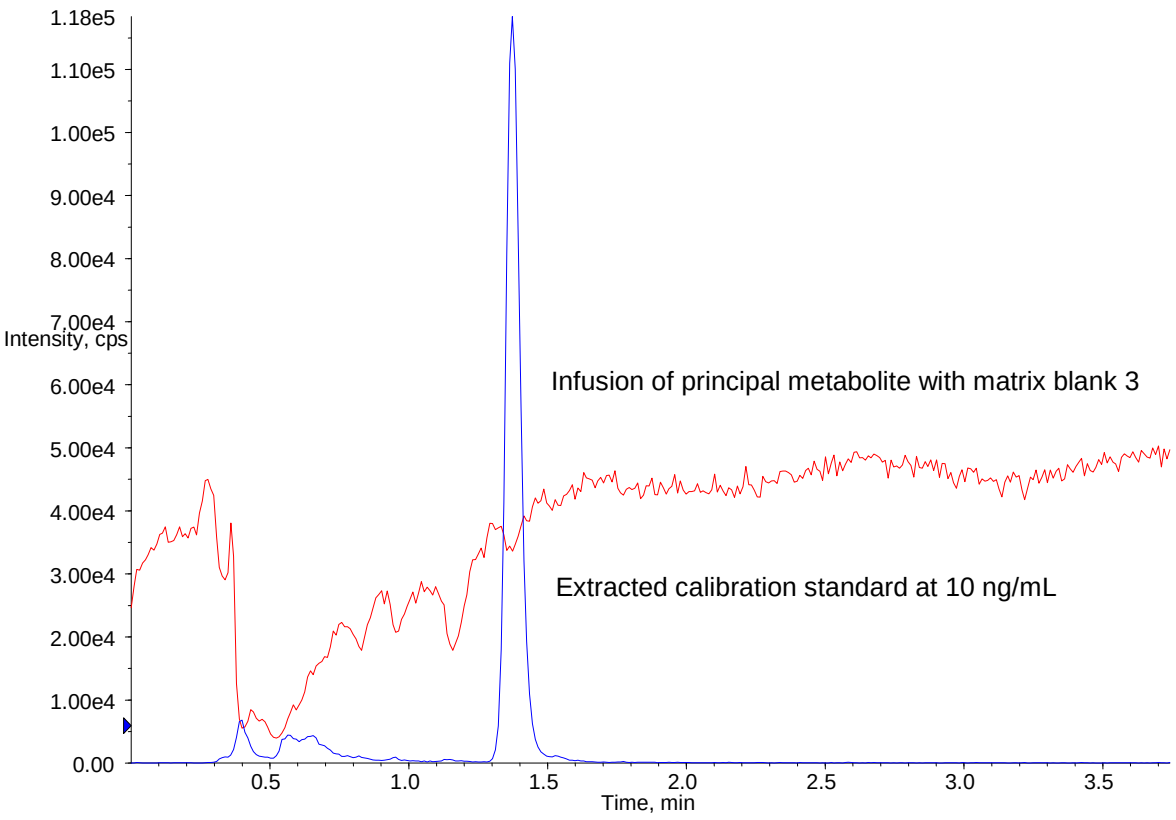


Figure 2.9 Ion suppression profile for matrix blank 3 (principal metabolite) using TurboIonSpray. HPLC system: PE Sciex API 4000 and PE Series 200 pumps, SIM for the principal metabolite. Column Hypersil Cyano 50 x 4.6 mm, 5 µm particles. Mobile phase acetonitrile - ammonium acetate (10 mM; pH 4) (35:65, v/v), flow rate 1 $mL.min^{-1}$. The principal metabolite was added to the system by infusion at approximately 10 $\mu L.min^{-1}$ using the set-up illustrated in Figure 2.3 and matrix blank 3 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (just) before the metabolite retention time (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore little or no ion suppression would be expected at the metabolite retention time.

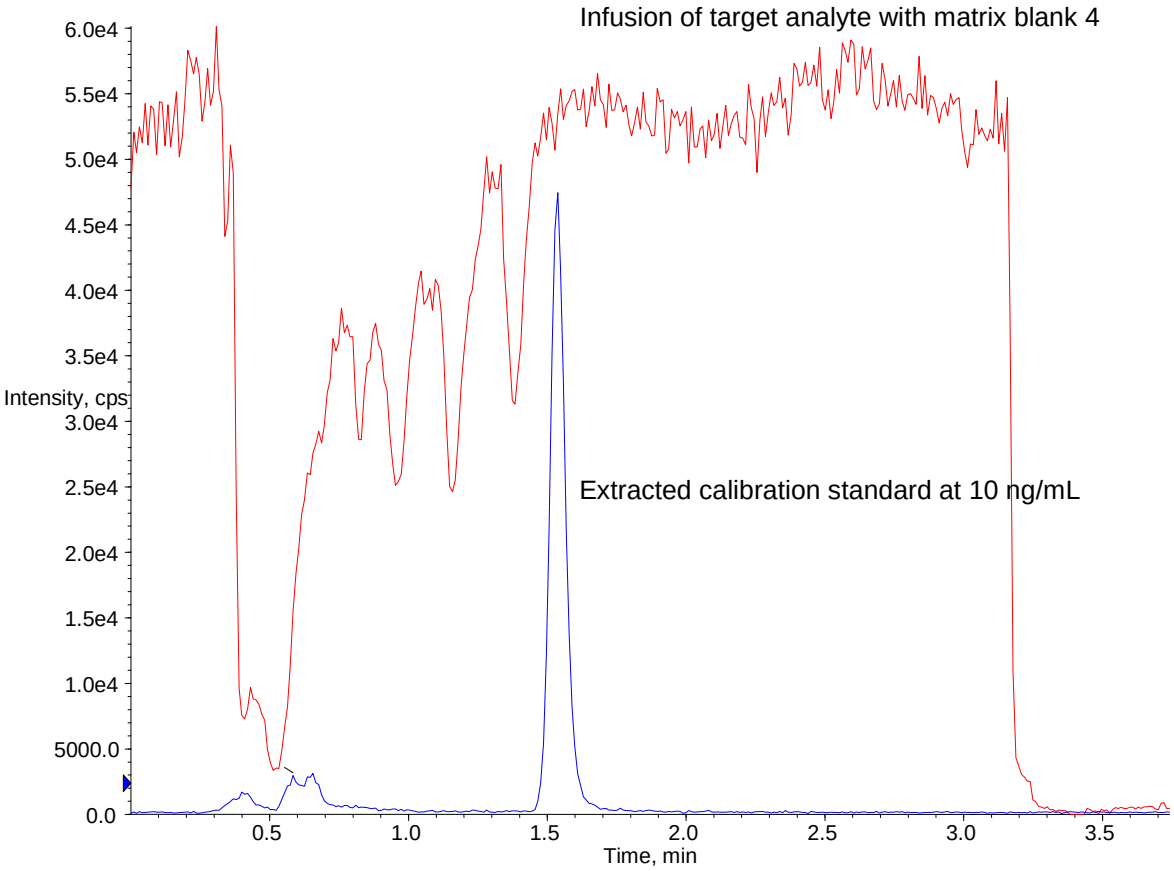


Figure 2.10 Ion suppression profile for matrix blank 4 (target analyte) using TurboIonSpray. HPLC system: As Figure 2.4. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 4 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time. N.B. Although at a retention time before the target analyte peak there is appreciably greater amount of suppression than for matrices 1-3.

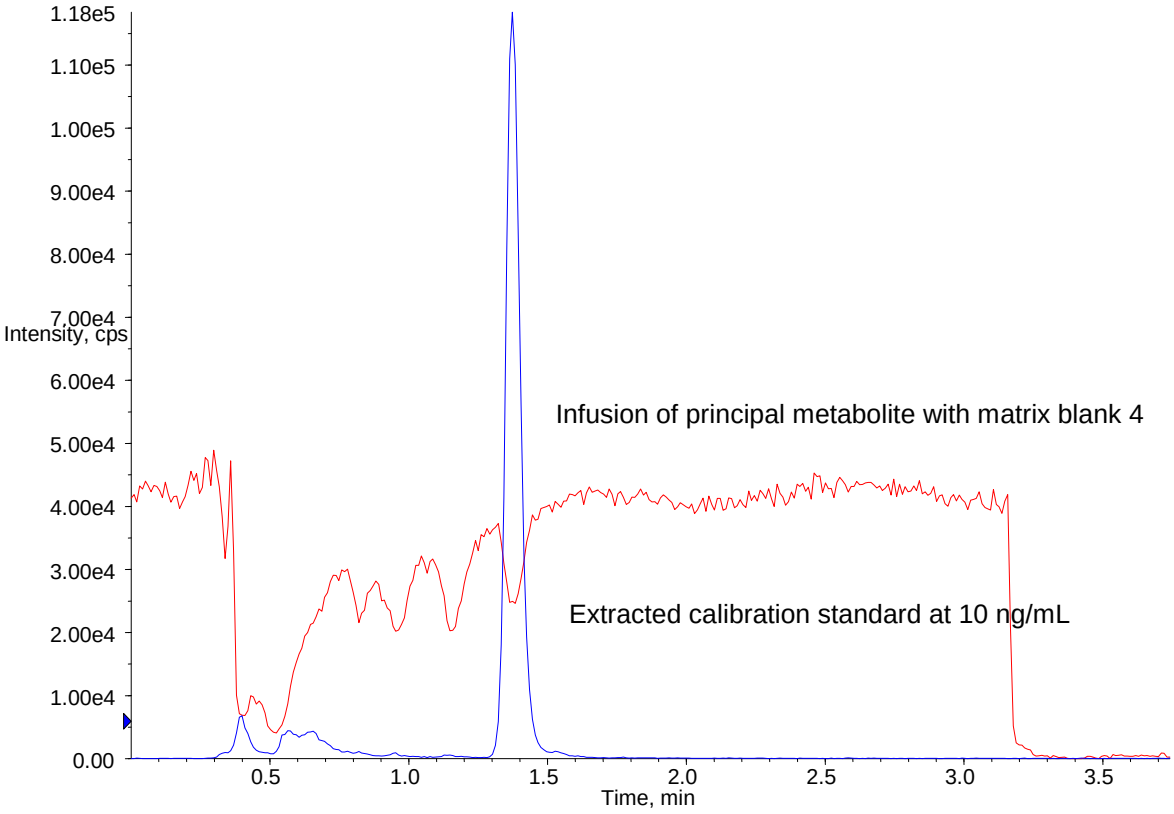


Figure 2.11 Ion suppression profile for matrix blank 4 (principal metabolite) using TurboIonSpray. HPLC system: As Figure 2.5. The principal metabolite was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 4 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The metabolite retention time exactly corresponds to one of the suppression areas (at 1.4 min); therefore significant ion suppression is present.

As the suppression regions were found all around the analytes of interest (particularly with "matrix 4", it was determined that it would be difficult to chromatographically

resolve this problem. As the sample preparation method used was fairly specific, using mixed-mode solid phase extraction, it was determined that a different way of overcoming the problem was required to changing the sample preparation method.

Accordingly it was decided to investigate a change of the mode of ionisation from TurboIonSpray to atmospheric pressure chemical ionisation (APCI). It was expected that this might result in a reduction or elimination of the matrix effects observed as APCI is considered to be far less susceptible to matrix-related modification of ionisation than TurboIonSpray [75]. The same matrix extracts that were used for the experiment illustrated in Figures 2.6 through 2.11 were used in a similar post-column infusion experiment, but with the mass spectrometer in APCI mode. This confirmed that there was far less modification of ionisation due to matrix in general and that the effect on the analytes of interest could be eliminated by this change of interface (Figures 2.12 - 2.19). As there was sufficient sensitivity to exceed the required limit of detection using APCI, this simple change of ionisation mode was used for the full validation of the analytical method. The lack of matrix-related modification of ionisation is illustrated in Tables 2.3 and 2.4, where the accuracy and precision values at each concentration are well within the acceptance criteria (85 - 115% for accuracy;≤15% for precision) for each analyte (in contrast to the values illustrated in Table 2.1 obtained when analysing similar samples using TurboIonSpray as the mode of ionisation.

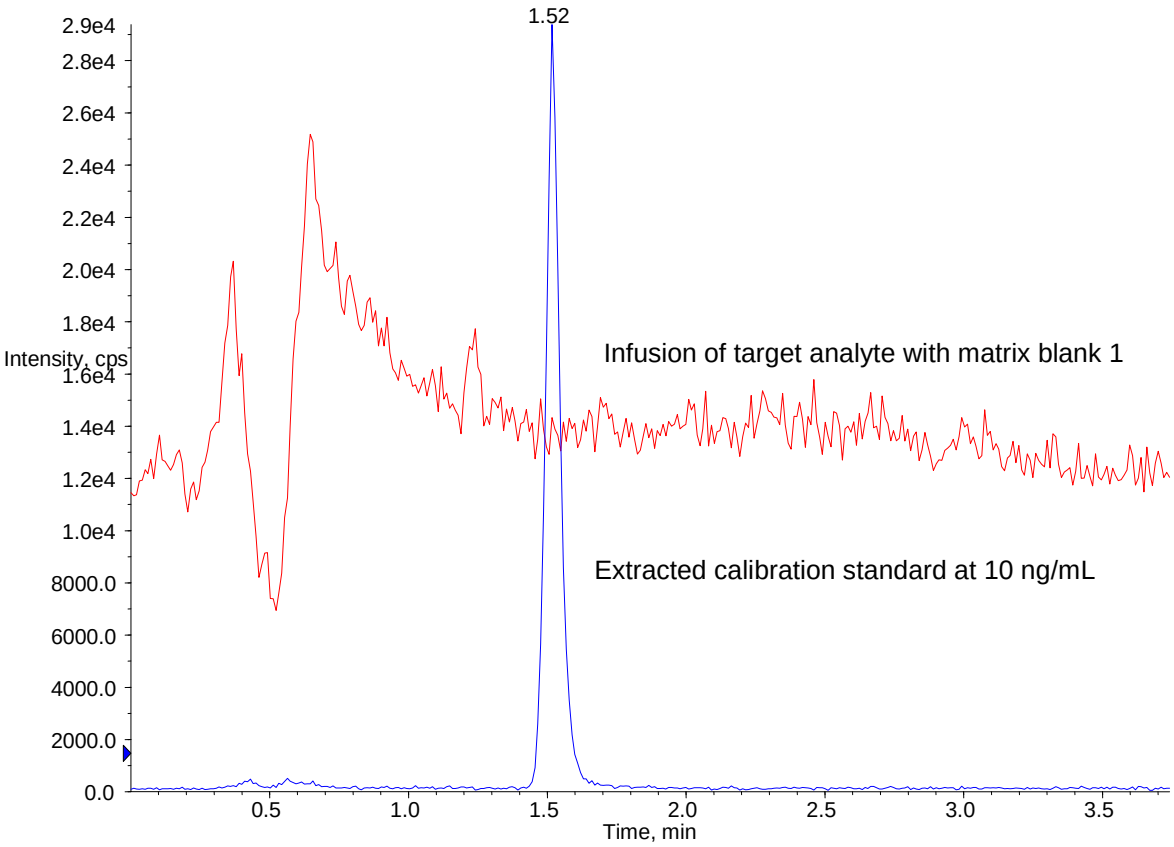


Figure 2.12 Ion suppression profile for matrix blank 1 (target analyte) using APCI. HPLC system: As Figure 2.4. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 1 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time. N.B. The region of suppression occurring at around 0.5 min is significantly less pronounced than for the same matrix using TurboIonSpray.

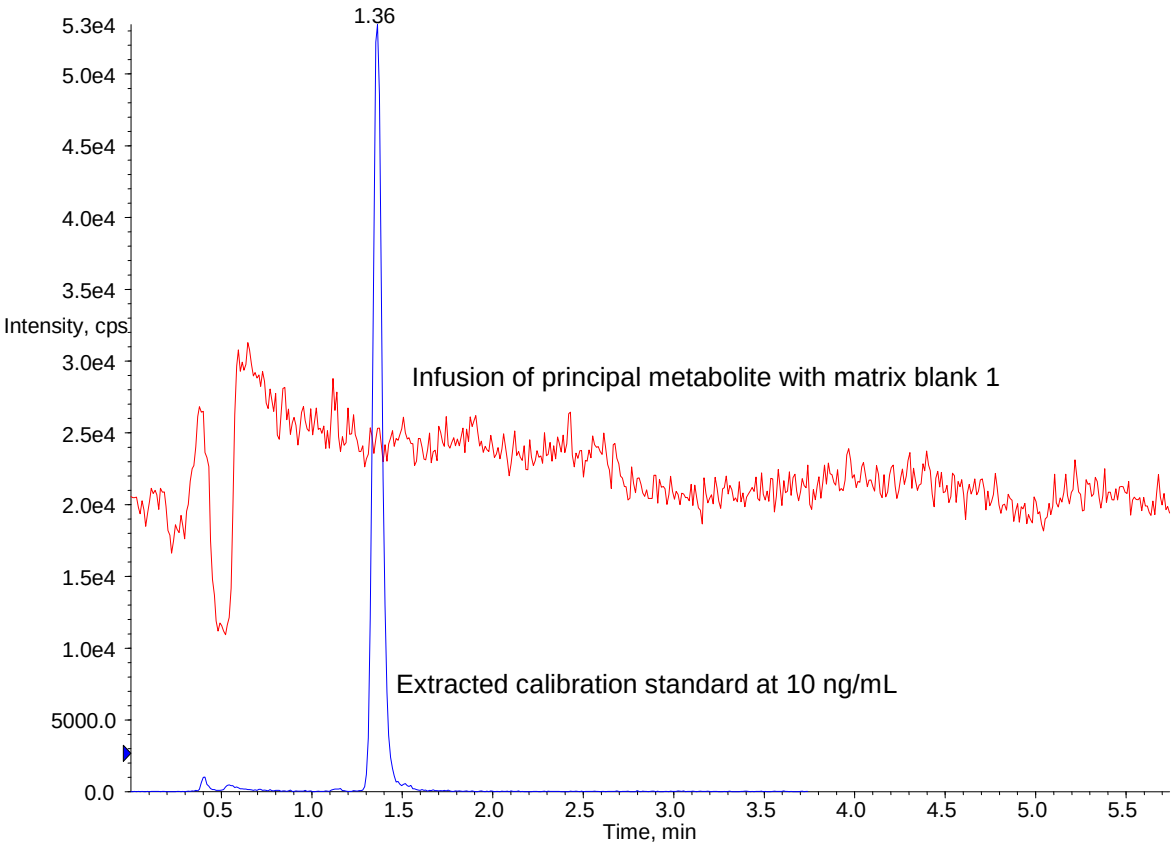


Figure 2.13 Ion suppression profile for matrix blank 1 (principal metabolite) using APCI. HPLC system: As Figure 2.5. The principal metabolite was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 1 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the metabolite retention time. N.B. The region of suppression occurring at around 0.5 min is significantly less pronounced than for the same matrix using TurboIonSpray.

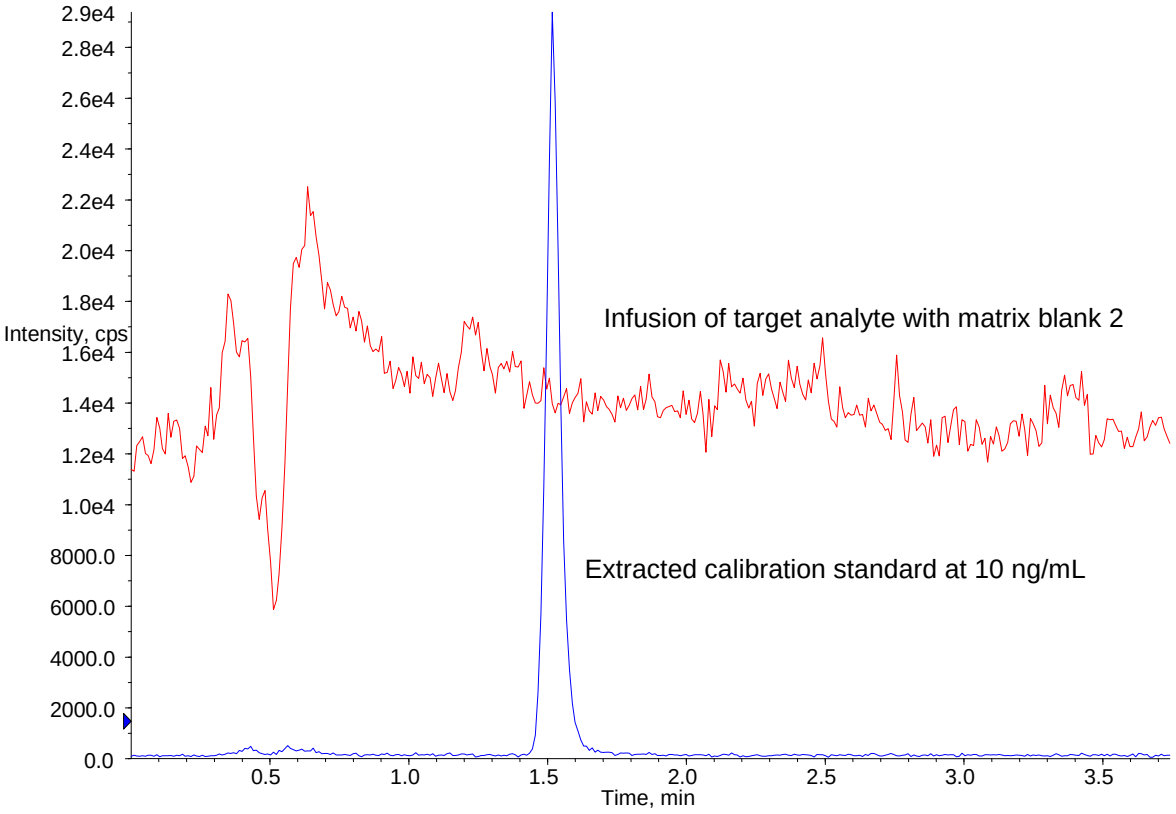


Figure 2.14 Ion suppression profile for matrix blank 2 (target analyte) using APCI. HPLC system: As Figure 2.4. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 2 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time. N.B. The region of suppression occurring at around 0.5 min is significantly less pronounced than for the same matrix using TurboIonSpray.

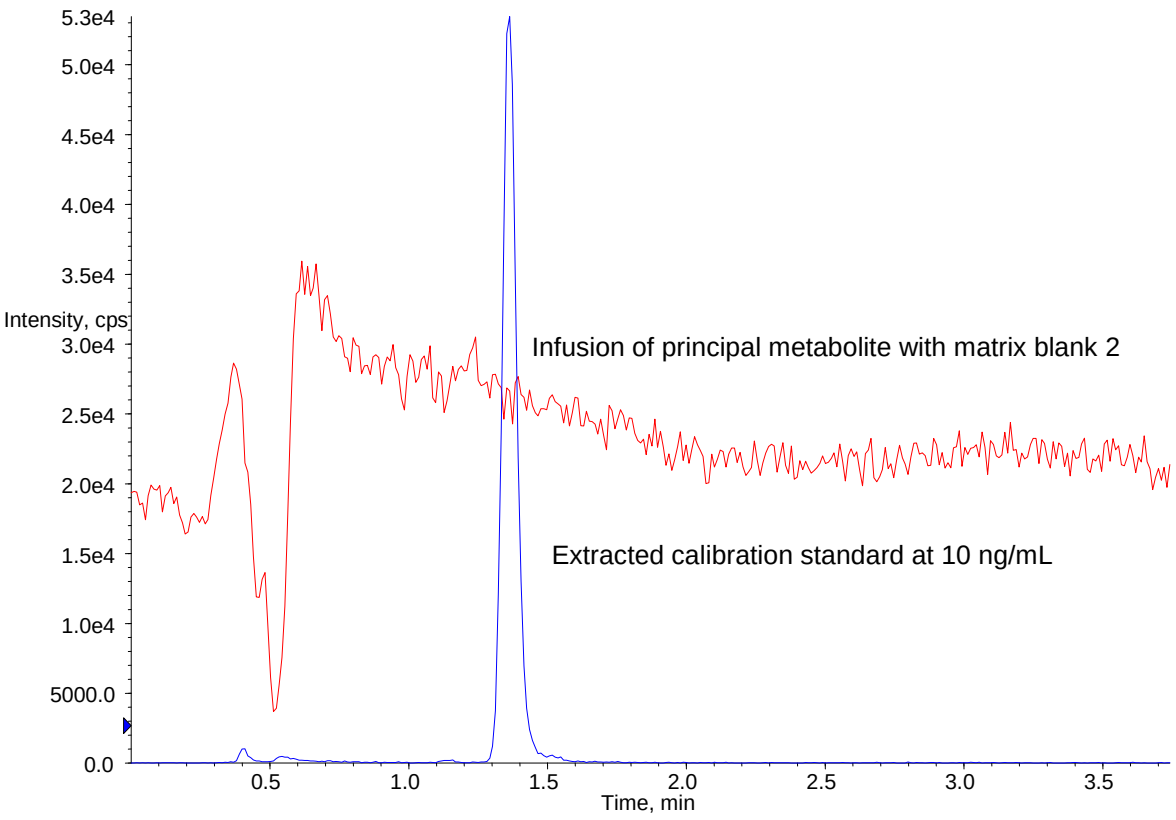


Figure 2.15 Ion suppression profile for matrix blank 2 (principal metabolite) using APCI. HPLC system: As Figure 2.5. The principal metabolite was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 2 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the metabolite retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the metabolite retention time. N.B. The region of suppression occurring at around 0.5 min is significantly less pronounced than for the same matrix using TurboIonSpray.

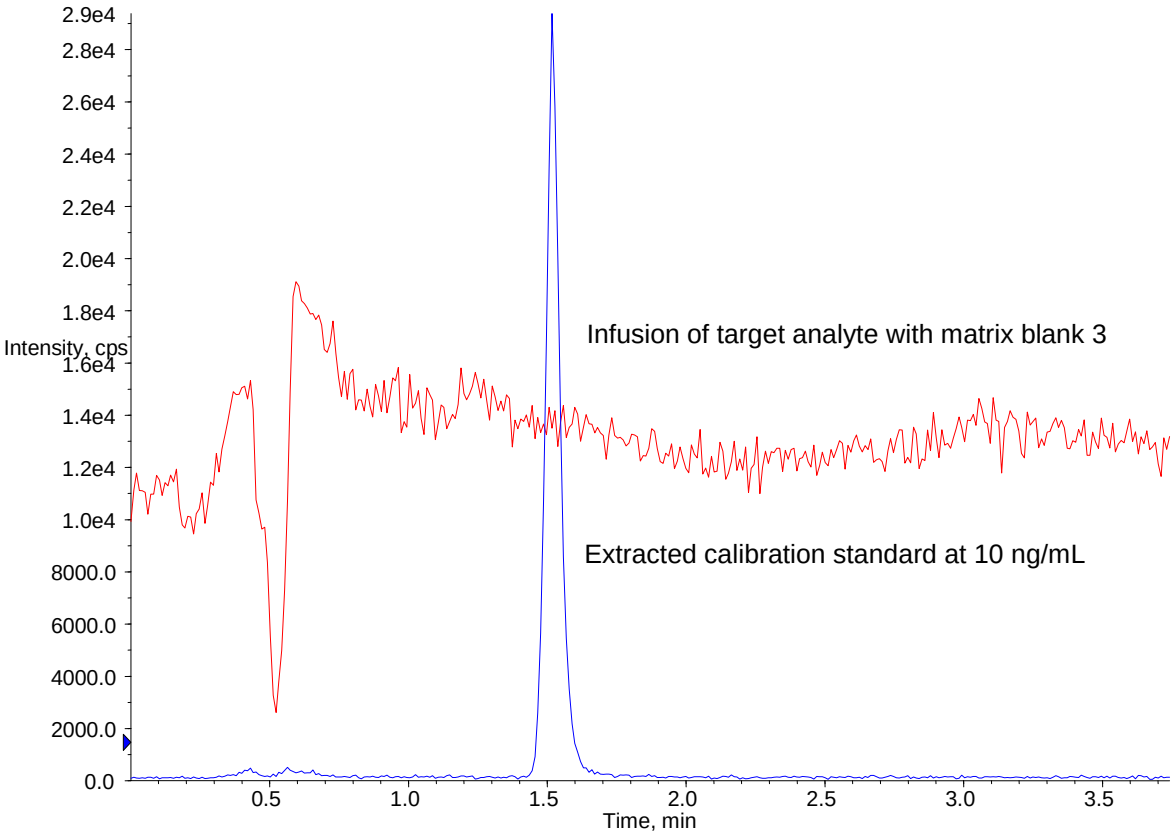


Figure 2.16 Ion suppression profile for matrix blank 3 (target analyte) using APCI. HPLC system: As Figure 2.4. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 3 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time. N.B. The region of suppression occurring at around 0.5 min is significantly less pronounced than for the same matrix using TurboIonSpray.

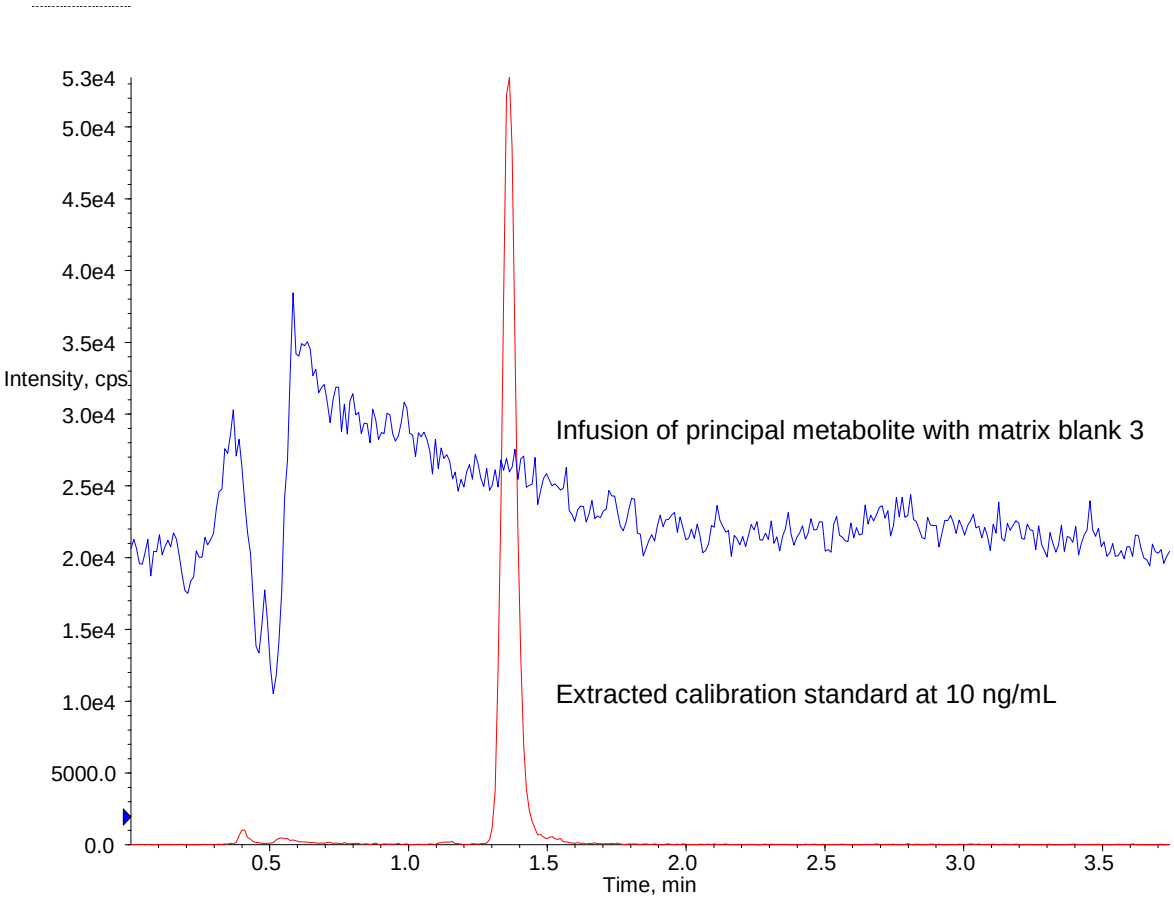


Figure 2.17 Ion suppression profile for matrix blank 3 (principal metabolite) using APCI. HPLC system: As Figure 2.5. The principal metabolite was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 3 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the metabolite retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the metabolite retention time. N.B. The region of suppression occurring at around 0.5 min is significantly less pronounced than for the same matrix using TurboIonSpray.

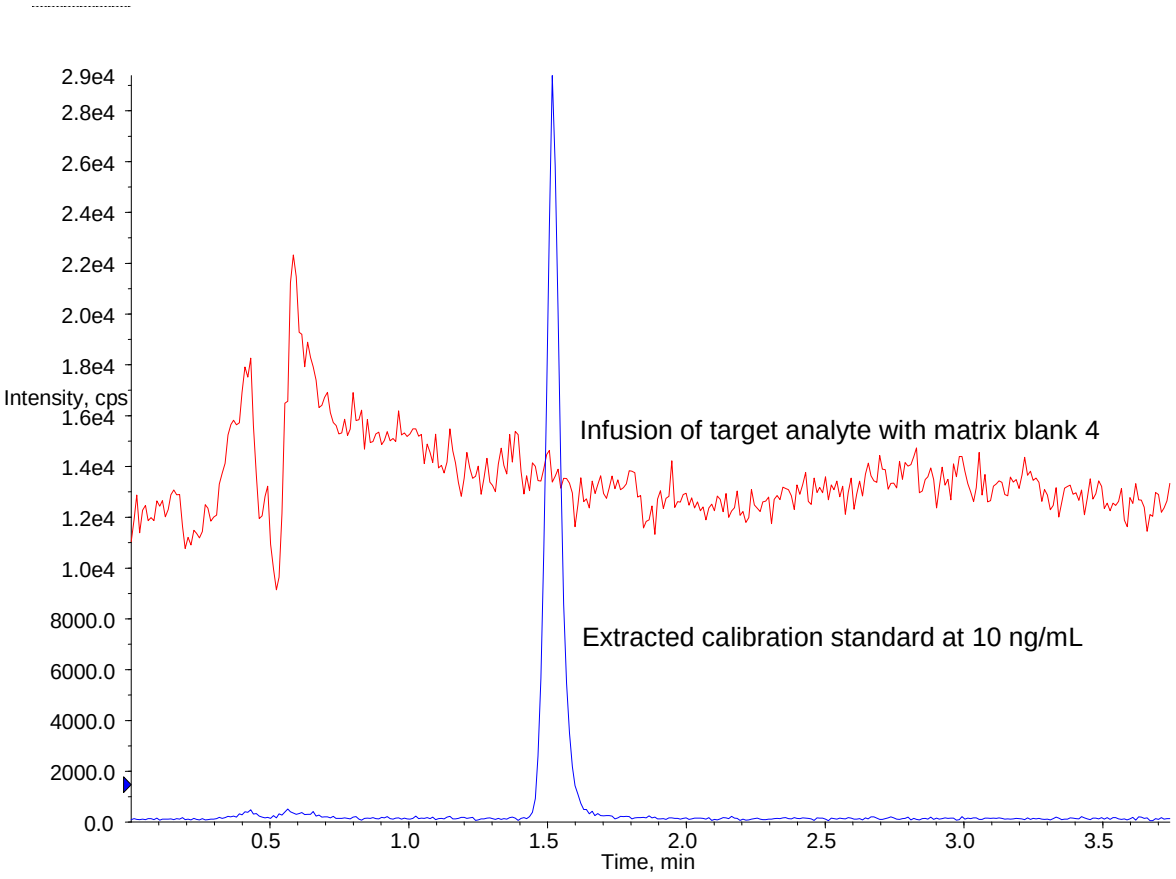


Figure 2.18 Ion suppression profile for matrix blank 4 (target analyte) using APCI. HPLC system: As Figure 2.4. The target analyte was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 4 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time. N.B. The region of suppression occurring at around 0.5 min is significantly less pronounced than for the same matrix using TurboIonSpray.

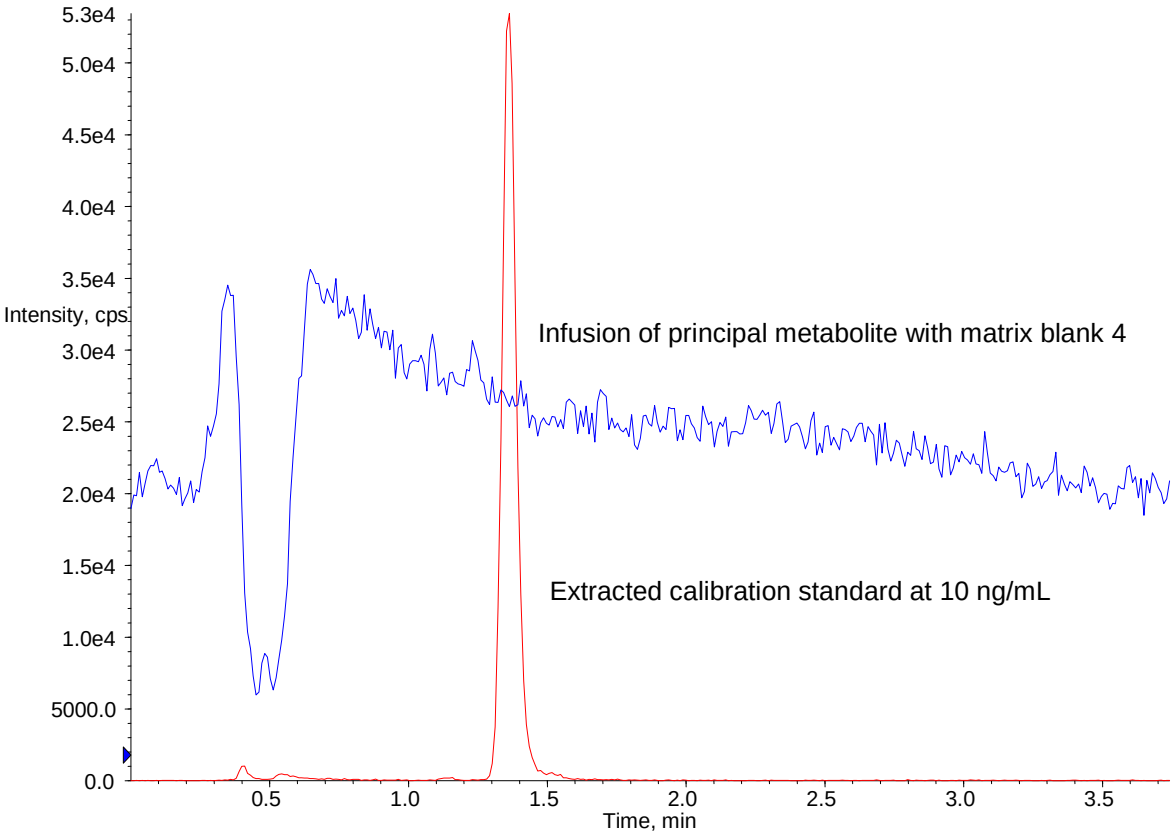


Figure 2.19 Ion suppression profile for matrix blank 4 (principal metabolite) using APCI. HPLC system: As Figure 2.4. The principal metabolite was added to the system by infusion at approximately 10 µL.min^{-1} using the set-up illustrated in Figure 2.3 and matrix blank 1 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the metabolite retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the metabolite retention time. In this example the region of suppression that directly coincided with the analyte retention time when using TurboIonSpray has been

completely eliminated. Only a small region of suppression (occurring at around 0.5 min) is present.

Table 2.3 Accuracy and precision of individual matrix effect samples using APCI (target analyte).

Nominal concentration (ng/mL)	Control matrix ID	Observed concentration (ng/mL)	Accuracy (%)	Mean accuracy (%)	Coefficient of variation (%)	n
0.100	1	0.0917	91.7	104.1	6.9	6
	2	0.113	113.0			
	3	0.103	103.0			
	4	0.105	105.0			
	5	0.109	109.0			
	6	0.103	103.0			
25.0	1	23.7	94.8	98.5	5.6	6
	2	23.6	94.4			
	3	23.6	94.4			
	4	25.7	102.8			
	5	26.9	107.6			
	6	24.2	96.8			

Table 2.4 Accuracy and precision of individual matrix effect samples using APCI (principal metabolite).

Nominal concentration (ng/mL)	Control matrix ID	Observed concentration (ng/mL)	Accuracy (%)	Mean accuracy (%)	Coefficient of variation (%)	n
0.100	1	0.0904	91.7	100.4	7.4	6
	2	0.0905	90.5			
	3	0.103	103.0			
	4	0.107	105.0			
	5	0.105	109.0			
	6	0.103	103.0			
25.0	1	21.9	87.6	95.4	6.4	6
	2	23.6	94.4			
	3	22.9	91.6			
	4	25.0	100.0			
	5	26.2	104.8			
	6	23.5	94.0			

2.1.4 Conclusions

There are several accepted ways of investigating matrix effects, but a full understanding of the techniques being employed is essential if significant findings are not to be ignored. It has been demonstrated clearly by this illustrative example that the ubiquitous technique of determining the effect on a matrix blank of post-column infusion of analyte has limitations, if an understanding of the variability of the matrix is not considered. In general on the basis of this example, it is suggested that the matrix effect trial using post-column infusion should be performed for at least six separate sources of individual matrix and it should be performed for all analytes being investigated, including the internal standard.

As urine varies extremely widely in both concentration and content [85, 86] it is particularly important to perform such trials on a range of individuals, in order to avoid erroneous conclusions about the absence of analytical issues.

Amongst the strategies normally employed to minimise matrix-related modification of ionisation is the use of stable isotopically-labelled internal standards. As well as the difficulties associated with obtaining such standards, particularly at the early stages of drug development, there are other problems associated with this approach. For many assays there are several target analytes (e.g. a compound and its metabolites) and the labelled internal standard may only be available for one of these [73]. Another problem can be that the internal standard will actually mask the problem at an early stage, with the suppression of ionisation not immediately apparent [87]. This may become a more significant problem when samples are analysed from clinical studies, where the variation between matrices from the "perfect" matrix that tends to be used for validation studies may result in erroneous results. Finally, if the matrix effect occurs at the retention time of the internal standard, the ratio of analyte : internal standard may be affected in different ways at different concentrations.

Each of the problems listed for stable isotopically-labelled internal standards are potentially magnified when using structural analogues as internal standard. It is therefore essential to perform the suppression investigation performed in this illustrative example on any internal standard which is a structural analogue of the target analyte(s). In the

illustrated example a structural analogue was in fact used as internal standard for both target analyte and principal metabolite. This analogue had a later retention time than either the target analyte or principal metabolite (approximately 2.5 min) and investigation suggested no region of suppression near the internal standard retention time.

The phenomenon of matrix-related modification of ionisation is a well established one. Although this problem was well documented before the reported work, the above example represents very good illustrative evidence of the possible extent of the problem, and the limitations of the "standard" method for overcoming it. This example has provided a good example which demonstrates the effectiveness of the option on changing the mode of ionisation.

In summary of the strategies discussed in this chapter, matrix effects can often be both greater and of more complexity than first envisaged, but with due care and attention and carefully thought out development protocols they can be overcome with relative ease, without resorting to stable-isotopically labelled internal standards, an option that will not always be available anyway.

Chapter 3

Post-Column Modification of Mobile Phase

3.1 Introduction

Although LC-API/MS is the dominant technique used for drug bioanalysis [16], the determination of enantiomers in biological fluids is one case where the application of LC-API/MS is not always straightforward. This is because for many methods for the separation of enantiomers the ideal chromatographic conditions are not readily compatible with mass spectrometric detection [88, 89]. For example, in the vast majority of chiral separations carried out on the most popular commercially available LC chiral stationary phases (CSP) i.e. derivatised polysaccharides, the mobile phase used, often n-hexane – propan-2-ol, with a basic or acidic additive, is non-aqueous [90] and does not lend itself readily to API/MS detection. Similarly, the 'polar-organic' mobile phases frequently used with cyclodextrin or macrocyclic antibiotic CSP [91] are usually not API/MS-friendly.

One solution to this type of problem is to ‘re-invent’ the chiral chromatography and indeed this approach was adopted for chiral separations which had been carried out using the polar-organic mode by developing a polar-ionic® separation [92] in which the ratio of amine to acid mobile phase modifier was adjusted so that atmospheric pressure ionisation became possible and there was still a chiral separation. Clearly though, it would be preferable to be able to proceed with pre-existing optimised conditions for the chiral separation. This is especially the case given the current scenario in which, almost exclusively, new chiral drugs are developed in a single enantiomer form [93]. In this situation enantioselective drug bioanalysis may be carried out strategically, rather than routinely in the Discovery phase, when it is being ascertained which enantiomer is the most suitable to develop, and during the Development phases, when checks for *in vivo* racemisation or chiral inversion are being made. It is therefore often difficult to justify more extensive method development, or in many cases there may be no choice about the chiral separation - there may be only one optimised separation, with no option to modify the mobile phase. Accordingly it was sought here to explore the possibility of adopting a less elegant, but easier to develop, approach of persisting with the non-API/MS-friendly mobile phase used for the chiral separation and employing post-column solvent addition to bring about conditions suitable for more optimal atmospheric pressure ionisation. This was originally investigated in a preliminary study for a number of dihydropyridine calcium channel blockers and fully validated for the specimen dihydropyridine felodipine (Figure 3.1), and has now been investigated for the detection of the enantiomers of a number of chiral drugs from a range of therapeutic categories.

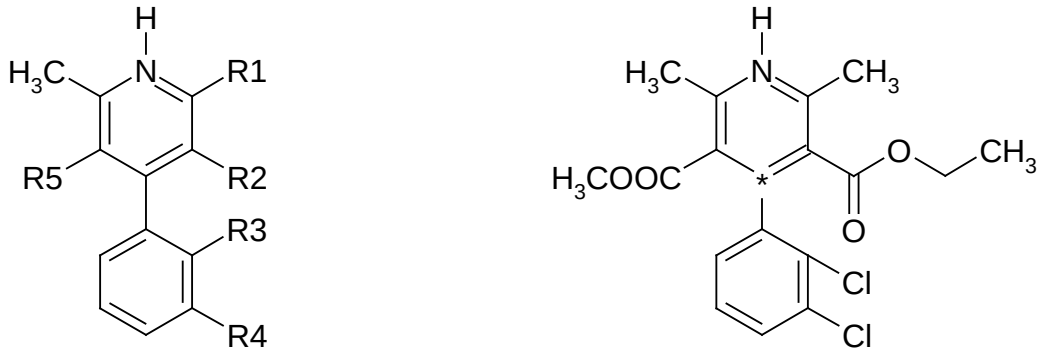


Figure 3.1 Structures of a Generic Dihydropyridine Calcium Channel Blocker and Felodipine. The "R" groups vary between dihydropyridines.

3.2 Experimental

3.2.1 Materials

A column packed with AGP-CSP (Chiral AGP, 100 mm x 4.0 mm, 5 µm) was supplied by Chromtech Ltd. (Congleton, Cheshire, UK). A column packed with a β-cyclodextrin-immobilised stationary phase (Cyclobond I, 250 mm x 4.0 mm, 5 µm) was supplied by ASTEC (Congleton, Cheshire, UK). Analytical grade reagents were supplied by Fisher Scientific (Loughborough, Leicestershire, UK). All compounds were supplied by Sigma Aldrich (Poole, Dorset, UK).

3.2.2 Instrumentation

The system used for this work consisted of a PE Series 200 Micro-pump (Perkin Elmer, Thornhill, Ontario, Canada), an online DGU-14A degasser (Shimadzu, Kyoto, Japan),

and a PE Series 200 Autosampler (Perkin Elmer, Thornhill, Ontario, Canada) connected to a Perkin Elmer API 3000 triple quadrupole mass spectrometer. Chromatographic system control, data acquisition and analysis were performed by means of Analyst software version 1.2 (Applied Biosystems – MDS Pharma, UK).

3.2.3 Chromatographic Conditions

The mobile phase for optimum enantiomeric separation was taken from literature methods and varied from 0-16% organic (0% terbutaline [94], ephedrine [95], 0.2% ondansetron [96], 0.5% propranolol [97], 1% pheniramine [98], 1.3% terfenadine [99], 2% brompheniramine [100], 5% mianserin [101], mexiletine [102] 16% organic (ketamine [103]) delivered at a rate of 1.0 $mL.min^{-1}$ through the analytical column. Conditions for enantiomeric resolution were again confirmed experimentally prior to investigation of post-column modification of ionisation.

3.2.4 Tandem Mass Spectrometric Detection

A Perkin Elmer API 3000 triple quadrupole mass spectrometer was used in positive ion, multiple reaction monitoring mode using a pneumatically and thermally assisted electrospray interface (TurboIonSpray) with the drying gas set at 475°C. The mass spectrometer voltage settings were optimised for each analyte being investigated.

The mobile phase was introduced at 1.0 mL.min^{-1} directly through the analytical column. A second pump was connected to the chromatographic system post column, *via* a tee piece, which allowed the introduction of acetonitrile to enable mixing with the column eluent. Following this tee piece a further tee was connected to act as a split to allow some of the combined mobile phase and acetonitrile to flow to waste, thereby optimising the flow to the mass spectrometer (Figure 3.2).

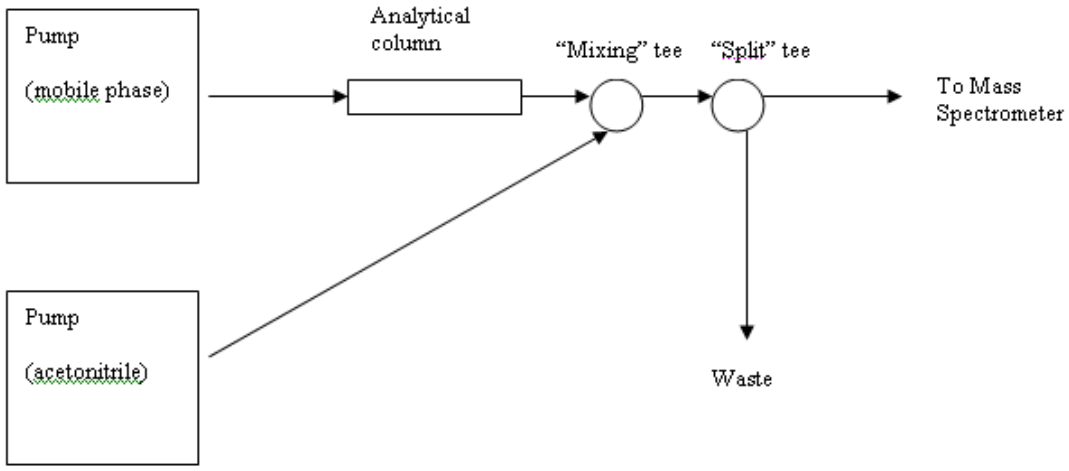


Figure 3.2 LC configuration used for post-column optimisation. Acetonitrile is added *via* a tee-piece ("mixing tee") after the analytical column, which results in a large total flow rate of greater than 1.0 mL.min^{-1}, which is split to waste, using a second tee-piece ("split tee").

3.3 Results and Discussion

Optimal enantioseparation for a range of compounds investigated used predominantly aqueous mobile phases (with less than 10% v/v organic modifier), when using mobile phases that are suitable for API/MS. As one of the principal determinants for sensitivity in the mass spectrometer is ion evaporation [104], these mobile phases are not well suited for high sensitivity. In order to overcome the problems associated with poor evaporation, the effect of post-column addition of organic modifier on sensitivity was investigated.

3.3.1 Percentage of Organic Modifier

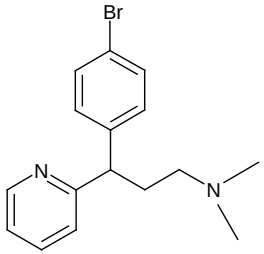
The amount of acetonitrile added post-column was gradually increased, without varying total flow rate, sample concentration and volume. This was achieved by increasing the amount of acetonitrile flowing through the mixing tee, whilst simultaneously reducing the final volume reaching the mass spectrometer using the split tee (as illustrated in Figure 3.2). The peak area obtained against the concentration of acetonitrile in the final mobile phase arriving at the MS interface is shown in Table 3.1. Due to the system used for adjusting the percentage of organic modifier, the amount of analyte reaching the mass spectrometer is being reduced; therefore the overall increase in sensitivity reported is actually significantly smaller than the absolute increase obtained if measured per unit mass of analyte reaching the mass spectrometer (N.B. the flow to the mass spectrometer was kept constant, by increasing the split to waste with increased flow rate).

Table 3.1 Peak area in relation to acetonitrile concentration.

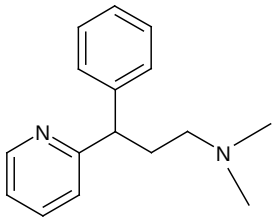
Compound	Average Response (Mean of 3 replicate injections)												
	5%	**10%**	**15%**	**20%**	**25%**	**30%**	**40%**	**50%**	**60%**	**70%**	**80%**	**90%**	**95%**
Brompheniramine	8166	9426	9875	9723	11322	12045	13782	15128	17099	19722	22412	27196	28884
Pheniramine	201707	202525	227847	218980	235864	205069	288481	311377	384474	458184	560267	699101	839527
Ephedrine	117405	111444	113028	129569	149841	182200	203400	279685	356394	429386	550363	714273	806965
Ketamine	1961	2068	2291	2489	2425	2707	3181	3561	4147	4756	5540	6764	7272
Mexiletine	836	892	919	1242	1370	1713	1993	2678	3679	4868	6056	7619	8364
Mianserin	7787	8756	11133	12472	14526	15842	18444	21158	24004	29088	34008	41422	43581
Ondansetron	6299	6961	7839	8174	8109	8897	9790	9940	11240	12490	14103	15077	13561
Propranolol	1233	1408	1618	1765	2084	2252	2706	3461	4398	5432	6986	9313	10669
Terbutaline	4389	4351	5245	6156	7194	7458	9540	11556	14518	18627	23134	28781	29323
Terfenadine	46196	74638	N/A	200712	N/A	346137	439655	571733	756474	1007148	1500840	2651602	N/A

N/A = not applicable – not investigated at the stated acetonitrile concentration

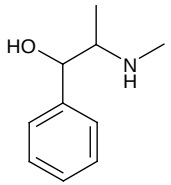
Brompheniramine
(Anithistamine)



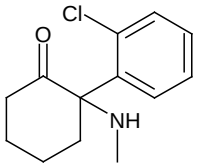
Pheniramine
(Antihistamine)



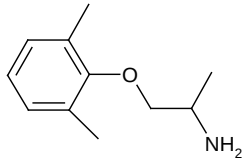
Ephedrine
(Stimulant, Decongestant)



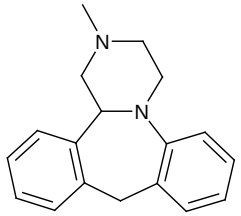
Ketamine
(Anaesthetic)



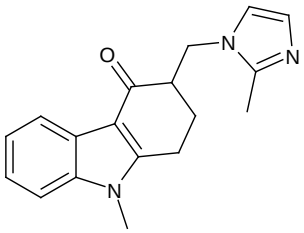
Mexiletine
(Antiarrythmic)



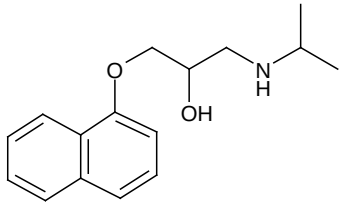
Mianserin
(Antidepressant)



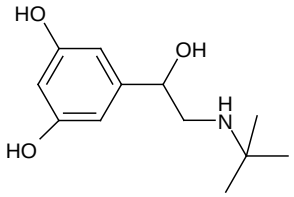
Ondansetron
(Antiemetic)



Propranolol
(Beta Blocker)



Terbutaline
(Bronchodilator)



Terfenadine
(Antihistamine)

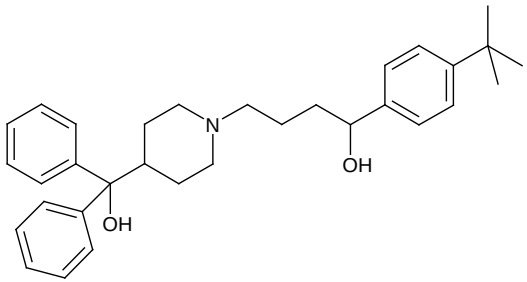


Figure 3.3 Structures of analytes investigated using post-column modification of mobile phase.

Rather than tabulate and graph with multiple scales, due to the differences in response for the various analytes, a correction factor was applied for each analyte to bring the average response with 5% acetonitrile to a similar value. This involved division of the observed response by the following factors: 4, 100, 50, 600, 0.4, 130, 3, 140, 2 and 20 for brompheniramine, pheniramine, ephedrine, ketamine, mexiletine, mianserin, ondansetron, propranolol, terbutaline and terfenadine, respectively. These normalised responses are shown in Table 3.2.

These changes in response are better illustrated in Figure 3.4. The response for terfenadine showed a far greater increase with increase in organic modifier concentration than any of the other compounds investigated, possibly due to it being by far the most non-polar of the compounds. A further graph with terfenadine removed is presented in Figure 3.5 to illustrate the general trend in more detail.

Table 3.2 A comparison of the change in response with increased acetonitrile concentration.

Compound	Average Response (Mean of 3 replicate injections)													Maximum increase (x lowest)
	5%	10%	15%	20%	25%	30%	40%	50%	60%	70%	80%	90%	95%	
Brompheniramine	2042	2357	2469	2431	2831	3011	3446	3782	4275	4931	5603	6799	7221	3.5
Pheniramine	2017	2025	2278	2190	2359	2051	2885	3114	3845	4582	5603	6991	8395	4.2
Ephedrine	2348	2229	2261	2591	2997	3644	4068	5594	7128	8588	11007	14285	16139	6.9
Ketamine	1961	2068	2291	2489	2425	2707	3181	3561	4147	4756	5540	6764	7272	3.7
	2383	2680	N/A	3359	N/A	4368	5638	6880	8320	10052	11966	13766	N/A	5.8
Mexiletine	2089	2230	2297	3105	3425	4282	4984	6696	9198	12170	15141	19048	20909	10.0
Mianserin	2049	2304	2930	3282	3823	4169	4854	5568	6317	7655	8950	10901	11469	5.6
	2048	2787	N/A	3949	N/A	5161	6589	8161	10115	12630	15897	21140	N/A	10.3
Ondansetron	2100	2320	2613	2725	2703	2966	3263	3313	3747	4163	4701	5026	4520	2.2
Propranolol	2096	2394	2751	3000	3542	3828	4601	5884	7477	9234	11876	15831	18137	8.7
	2024	2390	N/A	2967	N/A	3888	5055	6442	8426	10863	14432	21140	N/A	10.4
Terbutaline	2195	2176	2622	3078	3597	3729	4770	5778	7259	9314	11567	14391	14662	6.7
Terfenadine	2310	3732	N/A	10036	N/A	17307	21983	28587	37824	50357	75042	132580	N/A	57.4

N/A = not applicable – not investigated at the stated acetonitrile concentration

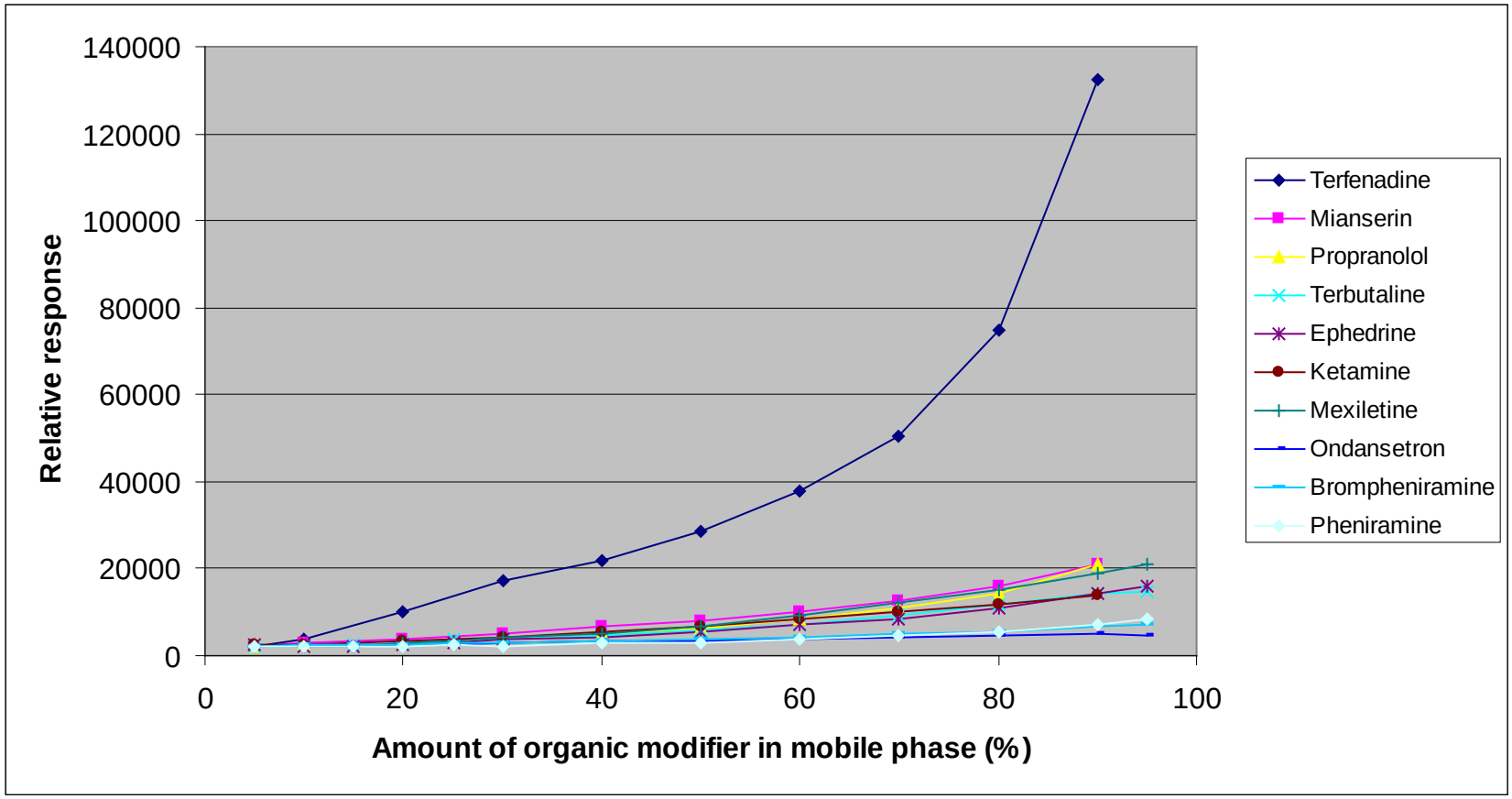


Figure 3.4 Response of analyte against percentage of organic in mobile phase at mass spectrometer. In all cases the response increased significantly with increasing concentration of the organic modifier.

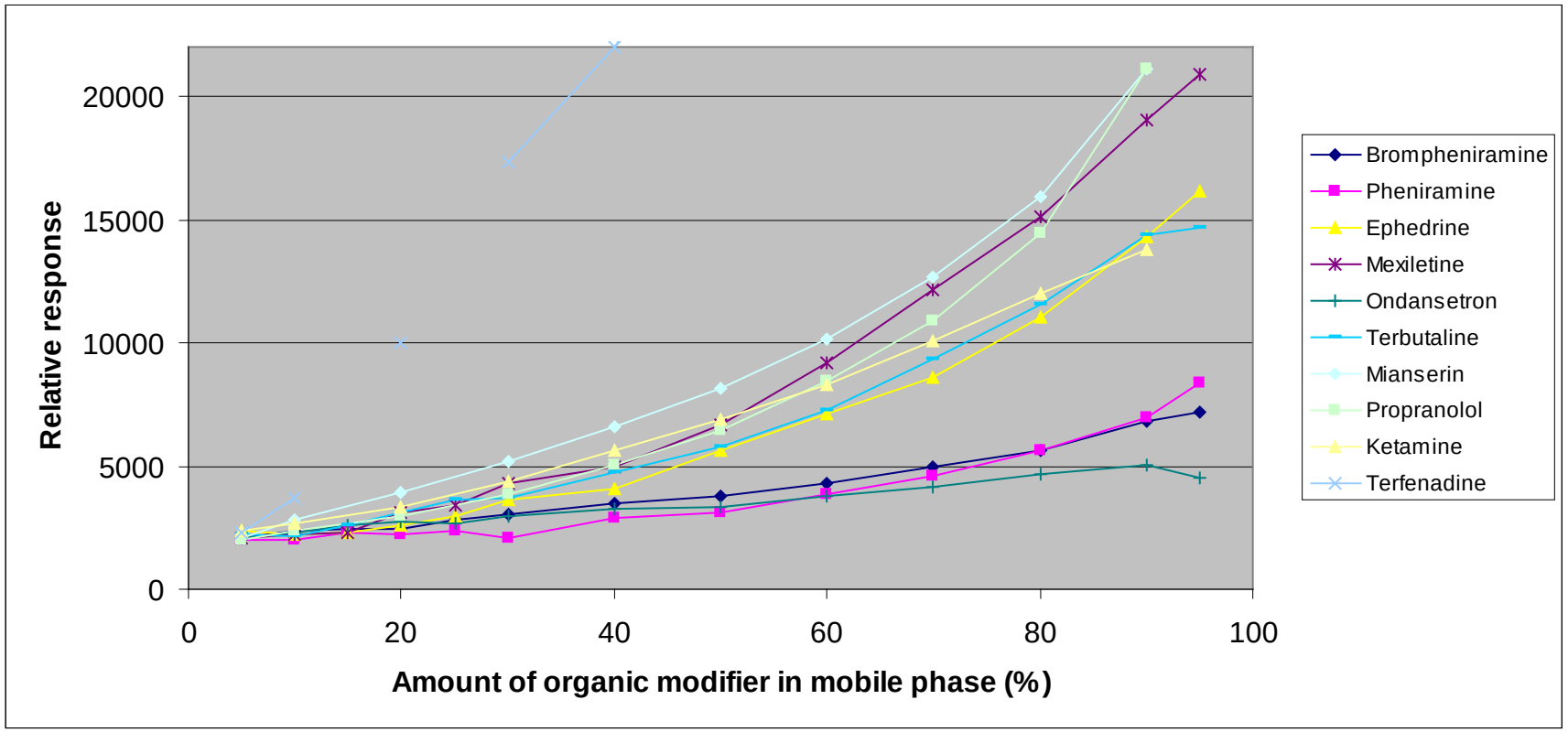


Figure 3.5 Response of analyte against percentage of organic in mobile phase at mass spectrometer, excluding terfenadine. Excluding terfenadine, which had a far larger change in response than the other compounds investigated, this highlights that there was an increase in response from two-fold to about an order of magnitude. When dealing with difficult to achieve limits of detection this can be the difference between meeting the required range and failing to do so.

3.3.2 Split Ratio

In addition to the volatility of the mobile phase reaching the MS interface, as controlled by the proportion of acetonitrile incorporated, the effect of flowrate of mobile phase reaching the interface was also examined.

The effect of flowrate at the MS interface on peak area of an example compound (felodipine) is shown in Figure 3.6. These results were obtained using a mobile phase split of approximately 20% to the MS (the remainder to waste).

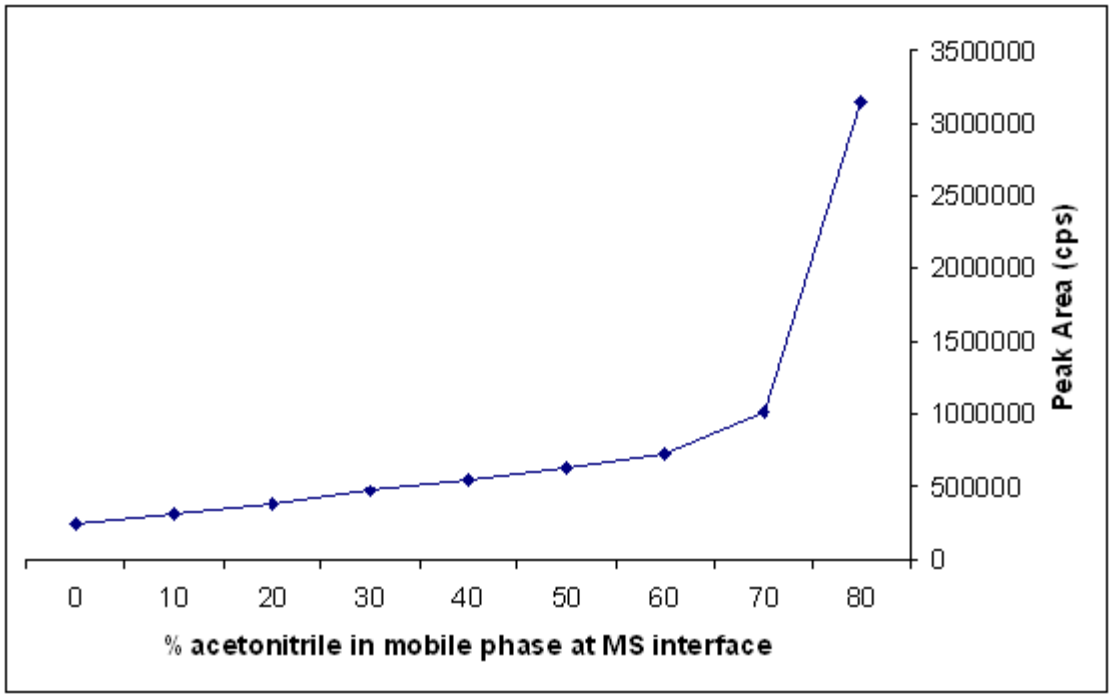


Figure 3.6 Split Optimisation. This illustrates the increase in response (peak area) for felodipine with increasing proportion of acetonitrile in the mobile phase that reaches the mass spectrometer.

Optimisation for felodipine resulted in the following conditions. The flow rate used for the acetonitrile was 0.9 $mL.min^{-1}$, with the split allowing 0.2 $mL.min^{-1}$ to the mass spectrometer. This produced a final proportion of only 50% acetonitrile in the final eluent reaching the mass spectrometer, which was lower than the optimum observed in the experiment illustrated by Figure 3.6. This was due to difficulties in the reliability of the mixing of the mobile phase and acetonitrile, which reduced the precision of repeated injections, if larger total flow rates were used.

The concentration of analyte reaching the mass spectrometer is effectively being reduced (by the post-column addition of organic mobile phase). However, the increase in sensitivity achieved using this technique more than compensated for this reduction in concentration.

These conditions gave rise to an approximate ten-fold increase in sensitivity over the original conditions investigated without post-column modification of mobile phase.

The degree of enhancement was investigated for a number of chiral compounds to determine whether the limits of detection under existing optimised conditions for enantioseparation could be improved to a significant (i.e. order of magnitude) degree. In addition the technique has been further investigated to establish whether or not a method involving this post-column solvent addition would stand up to the rigours of a full method validation [105].

For the evaluation of post-column modification of mobile phase as a generic method for the improvement in sensitivity for a range of analyte, the flow rate reaching the mass spectrometer interface was set at approximately 0.5 $mL.min^{-1}$.

It was clear from the study of post-column modification that this simple method for the enhancement of sensitivity which had enabled the validation of a method for the determination of a specimen dihydropyridine [105] to rigorous internationally recognised standards, was likely to be applicable not only to dihydropyridines but also to a wide range of basic drugs.

A major problem with many methods for the separation of enantiomers is that the ideal chromatographic conditions are not readily compatible with mass spectrometric detection. This is due to the nature of the mobile phase required for optimum separations and is particularly true for the protein based CSPs, such as the AGP-CSP used here.

3.4 Conclusions

The approach to overcoming the problem presented here involved the post-column addition of an organic modifier in sufficient quantities to enhance ion evaporation. The concentration of analyte reaching the mass spectrometer is effectively being reduced (by the post-column addition of organic mobile phase). However the increase in sensitivity achieved using this technique more than compensates for this reduction in concentration.

The proportion of the organic component required for the optimum increase in sensitivity varies widely depending on the compound type. This may be due to the relative affinity of the compounds to the droplet surface. It is possible to further enhance sensitivity by the optimisation of the split ratio, which controls the flow rate of mobile phase to the mass spectrometer.

This simple artefact enabled a ten-fold increase in sensitivity over the original conditions investigated without post-column modification of mobile phase to be used for the validation of a method for one of the analytes investigated.

While there a number of interesting features of the illustrative method described, the main conclusions of this study are very clear. The device of using post-column addition of organic solvent for chiral drug bioanalysis by LC-API/MS using highly aqueous mobile phases proved to be easy to set up, robust in use and led to significantly improved sensitivity and a rapidly developed method. It was a very practical solution to the problem of poor sensitivity when using highly aqueous mobile phases in that it allowed previously published optimum conditions for the chiral separations to be used.

Further, it would be anticipated that a similar approach could be successfully employed in other instances, such as when using “polar-organic” and hexane - propan-2-ol mobile phases mentioned in Section 3.1, of achieving chiral separations using MS-unfriendly mobile phases. Indeed, at a recent UK symposium, a Pfizer speaker (P Ferguson,

Hichrom Chiral HPLC Symposium, Sandwich, 25 Sept 2006) gave a presentation of the use of post-column modification of mobile phase to solve a sensitivity problem in “straight-phase” chiral work. This mainly used illustrative examples to show that the use of the post-column modification of mobile phase discussed in this chapter, works well as a general technique to improve sensitivity when struggling for sensitivity with other equally MS-unfriendly mobile phases.

Chapter 4

Strategies for dealing with quantitative determination of endogenous analytes

There are a number of reasons for the requirement for a robust method for the determination of an endogenous analyte. One of the principal ways of determining the effectiveness of a therapy can be by observing the effect of a dose of a drug substance on an endogenous compound in a biological system (i.e. a bio-marker [106]). In order to do this effectively a method for the quantitative analysis of the bio-marker would be required. Similarly bio-markers may be used as an indication of a disease state, where their presence/absence and concentration may provide an indication of the presence or progress of a disease.

The main problem associated with the quantitative analysis of endogenous analytes is associated with the provision of "control" matrix, i.e. a supply of the matrix to be analysed that does not contain the analytes under investigation. There are a number of strategies available to overcome this problem:

a) The use of a “surrogate” matrix, i.e. a matrix that is as similar to the sample matrix as possible, but that does not contain the target analyte [107]. For example, as will be described in Section 4.1, the use of gelding serum for testosterone analysis. The similarity of a surrogate to the sample matrix can be extremely good, but the main disadvantage of this approach is that there are many examples where a truly representative surrogate can not be obtained.

b) The use of non-matrix standards, where the calibration standards (and possibly quality control samples) are prepared in the injection solvent used for the analysis, without any matrix present at any time in the sample preparation procedure [108]. This is an extremely simple approach, but suffers from the difficulties associated with sample extraction recovery and matrix effects.

c) The adoption of the standard addition method for sample and standard preparation. Using this methodology a standard solution (i.e. a solution containing a known concentration of the analyte) is added to the unknown solution or sample matrix so that the endogenous amount can be accounted for in the analysis [109]. Knowledge of how the response changes before and after adding the standard solution allows extrapolation to determine the concentration initially in the sample. In many cases this can provide an excellent strategy for these analyses, as any matrix-related (extraction and ionisation) effects are minimised. The main disadvantage is that the response of an analyte may not be linear across the entire range, particularly if the endogenous level selected is relatively high within the dynamic range investigated.

d) For many matrices, e.g. plasma, charcoal-stripping may be utilised. This involves many compounds within the matrix being removed by passing the matrix over charcoal [110]. The main disadvantages of this technique are that it can result in incomplete removal of the analyte and it can be an expensive and time-consuming technique.

e) The use of a surrogate analyte, where for example a deuterated version of the target analyte is used to generate the calibration curve (rather than as an internal standard) and the sample concentrations of the target analyte are read off this calibration curve [111]. Difficulties in obtaining pure deuterated analyte, or the preference to use the deuterated version of the analyte as an internal standard make this a rare strategy of choice.

The advantages and disadvantages of these strategies may be dependent on the nature of the sample matrix or the nature of the target analyte. The choice of which of these strategies to adopt is likely to be dependent on practicability, based on the availability of a suitable surrogate (matrix or analyte), though in practice, it is often based on familiarity with a particular solution. All of these strategies can provide a suitable methodology for the preparation of a robust analytical method, but the essential thing is to prove the suitability of the strategy selected, through the course of the method evaluation and validation.

4.1 A Method for the Determination of Clinically Suppressed Levels of Testosterone in Human Serum

4.1.1 Introduction

Testosterone is the principal male sex hormone, which is responsible for the growth and development of masculine characteristics. It has a direct influence of the maturation of the male sexual organs, development of sperm within the testes, sexual drive, erectile function of the penis, and male secondary sexual characteristics including facial hair and pronounced musculature. Testosterone is produced in the testes. Testosterone adversely affects diseases of the prostate gland by increasing the growth of carcinomas of the prostate.

Chemical castration is a clinical aim in certain therapies, in particular therapies for the treatment of cancer of the prostrate gland. Chemical castration is considered to be defined as a serum testosterone level of below 100 $pg.mL^{-1}$ [112, 113]. The range normally found in male humans is 3500-10800 $pg.mL^{-1}$ [114]. In order to monitor the suppressed levels of testosterone in chemically castrated males a lower limit of quantification of 50 $pg.mL^{-1}$ was required.

One of the major problems in measuring such low levels is obtaining “blank” serum, with even normal serum concentrations in female humans being 150 to 700 $pg.mL^{-1}$[115]. The aim of this investigation was to assess the suitability of using equine (gelding) serum as a surrogate matrix to overcome the problem.

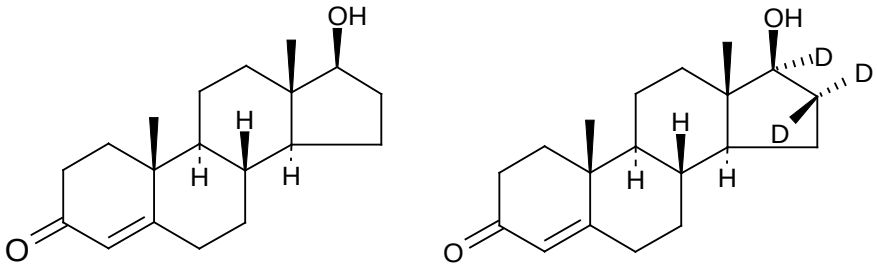


Figure 4.1.1 Structures of Testosterone and Testosterone-D_3

4.1.2 Experimental

4.1.2.1 Materials

Testosterone and testosterone-D_3 were supplied by Sigma-Aldrich (Poole, Dorset, UK). All reagents were of analytical grade and were supplied by Fisher Scientific UK (Loughborough, Leicestershire, UK). Genesis C18 columns (50 x 4.6 mm, 3 µm) were supplied by Grace Vydac (Carnforth, Lancashire, UK). Equine serum was supplied by B&K Universal (Aldbrough, Hull, UK). 96-well solid phase extraction plates (Isolute C18, 2 mL, 100 mg) were supplied by Argonaut (Hengoed, Glamorgan, UK).

4.1.2.2 Instrumentation

The system used for this work consisted of a PE Series 200 Micro-pump (Perkin Elmer, Thornhill, Ontario, Canada), an online DGU-14A degasser (Shimadzu, Kyoto, Japan), and a PE Series 200 Autosampler (Perkin Elmer, Thornhill, Ontario, Canada) connected

to a Perkin Elmer API 3000 triple quadrupole mass spectrometer. Chromatographic system control, data acquisition and analysis were performed by means of Analyst software version 1.2 (Applied Biosystems – MDS Pharma, UK).

4.1.2.3 Chromatographic Conditions

Testosterone and the internal standard, testosterone-D_3 were separated from matrix components using a Genesis C18 50 x 4.6 mm, 3 µm analytical column. The mobile phase was methanol - ammonium acetate (10 mM; pH native) (75:25, v/v), delivered at a rate of 1.0 $mL.min^{-1}$ through the analytical column. The retention times were approximately 2.0 min for both testosterone and testosterone-D_3.

4.1.2.4 Sample Preparation

A 500 µL sample was added to a polypropylene tube. 75 µL of acetonitrile were added to each sample. These were then vortex mixed, and left to stand for 10 min. The samples were then buffered with 1.0 mL ammonium acetate (10 mM; pH native), containing internal standard at 5 $ng.mL^{-1}$, vortex mixed and then centrifuged.

An Isolute C18 solid phase extraction plate was conditioned using 1 mL methanol, and then equilibrated using 1.0 mL of ammonium acetate (10 mM; pH native). The samples were then added to the plate and a very low vacuum applied to slowly load the sorbent material. The plate was then washed with 1 mL ammonium acetate (10 mM; pH native)

and the plate was fully dried. The plate was then washed with 1 mL hexane and the plate was again fully dried. The samples were eluted into a polypropylene collection plate using 2 aliquots of 800 µL methyl acetate and then evaporated to dryness under nitrogen at 40°C. The residue was redissolved in 200 µL methanol - ammonium acetate (10 mM; pH native) (50:50, v/v). The plate was then sealed, vortex mixed gently, centrifuged, and then submitted for LC-MS/MS analysis.

4.1.2.5 Mass Spectrometric Detection – Starting Conditions

A Perkin Elmer API 3000 triple quadrupole mass spectrometer was used in positive ion mode to generate protonated molecular ions $[M+H]^+$, using a pneumatically and thermally assisted electrospray interface (TurboIonSpray). The mass spectrometer was set up in multiple reaction monitoring mode to detect the protonated pseudo-molecular ions at *m/z* 289.2 (testosterone) and 292.2 (testosterone-D_3) *via* the first quadrupole filter (Q1), with collisionally induced fragmentation (using nitrogen) at Q2 (collision energy 40eV) and monitoring the most abundant product ions *via* Q3 at *m/z* 97.1 (testosterone) and 97.1 (testosterone-D_3). In order to aid evaporation, to optimise sensitivity of the assay, the TurboIonSpray drying gas was set at 400°C. Chromatographic peak areas were obtained for the transitions *m/z* 289.2→97.1 (testosterone) and *m/z* 292.2→97.1 (testosterone-D_3). Linear regression of response (peak area ratio testosterone:testosterone-D_3) against concentration, employing the reciprocal of concentration as weighting, was used to generate the calibration function

4.1.3 Results and Discussion

4.1.3.1 Analytical Approach

Testosterone-D_3 was used as the internal standard to help compensate for any potential inter-species variations in recovery, or matrix related modification of ionisation.

In order to compensate for the low levels of testosterone in anticipated sample studies, equine (gelding) serum was used as the matrix for the preparation of the calibration samples and for the LLOQ QC (50 $pg.mL^{-1}$). A number of samples of female human serum were screened and one with a relatively low concentration was selected as the low QC. This serum had additional quantities of testosterone added to it to provide the other QC concentrations. All QC tables include compensation for the endogenous amount of testosterone in this serum.

The validation consisted of three different batches run on separate days used for the accuracy and precision data and a fourth batch (also run on a separate day) to assess matrix related modification of ionisation.

4.1.3.2 Accuracy and Precision

The inter-batch precision of the analytical method was defined as the coefficient of variation of the observed concentration for all QC replicates analysed within batches designated as being part of the assessment of accuracy and precision, at each concentration level. For this to be acceptable, it was required to be less than, or equal to, 15% at all levels, other than at the LLOQ, at which precision was required to be less than, or equal to, 20%. The mean inter-batch accuracy was required to be 85 to 115% of the nominal concentration at all levels, other than at the LLOQ, at which accuracy could have been 80 to 120% of the nominal concentration.

Inter-batch precision ranged between 4.0% and 14.1%. Inter-batch accuracy ranged between 93.6% and 106.2%. The method was therefore acceptable at all QC levels. (Table 4.1.1).

Table 4.1.1 Inter-batch precision and accuracy of quality control sample data. N.B. The QC 2000, 12000 and 25000 have been corrected for the endogenous level (i.e. the mean endogenous level has been subtracted from the observed concentration).

Inter-batch precision and accuracy of quality control sample data										
	50 ($pg.mL^{-1}$)		E* ($pg.mL^{-1}$)		E* + 2000 ($pg.mL^{-1}$)		E* + 12000 ($pg.mL^{-1}$)		E* + 25000 ($pg.mL^{-1}$)	
Batch 1	55.5	111.0	372	-	2080	104.0	13750	110.0	27750	111.0
	53.8	107.6	352	-	2110	105.5	13450	107.6	27450	109.8
	48.0	96.0	322	-	2110	105.5	13450	107.6	27350	109.4
	38.9	77.8	349	-	2110	105.5	13750	110.0	26650	106.6
	50.2	100.4	439	-	2210	110.5	13050	104.4	27950	111.8
	43.5	87.0	358	-	2200	110.0	13550	108.4	27750	111.0
Batch 2	59.3	118.6	348	-	2050	102.5	10650	85.2	27050	108.2
	NR	-	325	-	1980	99.0	12550	100.4	26750	107.0
	32.8	65.6	360	-	1990	99.5	11850	94.8	27250	109.0
	48.2	96.4	304	-	1990	99.5	12450	99.6	25950	103.8
	40.7	81.4	313	-	2030	101.5	12450	99.6	26750	107.0
	54.2	108.4	319	-	1990	99.5	12150	97.2	26450	105.8
Batch 3	45.7	91.4	349	-	1990	99.5	12250	98.0	27050	108.2
	45.3	90.6	301	-	1980	99.0	12150	97.2	26050	104.2
	43.8	87.6	320	-	1850	92.5	12150	97.2	24650	98.6
	41.8	83.6	356	-	1870	93.5	12050	96.4	25650	102.6
	47.1	94.2	267	-	1860	93.0	12050	96.4	24350	97.4
	46.8	93.6	261	-	1850	92.5	11950	95.6	25250	101.0
Mean	46.8		334.2		2013.9		12538.9		26561.1	
S.D.	6.6		40.6		111.0		815.2		1055.5	
Precision (%)	14.1		12.1		5.5		6.5		4.0	
Accuracy (%)	93.6		N/A		100.7		104.5		106.2	

E* = Endogenous level in selected control female human serum. The mean has been subtracted from the higher QC levels to enable the calculation of accuracy.

4.1.3.3 Lower Limit of Quantification

The lower limit of quantification (LLOQ), which was defined as the lowest QC at which accuracy was within 20% of nominal and precision was no greater than 20% [116], was 50 pg.mL^{-1} (Figure 4.1.2). This range was suitable for the analysis of both "normal" and clinically suppressed levels of testosterone in samples.

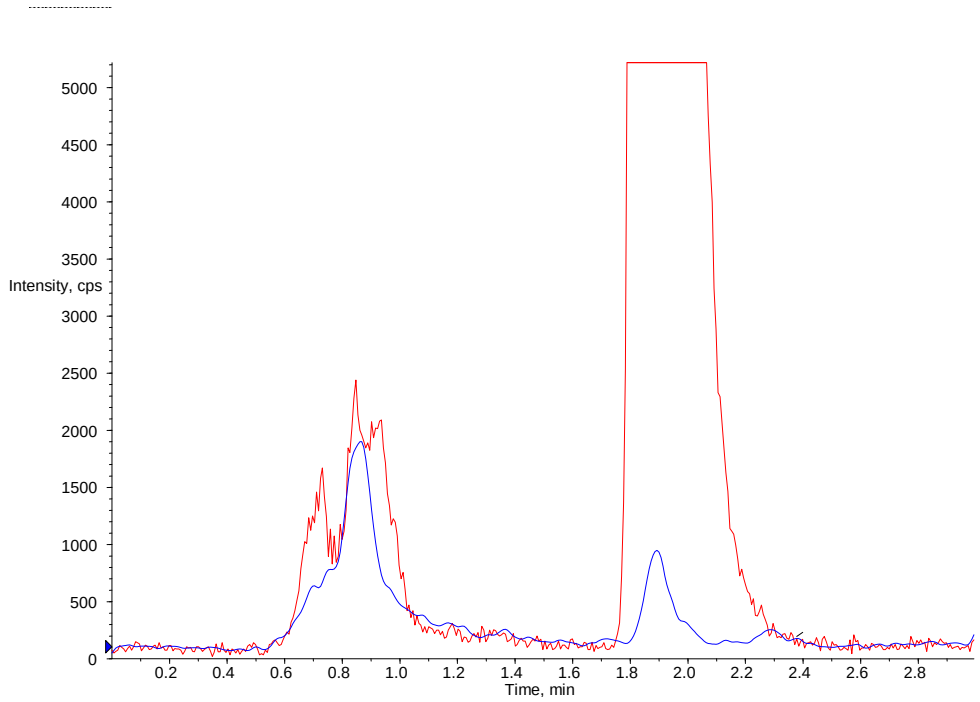


Figure 4.1.2 MRM chromatogram resulting from the analysis of a quality control sample at 50 pg.mL^{-1} HPLC system: PE Sciex API 3000 and PE Series pumps, SIM for testosterone and internal standard. Column Genesis C18 50 x 4.6 mm, 3 µm particles Mobile phase methanol - ammonium acetate (pH native; 10 mM), 75:25, v/v, flow rate 1 mL.min^{-1}. The blue trace shows the response for testosterone at the LLOQ, with the red trace showing the deuterated internal standard.

4.1.3.4 Linearity

The range was selected following the preliminary analysis data. Linearity was demonstrated over the range 50 $pg.mL^{-1}$ to 25000 $pg.mL^{-1}$, using a linear regression of peak area ratio (testosterone:internal standard) versus concentration, using the reciprocal of concentration as weighting, as this is considered likely to provide the best fit of data over the dynamic range investigated [117, 118].

All coefficients of determination (r^2) of the calibration lines obtained during the validation were equal to, or better than, 0.9962.

Linearity data for all calibration lines used in the assessment of inter-batch precision and accuracy are shown in Table 4.1.2.

Table 4.1.2 Inter-batch precision and accuracy of calibration sample data.

N.B. All calibration samples are prepared in equine (gelding) serum.

Inter-batch precision and accuracy of calibration sample data								
Nominal Concentration ($pg.mL^{-1}$)	50	100	500	2000	10000	15000	22500	25000
Batch 1	40.7	101	541	2010	11400	15300	21500	24300
Batch 2	51.0	106	492	1850	10000	15000	22900	24800
Batch 3	52.5	98.1	524	1870	9580	15000	24000	23900
Mean ($pg.mL^{-1}$)	48.1	102	519	1910	10300	15100	22800	24300
Standard Deviation (n-1)	6.42	4.00	24.9	87.2	953	173	1250	451
Precision (%)	13.3	3.9	4.8	4.6	9.3	1.1	5.5	1.9
Accuracy (%)	96.2	102.0	103.8	95.5	103.0	100.7	101.3	97.2

Curve parameters			
	Gradient (m)	Intercept (c)	Coefficient of Determination
Batch 1	0.000089	0.00174	0.9962
Batch 2	0.000098	0.00198	0.9998
Batch 3	0.000100	0.00263	0.9976

4.1.3.5 Stability

Freeze / Thaw Stability

Stability investigations were performed on serum that had undergone an additional three freeze / thaw cycles (above the single cycle that all quality control samples were given following preparation). Comparison of the freeze / thaw investigation samples with samples which had only undergone a single cycle (run in the same analytical batch) showed changes from baseline of ≤8.8%. Each freeze/thaw cycle consisted of a thaw of not less than 2 hours, where the plasma was visibly confirmed to have been thawed and a freeze of not less than 24 hours at -20°C.

These results showed that testosterone is stable in serum, when exposed to at least four freeze / thaw cycles.

Freeze/thaw stability data are shown in Table 4.1.3.

Table 4.1.3 Freeze/thaw stability of testosterone. Baseline QC samples underwent a single freeze/thaw cycle, with the stability QCs being subjected to an additional 3 freeze/thaw cycles (i.e. a total of 4 cycles). All samples were extracted and run in the same analytical batch.

Quality control level	Baseline QC samples		QC samples subjected to 3 additional freeze/thaw cycles prior to extraction		Difference from baseline (%)
	Observed concentration ($pg.mL^{-1}$)	Mean ($pg.mL^{-1}$) (CV%)	Observed concentration ($pg.mL^{-1}$)	Mean ($pg.mL^{-1}$) (CV%)	
LoQC	372 352 322 349 439 358	365 (10.8)	329 330 307 357 325 351	333 (5.5)	-8.8
MeQC	2430 2460 2460 2460 2560 2550	2490 (2.2)	2520 2540 2480 2590 2540 2480	2530 (1.7)	1.6
HiQC	14100 13800 13800 14100 13400 13900	13900 (1.9)	14400 14100 14000 14000 13700 14000	14000 (1.6)	0.7

Room Temperature In-Matrix Stability

The stability of testosterone in serum, stored at room temperature for 24 h before being frozen, was assessed. Comparison of the concentrations of testosterone observed in investigational samples stored at room temperature with those observed in samples which were frozen immediately after preparation (run in the same analytical batch) showed changes from baseline of ≤6.7%. These results showed that testosterone is stable in serum for at least 24 h when stored at room temperature.

Room temperature in-matrix stability data are shown in Table 4.1.4.

Table 4.1.4 Room temperature stability of testosterone. Baseline QC samples were frozen immediately following preparation, with the stability QCs being stored at room temperature for 24 hours prior to storage. All samples were extracted and analysed in the same analytical batch.

Quality control level	Baseline QC samples		QC samples subjected to approximately 24 hours storage at room temperature		Difference from baseline (%)
	Observed concentration ($pg.mL^{-1}$)	Mean ($pg.mL^{-1}$) (CV%)	Observed concentration ($pg.mL^{-1}$)	Mean ($pg.mL^{-1}$) (CV%)	
LoQC	348 325 360 304 313 319	328 (6.6)	321 420 322 359 319 358	350 (11.2)	6.7
MeQC	2400 2330 2340 2340 2380 2340	2360 (1.2)	2370 2510 2290 2270 2430 2480	2390 (4.1)	1.3
HiQC	11000 12900 12200 12800 12800 12500	12400 (5.8)	12700 12300 12400 12400 13100 12600	12600 (2.3)	1.6

Stability of Prepared Samples

Samples that had undergone the sample preparation procedure were left at room temperature for 1 week before being subjected to the LC-MS/MS analysis. Comparison of stored, prepared samples with freshly prepared samples (run in the same analytical run, with a freshly prepared calibration line) showed changes from baseline of ≤0.9%. Prepared samples were therefore stable when stored at room temperature, for at least one week.

Stability data for samples stored at room temperature for 1 week before analysis is shown in Table 4.1.5.

Table 4.1.5 Extract stability of testosterone. Baseline QC samples were injected immediately following preparation. Stability QC sample extracts were stored at room temperature for 7 days prior to injection (in the same analytical batch).

Quality control level	Baseline QC samples		QC sample extracts subjected to 7 days' room temperature storage		Difference from baseline (%)
	Observed concentration ($pg.mL^{-1}$)	Mean ($pg.mL^{-1}$) (CV%)	Observed concentration ($pg.mL^{-1}$)	Mean ($pg.mL^{-1}$) (CV%)	
LoQC	349	309 (13.0)	342	308 (13.8)	-0.3
	301		363		
	320		307		
	356		240		
	267		302		
	261		295		
MeQC	2340	2250 (2.9)	2390	2270 (3.7)	0.9
	2330		2330		
	2200		2230		
	2220		2290		
	2210		2210		
	2200		2160		
HiQC	12600	12500 (0.8)	12800	12600 (2.4)	0.8
	12500		13100		
	12500		12400		
	12400		12300		
	12400		12400		
	12300		12700		

4.1.3.6 Specificity

The limitations imposed by investigating specificity with an endogenous analyte, principally involving the problems associated with getting a sample clear of the analyte, were considered when designing the specificity investigation. Taking these into account equine (gelding) serum blanks that had been prepared in the same manner as quality control samples were investigated for the presence of interfering peaks in the MRM chromatograms at retention times corresponding to testosterone or testosterone-D_3. No interfering peaks were detected.

Potential interference from other endogenous and commonly administered steroids was considered based on structural characteristics. The compounds, with their molecular masses for mass spectrometric determination in brackets, considered were aldosterone (360), 5β-androstan-3α,17β-diol (292), androstenedione (286), 5α-androstan-3β,17β-diol (292), 5α-androstan-3,17-dione (288*), 5-androsten-3β,17β-diol (290), androsterone (290), corticosterone (346), cortisol (362), cortisone (360), danazol (337), 11-deoxycortisol (346), dexamethasone (392), dehydroepiandrosterone (288*), dehydroepiandrosterone sulphate (288*), 5α-dihydrotestosterone (290), estradiol (272), estrone (270), ethisterone (312), fluoxymesterone (336), 11-ketotestosterone (304), methyltestosterone (302), norethindrone (298), norethynodrel (298), prednisone (358), progesterone (314), spironolactone (416) and triamcinolone (394). These were considered as they are the steroids specified in standard immuno-assay diagnostic tests for testosterone [119, 120, 121]. Where the molecular mass is at least 1 mass unit

different to testosterone (288) or testosterone-D_3 (291) there is no significant likelihood of producing any parent ion that would interfere with these analytes. Where the steroid's molecular mass is within 1 mass unit (those indicated by *), the structures indicate that they would not produce the product ion (containing the double bond at carbon 4) with a significant response.

These investigations indicated that the method was specific enough for the determination of testosterone in human serum.

4.1.3.7 Matrix-related modification of ionisation

A calibration line was prepared in each of six different human (female) sera. They were analysed according to the method, and their regression characteristics were calculated. The slopes of the lines were compared with that from a calibration line prepared in control equine (gelding) serum. Differences of less than or equal to ±15% were considered to be acceptable, within the predefined acceptance criteria.

All of the individual calibration lines met this acceptance criterion (a maximum of 5.9% difference was observed).

Matrix-related modification of ionisation data are shown in Table 4.1.6.

Table 4.1.6 Matrix-related modification of ionisation. A comparison of the regression lines produced by preparing calibration standards in a range of female human urines with a line compared in equine (gelding) serum.

Curve parameters					
Matrix	Endogenous level ($pg.mL^{-1}$)	Gradient (m)	Intercept (c)	Coefficient of Determination (r^2)	Difference in slope from equine line (%)
Eq/S/01/002	-	0.000096	0.0018	0.9988	-
Hu/S/01/001	335	0.000100	0.0335	0.9986	4.0
Hu/S/01/002	471	0.000100	0.0470	0.9996	4.0
Hu/S/01/003	199	0.000100	0.0199	1.0000	4.0
Hu/S/01/004	387	0.000099	0.0382	0.9994	3.0
Hu/S/01/005	202	0.000102	0.0206	0.9994	5.9
Hu/S/01/006	248	0.000100	0.0248	0.9992	4.0

4.1.4 Conclusion

The LC-MS/MS method developed for the rapid and simple determination of testosterone in human serum not only complied with international standards [122] on all aspects of its validation but also delivered the anticipated advantages over existing methodology [123] with respect to sensitivity for measuring clinically suppressed levels of testosterone. This would be useful in dealing with studies of appropriate size to give convincing statistical power and, indeed, the method has already been used in the analysis of samples from a large clinical study, in which testosterone levels have been measured in patients undergoing treatment for prostate cancer. As a result of this major clinical study, the method has been shown to be suitable for the routine monitoring of clinically suppressed serum testosterone levels.

The results of this investigation provide good evidence to suggest that adoption of this approach to overcoming the problem of developing a robust method for the analysis of an endogenous analyte was both suitable in this case and may be applicable for more widespread use, where the target analyte can be isolated by use of a surrogate matrix. Due to difficulties in obtaining suitable surrogate matrices possessing significant similarities to the test matrix, but with the target analyte either absent, or present in insignificant levels, this is likely to remain a relatively rare, though extremely useful when applicable, solution to the problem of analysis of endogenous analytes.

4.2 Development and Validation of an Analytical Method for the Determination of Indolyl-3-AcryloylGlycine in Human Urine using LC-MS/MS

4.2.1 Introduction

Indolyl-3-acryloylglycine (IAG) (Fig. 4.2.1) has been implicated in learning disorders such as core autism, atypical autism and Asperger's Syndrome (a range of developmental disorders in that sufferers display abnormal and/or impaired development in social interaction, communication, and restrictive, stereotyped, repetitive behaviour). Atypical autism is diagnosed most readily in profoundly retarded individuals whose very low level of functioning provides little scope for exhibition of the specific deviant behaviours required for the diagnosis of autism. Persons with Asperger's Syndrome show marked deficiencies in social skills and have difficulties with changes in routine or environment. They frequently have obsessive routines and are obsessive about a particular subject of interest. They have a great deal of difficulty in interpreting body language.

There has been significant research on trying to establish biomarkers for these conditions [124] in order to both investigate the disorders at an early stage and to try to determine the metabolic causes of the disorders. It has been postulated that high urine levels of IAG, resulting from abnormal tryptophan metabolism, indicates an increase in the permeability of the gut wall and blood brain barrier to exogenous peptides [125, 126]. According to this hypothesis the increase in these exogenous peptides may result in

effects which are opioid-like in nature, leading to learning difficulties. Presence of an elevated level of IAG in the urine has been reported in autistic children [127] and it has been postulated that IAG may be diagnostic for these conditions [128]. The aim of this study therefore was to develop an analytical method suitable for monitoring levels of IAG in urine in the types of large clinical studies that would be needed to establish once and for all whether IAG is a biomarker for autism spectrum disorders. Such a method would need to be not only specific to IAG but also be sufficiently sensitive to be able to quantify IAG in both anticipated elevated levels in samples from autistic children and possible lower levels in samples from non-autistic "controls".

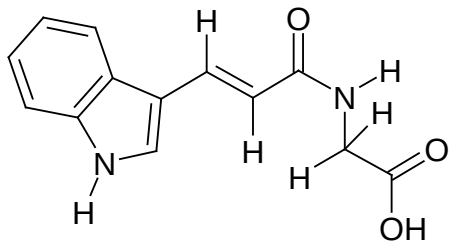


Figure 4.2.1 Structure of indolyl-3-acryloylglycine

4.2.2 Experimental

4.2.2.1 Materials

A research group based at the Child and Adolescent Mental Health Unit, University of York, which included representatives of the University of York Chemistry and Biochemistry departments was formed for a range of investigations into the biochemical pathways of autism spectrum disorders. IAG was synthesised by Professor D.K. Smith of the University of York, using a method which involved esterification of indole-3-acrylic acid with glycine methyl ester using dicyclohexylcarbodiimide and hydroxybenzotriazole in dichloromethane [129].

In addition, a dideuterated analogue of IAG (d_2-IAG) was synthesised using the same route from glycine-2,2-d_2 methyl ester, which was synthesised from glycine-2,2-d_2 [116].

4.2.2.2 Instrumentation

The system used for this work consisted of a PE Series 200 Micro-pump (Perkin Elmer, Thornhill, Ontario, Canada), an on-line DGU-14A degasser (Shimadzu, Kyoto, Japan), and an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) connected to a Perkin Elmer API 3000 triple quadrupole mass spectrometer (Perkin Elmer, Thornhill, Ontario, Canada). Chromatographic system control, data acquisition and analysis were performed by means of Analyst software version 1.2 (Applied Biosystems – MDS Sciex, UK).

4.2.2.3 Chromatographic Conditions

Indolyl-3-acryloylglycine and d_2-indolyl-3-acryloylglycine were separated from matrix components using a Luna phenyl-hexyl analytical column (50 mm x 4.6 mm, 3 µm particle, Phenomenex, UK). The mobile phase was a mixture of 30% acetonitrile and 70% water containing 0.1% formic acid and was delivered at a flow rate of 1 $mL.min^{-1}$. The retention time for both IAG and d_2-IAG was 1.6 min.

4.2.2.4 Sample Preparation

An aliquot of 20 µL of urine was taken from a thoroughly mixed sample tube and added to a polypropylene tube. Internal standard (d_2-IAG, 20 µL of a 10 $\mu g.mL^{-1}$ solution) was added to each tube. 1 mL of mobile phase (acetonitrile - 0.1% *v/v* formic acid, 30:70, *v/v*) was added to each tube. The tubes were then vortex mixed and an aliquot transferred to a 96-well polypropylene injection plate ready for LC-MS-MS analysis. All sample preparation was performed under monochromatic (sodium) lighting to prevent isomerisation between the *trans* and *cis* forms of IAG.

4.2.2.5 Tandem Mass Spectrometric Detection

A Sciex API 3000 triple quadrupole mass spectrometer was used with a TurboIonSpray (temperature and pneumatically assisted electrospray) interface used in the negative ion

mode to generate deprotonated molecular ions $[M-H]^+$. The mass spectrometer was set up in multiple reaction monitoring mode to detect the deprotonated pseudo-molecular ions at *m/z* 243.2 (IAG) and 245.2 (d_2-IAG) *via* the first quadrupole filter (Q1), with collisionally induced fragmentation (using nitrogen) at Q2 (collision energy 26eV) and monitoring the product ions *via* Q3 at *m/z* 142.0 (IAG) and 142.0 (d_2-IAG). In order to aid ion evaporation, to optimise sensitivity of the assay, the TurboIonSpray was set at 400°C. Chromatographic peak areas were obtained for the transitions *m/z* 243.2→142.0 (IAG) and *m/z* 245.2→142.0 (d_2-IAG). Linear regression of response (peak area ratio IAG:d_2-IAG) against concentration, employing the reciprocal of concentration as weighting, was used to generate the calibration function.

4.2.3 Results and Discussion

4.2.3.1 Analytical Approach

A routine assay for indolyl-3-acryloylglycine in urine had been reported [129], which employed HPLC with UV detection at 326 nm. However, given the power of modern methods in LC-MS/MS it was felt that there was considerable scope for improving upon this method for bioanalysis. For example, although indolyl-3-acryloylglycine provides a strong signal at 326 nm there are many potential interfering species which may not be discriminated against with this relatively non-specific technique. LC-MS/MS is a highly specific technique, with extremely high sensitivity, which would be expected to provide a robust assay, which would dramatically reduce the likelihood of false readings being

reported when analysing patient samples. Because of this it would more likely be possible to fulfil the objective of developing and validating a method to match and hopefully exceed rigorous, internationally recognised, standards [74].

Perhaps as importantly, one of the main advantages of using LC-MS/MS is the ability to utilise low run times, with the associated high throughput, which may be required for studies with large numbers of samples, which are required to provide appropriate statistical power. A further advantage of this methodology is the availability of dideuterated IAG to be used as the internal standard. Using a stable, isotopically labelled analogue of the primary analyte as the internal standard would provide a huge degree of confidence that most of the potential causes of inaccuracy (such as extraction, adsorption and particularly modification of ionisation issues) would be eliminated.

Sample preparation was kept to a minimum, as the instrument response for the analytes was extremely high. Therefore there was no requirement to remove interfering species and simple dilution was used.

Preliminary analyses indicated that, as had been experienced by Mills *et al* [129], IAG was found in all human urine samples (autistic and non-autistic). The preliminary analyses involved over 20 different urines, from males and females, of a wide range of adult ages and a number of ethnic backgrounds. A range of 1.26 to 13.7 $\mu g.mL^{-1}$ was found in these non-autistic, adult samples. It was therefore necessary to employ non-matrix calibration standards, having first established that urine components did not

significantly affect the response of IAG (Section 4.2.4.7). In addition, the use of a dideuterated internal standard was adopted in order to assist in compensating for any variability in individual matrix sources, by eliminating any matrix-related modification of ionisation.

The validation exercise consisted of three different batches run on separate days used to assess the accuracy and precision of the method, a fourth batch (also run on a separate day) to assess matrix-related modification of ionisation and a fifth batch to assess the stability of IAG in urines at various pH values. This assessment of stability at various pH values was in excess of the requirements of the stated validation standards [116], but was included to ensure the robustness of the method.

4.2.3.2 Accuracy and Precision

The inter-batch precision of the analytical method was defined as the coefficient of variation of the observed concentration for all QC replicates analysed within batches designated as being part of the assessment of accuracy and precision, at each concentration level. For this to be acceptable, it was required to be less than, or equal to, 15% at all levels, other than at the LLOQ, at which precision was required to be less than, or equal to, 20%. The mean inter-batch accuracy was required to be 85 to 115% of the nominal concentration at all levels, other than at the LLOQ, at which accuracy could have been 80 to 120% of the nominal concentration.

Inter-batch precision ranged between 2.8% and 14.1%. Inter-batch accuracy ranged between 89.9% and 101.4%. The method was therefore acceptable at all QC levels (Table 4.2.1).

Table 4.2.1 Inter-assay accuracy and precision data for IAG in human urine. $^{+}$LoQC is blank urine (containing an endogenous level of 2.36 µg.mL^{-1}). QCs 30 and 70 are prepared from the same urine with the nominal concentration added.

Nominal concentration (µg.mL^{-1})	Mean observed concentration (µg.mL^{-1})	Precision (CV %)	Mean accuracy (%)	*n*
0.5	0.507	14.1	101.4	18
LoQC^{+}	2.36	9.0	n/a	18
30^{+}	29.1	10.9	89.9	18
70^{+}	70.8	7.5	97.8	18
100	101	2.8	101	18

$^{+}$ LoQC is blank urine (containing an endogenous level of 2.36 µg.mL^{-1}). QC 30 and QC 70 are prepared from the same urine with the nominal concentration added.

4.2.3.3 Lower Limit of Quantification

The lower limit of quantification (LLOQ), which was defined as the lowest QC at which accuracy was within 20% of nominal and precision was no greater than 20% [74], was 0.5 µg.mL^{-1} (Figure 4.2.2). This range comfortably encompassed the anticipated range of IAG in samples, which was determined based on a screen of samples from a range of subjects. (Sensitivity of the analytical technique was such that a far lower limit of

quantification could have been selected if the expected urine concentrations had been at a lower range.)

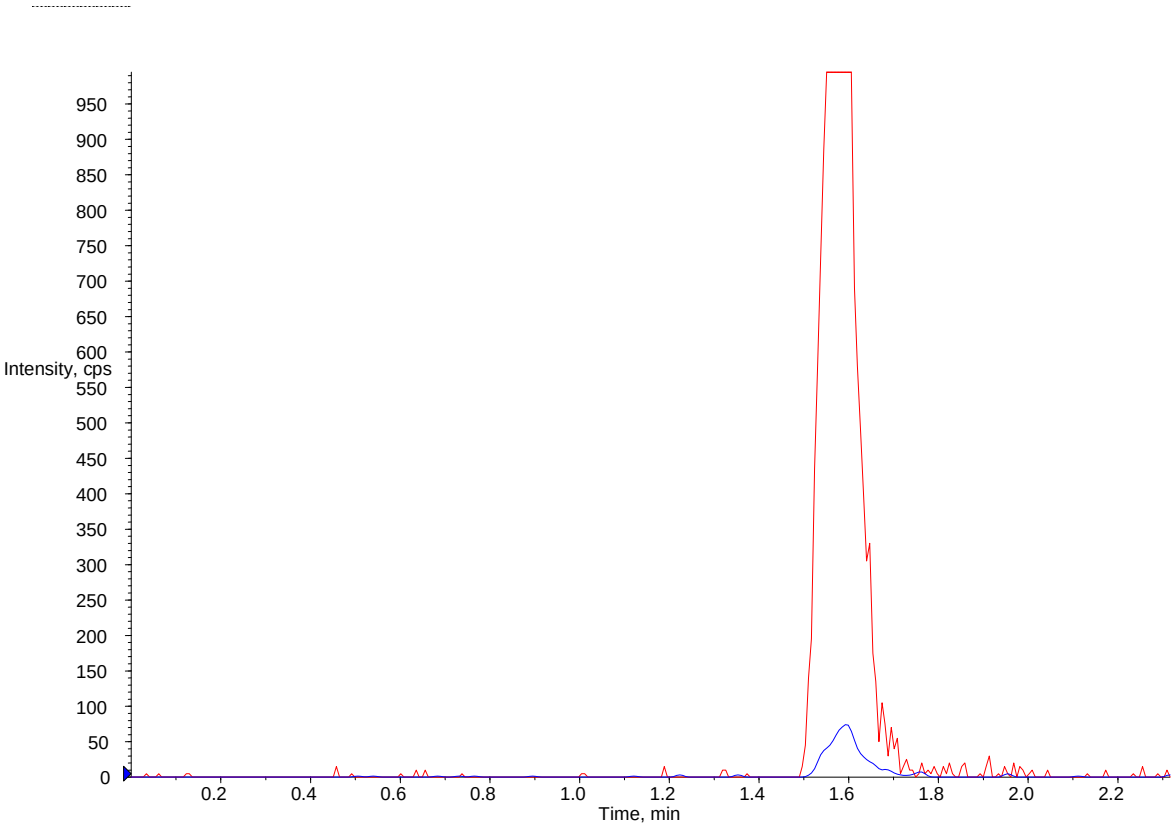


Figure 4.2.2 MRM chromatogram resulting from the analysis of a standard at 0.5 µg.mL^{-1} HPLC system: PE Sciex API III and PE Series 200 pumps, SIM for IAG and internal standard. Column Luna phenyl-hexyl 50 x 4.6 mm, 3 µm. Mobile phase acetonitrile - water - formic acid (30:70:0.1, v/v/v), flow rate 1.0 mL.min^{-1}. The trace shows the response for IAG at the LLOQ, with the red trace showing the deuterated internal standard.

4.2.3.4 Linearity

The range was selected following the preliminary analysis data. Linearity was demonstrated over the range 0.5 µg.mL^{-1} to 100 µg.mL^{-1}, using a linear regression of peak area ratio (IAG:internal standard) versus concentration, using the reciprocal of concentration as weighting as this is considered likely to provide the best fit of data over the dynamic range investigated [117].

Linearity data are shown in Table 4.2.2.

All coefficients of determination (r^2) of the calibration lines obtained during the validation were equal to, or better than, 0.9968.

Calibration curve data are shown in Table 4.2.3 and Figure 4.2.4.

Table 4.2.2 Precision and accuracy of calibration standard data for IAG in human urine.

Batch	Back-calculated concentrations (µg.mL^{-1})							
	Calibration level (µg.mL^{-1})							
	0.500	1.00	2.00	5.00	20.0	50.0	80.0	100
HuUV01	0.488	0.999	2.08	4.85	20.0	50.6	82.3	97.2
HuUV02	0.505	0.990	2.02	4.71	21.1	50.7	78.7	99.8
HuUV03	0.462	0.960	2.07	5.18	19.9	55.2	79.0	95.7
HuUV04	0.529	-	1.84	4.91	21	48.9	83.6	96.8
Mean (µg.mL^{-1})	0.496	0.983	2.00	4.91	20.5	51.4	80.9	97.4
Standard Deviation (n-1)	0.0282	0.0204	0.111	0.197	0.638	2.70	2.43	1.74
Precision (%)	5.7	2.1	5.6	4.0	3.1	5.3	3.0	1.8
Accuracy (%)	99.2	98.3	100.0	98.2	102.5	102.8	101.1	97.4

Table 4.2.3 Curve parameters of calibration standard regression lines for IAG in human urine.

Batch	Curve parameters		
	Gradient (m)	Intercept (c)	Coefficient of Determination
HuUV01	0.0849	0.007700	0.9994
HuUV02	0.0820	0.000176	0.9996
HuUV03	0.0821	0.012200	0.9968
HuUV04	0.0817	0.006100	0.9986
HuUV05i	0.0885	0.007260	0.9998

4.2.3.5 Stability

Freeze / Thaw Stability

Stability investigations were performed on urine that had undergone an additional three freeze / thaw cycles (above the single cycle that all quality control samples were given). Comparison of the freeze / thaw investigation samples with samples which had only undergone a single cycle (run in the same analytical batch) showed changes from baseline of ≤7.7%. These results showed that IAG is stable in urine, when exposed to at least four freeze / thaw cycles.

Freeze/thaw stability data are shown in Table 4.2.4.

Table 4.2.4 Stability of IAG in human urine following four freeze/thaw cycles.

Quality control level	Baseline QC samples		QC samples subjected to 3 additional freeze/thaw cycles prior to extraction		Difference from baseline (%)
	Observed concentration ($\mu g.mL^{-1}$)	Mean ($\mu g.mL^{-1}$) (CV%)	Observed concentration ($\mu g.mL^{-1}$)	Mean ($\mu g.mL^{-1}$) (CV%)	
LoQC	2.00 1.92 2.30 2.03 2.62 2.44	2.22 (12.6)	2.47 2.30 2.54 2.09 2.45 2.48	2.39 (7.0)	7.7
MeQC	34.6 35.7 31.8 30.1 36.1 33.5	33.6 (6.9)	30.8 - 33.6 34.5 26.9 33.9	31.9 (9.9)	-5.1
HiQC	79.6 80.2 64.5 71.3 73.5 72.0	73.5 (7.9)	56.8 72.8 69.0 76.3 69.7 67.6	68.7 (9.6)	-6.5

In addition, investigations were carried out into the stability of IAG in urines at pH 5, 6 and 7 following three additional freeze / thaw cycles. All differences from samples that had only undergone a single freeze / thaw cycle were ≤9.7%. These results showed that there was no significant change in the urinary IAG concentrations following these additional freeze / thaw cycles.

Room Temperature In-Matrix Stability

The stability of IAG in urine, stored at room temperature for 24 h before being frozen, was assessed. Comparison of the concentrations of IAG observed in investigational samples stored at room temperature with those observed in samples which were frozen immediately after preparation (run in the same analytical batch) showed changes from baseline of ≤3.8%. These results showed that IAG is stable in urine for at least 24 h when stored at room temperature.

Room temperature in-matrix stability data are shown in Table 4.2.5.

Table 4.2.5 Stability of IAG in human urine following storage at room temperature for 24 hours.

Quality control level	Baseline QC samples		QC samples subjected to 24 hours storage at room temperature		Difference from baseline (%)
	Observed concentration ($\mu g.mL^{-1}$)	Mean ($\mu g.mL^{-1}$) (CV%)	Observed concentration ($\mu g.mL^{-1}$)	Mean ($\mu g.mL^{-1}$) (CV%)	
LoQC	2.15 2.46 2.26 2.46 2.28 2.47	2.35 (5.8)	2.18 2.30 2.28 2.34 2.37 2.08	2.26 (4.8)	-3.8
MeQC	31.7 28.8 31.2 29.6 26.9 32.3	30.1 (6.8)	28.6 27.4 27.9 35.6 28.8 29.1	29.6 (10.2)	-1.7
HiQC	63.6 74.8 75.1 77.1 73.8 63.3	71.3 (8.6)	68.1 71.2 79.5 73.7 79.0 71.8	73.9 (6.1)	3.6

In addition, stability investigations were carried out in urines at pH 5, 6 and 7 which had been stored at room temperature for 24 h before freezing. All differences from samples that had been frozen immediately upon preparation were ≤6.1%. These results showed that there was no significant change in the urinary IAG concentrations following this additional room temperature storage.

The above investigations indicated that the pH of the urine (over the normal range expected in man) is not a significant factor in the stability of IAG.

Stability of Prepared Samples

Samples that had undergone the sample preparation procedure were left at room temperature for 1 week before being subjected to the LC-MS/MS analysis. Comparison of stored, prepared samples with freshly prepared samples (run in the same analytical run, with a freshly prepared calibration line) showed changes from baseline of ≤10.8%. Prepared samples were therefore stable when stored at room temperature, for at least one week.

Extract (prepared sample) stability data are shown in Table 4.2.6.

Table 4.2.6 Stability of IAG in human urine extracts following storage at room temperature for 1 week.

Quality control level	Baseline QC samples		QC sample extracts subjected to room temperature storage for 1 week		Difference from baseline (%)
	Observed concentration ($\mu g.mL^{-1}$)	Mean ($\mu g.mL^{-1}$) (CV%)	Observed concentration ($\mu g.mL^{-1}$)	Mean ($\mu g.mL^{-1}$) (CV%)	
LoQC	2.00	2.22 (12.6)	2.41	2.46 (4.3)	10.8
	1.92		2.65		
	2.30		2.50		
	2.03		2.39		
	2.62		2.36		
	2.44		2.47		
MeQC	34.6	33.6 (6.9)	29.2	30.3 (13.3)	-9.8
	35.7		31.9		
	31.8		35.3		
	30.1		33.4		
	36.1		27.6		
	33.5		24.3		
HiQC	79.6	73.5 (7.9)	73.4	73.8 (3.2)	0.4
	80.2		74.1		
	64.5		70.1		
	71.3		74.5		
	73.5		77.4		
	72.0		73.1		

4.2.3.6 Specificity

The limitations imposed by investigating specificity with an endogenous analyte, principally involving the problems associated with getting a sample clear of the analyte, were considered when designing the specificity investigation. Taking these into account reagent blanks that had been prepared in the same manner as quality control samples were investigated for the presence of interfering peaks in the MRM chromatograms at retention times corresponding to IAG or d_2-IAG. No interfering peaks were detected. This, combined with the investigations comparing a variety of matrices with samples prepared in solvent, which showed no differences in response, indicate that the method provided the required specificity for the determination of IAG in human urine.

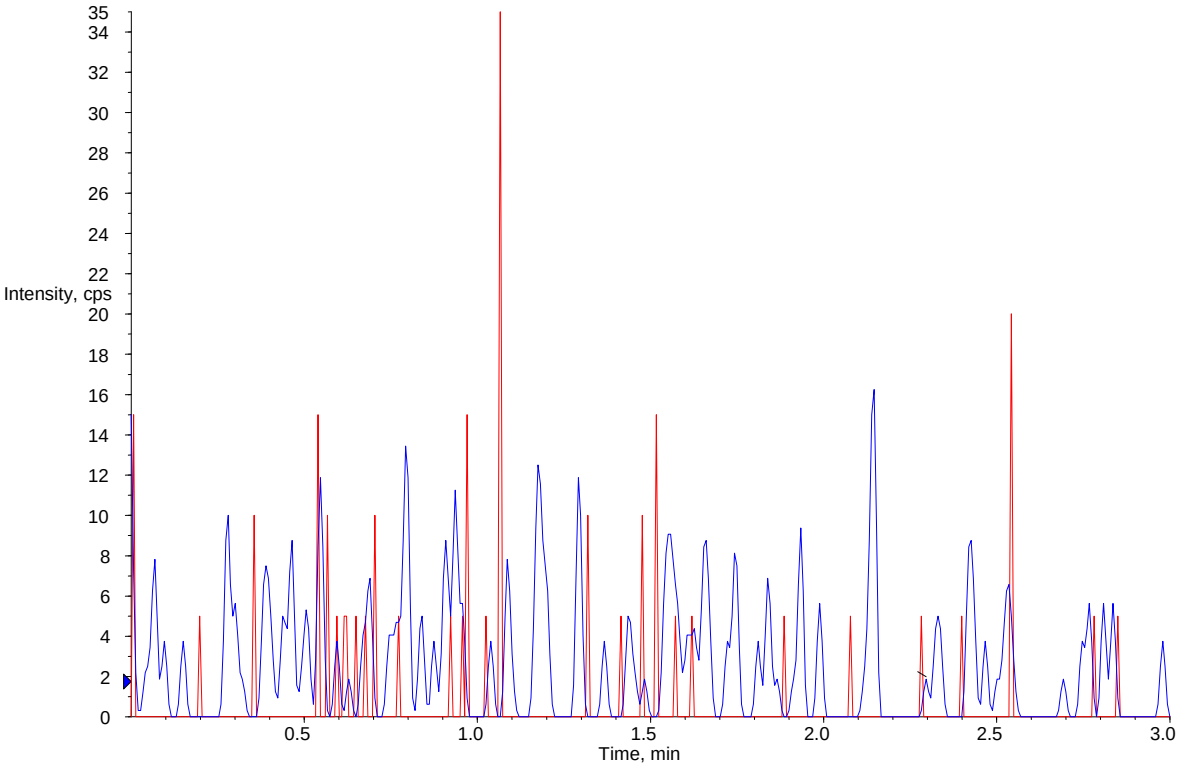


Figure 4.2.3 MRM chromatogram resulting from the analysis of a reagent blank. HPLC system: PE Sciex API III and PE Series 200 pumps, SIM for IAG and internal standard. Column Luna phenyl-hexyl 50 x 4.6 mm, 3 µm. Mobile phase acetonitrile - water - formic acid (30:70:0.1, v/v/v), flow rate 1.0 mL.min^{-1}. The blue trace shows the response for IAG at the LLOQ, with the red trace showing the deuterated internal standard.

4.2.3.7 Matrix-Related Modification of Ionisation

A calibration line was prepared in each of six individual human urines, with varying endogenous levels of IAG (the endogenous levels of IAG found in the six different urines

ranged from 2.98 to 14.7 µg.mL^{-1}), and the slope compared to that of a calibration line prepared in methanol – water (50:50, *v/v*). The slopes of all seven lines were extremely similar (the maximum difference in slope between the pure standard (calibration) line and the human urine lines was 4.1%), therefore providing evidence that there was no matrix-related modification of ionisation for IAG under the conditions being used (Figure 4.2.4, Table 4.2.7). This experiment also confirmed that the decision to use non-matrix calibration standards would provide calibration standards suitable for the determination of the levels of IAG in human urine samples.

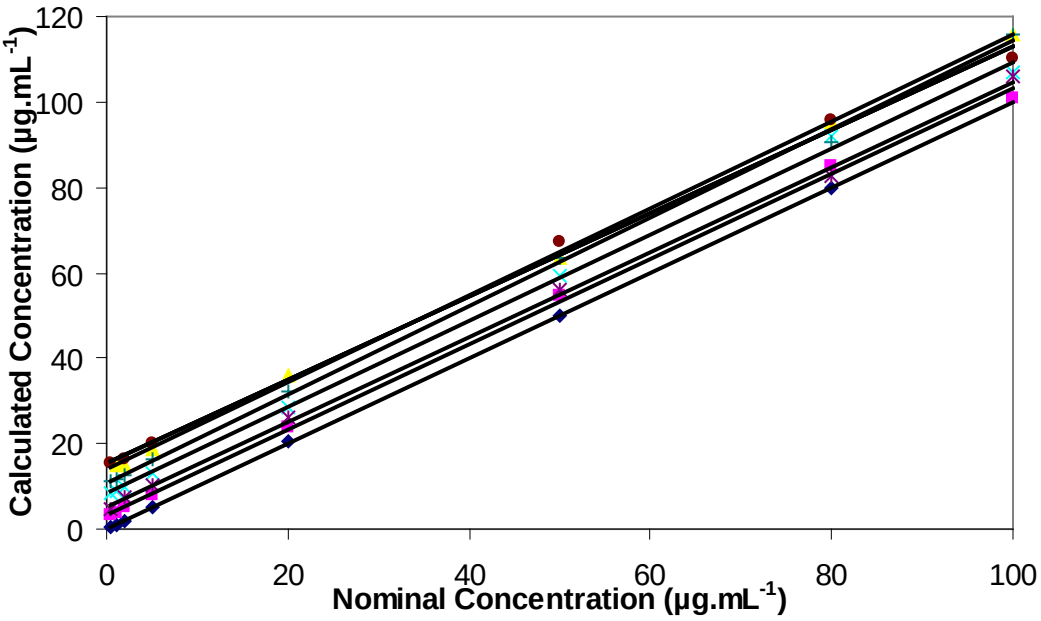


Figure 4.2.4 Calibration lines for matrix-related modification of ionisation test, illustrating similarity in slope at various endogenous levels. The similarity in the slopes shows that the response of IAG is linear with the same relationship in all cases. It is therefore acceptable to adopt the methodology of using non-matrix calibration standards.

Table 4.2.7 Matrix-related modification of ionisation data: comparison of calibration lines from different individual urines containing various endogenous levels of IAG.

Curve parameters					
Matrix	Endogenous level ($\mu g.mL^{-1}$)	Gradient (m)	Intercept (c)	Coefficient of Determination (r^2)	Difference from baseline (%)
Methanol-water, 1:1, *v/v*	n/a	0.0885	0.00726	0.9998	-
Urine 1	2.98	0.0894	0.271	0.9994	+1.0
Urine 2	13.7	0.0901	1.22	0.9976	+1.8
Urine 3	7.90	0.0905	0.706	0.9994	+2.3
Urine 4	4.57	0.0897	0.412	0.9974	+1.4
Urine 5	14.7	0.0881	1.31	0.9982	-0.5
Urine 6	10.7	0.0921	0.951	0.9992	+4.1

4.2.4 Conclusions

The LC-MS/MS method developed for the rapid and simple determination of IAG in human urine not only complied with international standards [74] on all aspects of its validation but also delivered the anticipated advantages over existing methodology [129] with respect to potential for high throughput, and freedom from potential interferences. This would be useful in dealing with studies of appropriate size to give convincing statistical power and, indeed, the method has already been used in the analysis of samples from a large clinical study, in which IAG levels have been compared between autistic children, parents and healthy siblings of autistic children, non-autistic learning disorder children and age/sex matched children from mainstream schools [130]. The method has been shown to be suitable for the routine monitoring of urinary IAG levels, if required. In principle a similar approach, i.e. using LC-MS/MS with a stable-isotopically-labelled internal standard and non-matrix calibration standards, could be used for biomarkers of other conditions.

While not the concern of this research programme this method has been used to indicate that the original premise, with regards to the relevance of IAG (at least on its own) was not supported.

Both approaches to the development of methods for the analysis of endogenous compounds described in this chapter have shown to be suitable for their respective analytes. Although most current research into endogenous analytes utilises immunoassay

methods, some work has been performed using LC-MS [131, 132]. These illustrate that the most important factor in choice of methodology is in the robustness of the resulting assay, which in the cases reported here was well within the accepted guidelines for the required analyses.

Chapter 5

Promotion of Ionisation: The Impact of Surfactants on the Ionisation Process

5.1 Introduction

A method for the determination of deltamethrin in bovine and ovine tissues had been developed and validated to comply with recognised regulatory requirements for the monitoring of residue levels of agricultural chemicals in animal tissues [133]. During the validation process nominal recovery values in excess of 100% had been observed in liver tissues for both species (when the actual recoveries in muscle, kidney and skin/fat had been in the region of 70% - as confirmed by absolute recovery determinations undertaken as part of the validation). Recoveries of over 100% can be the result of methodological errors or as a result of promotion of ionisation. As the phenomenon of promotion of ionisation is so rarely observed this was considered sufficiently unusual to warrant further investigation.

It had been postulated by the author that the reason for this phenomenon was that the high concentrations of surfactants that are present in the liver [134] affect the ionisation process, by affecting the properties of the solvent, interferents and/or analyte in solution, thereby allowing the promotion of ionisation relative to that for samples extracted from other tissues. The mechanism for this may have been that the purpose of the main group of liver surfactants – the bile salts – is to emulsify fats and oils into smaller droplets, which can then be broken down enzymatically. The presence of these fats and oils may have been affecting the ionisation process, causing ion suppression in the absence of these surfactants.

Almost all examples of matrix-related modification of ionisation in the current literature refer to suppression of the analyte signal caused by co-eluting or late eluting interferents [64, 135]; therefore this situation whereby matrix components might have been responsible for promotion of ionisation , if occurring would be extremely unusual. The first aim of this programme of investigations was to confirm that this method included promotion of ionisation. Once confirmed the aim was to establish the reason for the phenomenon. Finally, further investigation of this phenomenon was proposed to determine whether knowledge of the mechanism could be utilised as a generic mechanism for the promotion of ionisation of multiple analytes.

It was therefore sought to determine whether this premise had any validity. In order to fulfil this general aim a series of controlled experiments were performed.

5.2 Experimental

5.2.1 Materials

Deltamethrin was supplied by Sigma-Aldrich (Poole, Dorset, UK). Analytical grade acetonitrile, methanol and n-hexane were supplied by BDH Merck (Poole, Dorset, UK). Analytical grade ammonium acetate and anhydrous sodium sulphate were supplied by Fisher Scientific UK (Loughborough, Leicestershire, UK). A column packed with base-deactivated silica material, with phenyl side chains (Hypersil BDS phenyl, 50 x 4.6 mm, 5 μm) was supplied by ThermoHypersil (Runcorn, Cheshire, UK). Isolute C2 96-well extraction blocks were supplied by Argonaut (Hengoed, Pontypridd, UK).

Sodium 1-dodecanesulphonate, benzyldimethyl tetradecylammonium chloride, polyoxyethylene 6 lauryl ether and 3-(decyldimethylammonio) propanesulphonate inner salt (Zwittergent 3-10) were supplied by Sigma-Aldrich (Poole, Dorset, UK).

5.2.2 Instrumentation

The system used for this work consisted of a PE Series 200 Micro-Pump (Perkin-Elmer, Thornhill, Ontario, Canada), an on-line DGU-14A degasser (Shimadzu, Kyoto, Japan), and an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) connected to a Perkin Elmer API 3000 triple quadrupole mass spectrometer. Chromatographic system

control, data acquisition and analysis were performed by means of Analyst software version 1.2 (Applied Biosystems-MDS Sciex, UK).

5.2.3 Chromatographic conditions

Deltamethrin was separated from matrix components using a Hypersil BDS phenyl analytical column. The mobile phase was methanol – 10 mM aqueous ammonium acetate (78:22, v/v) and was delivered at a flow rate of 1 mL/min. The retention time for deltamethrin was 1.9 min.

5.2.4 Sample preparation

Sample preparation was in three stages, i.e. homogenisation, liquid extraction, and then solid phase clean-up. A standard procedure was used for each stage:

5.2.4.1 Sample Homogenisation

Control liver tissues were cut into small pieces (approximately 1 cm^2) and placed into a food processor to which dry ice was added. The food processor was turned on and the mixture left to run until the sample had been reduced to a fine powder. The sample was then transferred to a polypropylene storage pot and placed in a freezer at a nominal

temperature of -20°C, with the lid placed loosely to allow sublimation of the dry ice overnight.

5.2.4.2 Liquid Extraction

An aliquot of 1 g of tissue was weighed into a glass tube. To this was added 1 g of anhydrous sodium sulphate and the sample was then vortex mixed. 7 mL of acetonitrile was added to each tube, then the samples were macerated using an Ultra-Turrax mixer for approximately 30 seconds. The samples were then centrifuged at 3000 rpm for 5 minutes at 20°C. 4.9 mL of the supernatant was transferred into a fresh tube, then 0.5 mL of water was added to the tubes and the samples were evaporated in a TurboVap at 55°C until about 2-300 μL remained. 200 μL of acetonitrile was added to the tubes, which were then vortex mixed for approximately 30 seconds. Samples were then sonicated for 5 minutes, then 0.8 mL of de-ionised water was added and the tubes were sonicated for a further minute.

5.2.4.3 Solid Phase Extraction

The wells of an Isolute C2, 50 mg 96-well SPE plate were conditioned using 1 mL of acetonitrile, then equilibrated with 1 mL of acetonitrile – 10 mM ammonium acetate (80:20, v/v) without allowing the packing material to dry. The prepared samples were then added to the plate and the sample tubes were washed with 0.5 mL of water, which

was then added to each well and mixed with the sample using a Pasteur pipette. The samples were then drawn through the SPE packing material under gentle vacuum. The wells were then washed by the addition of 1 mL of acetonitrile – 10 mM ammonium acetate (80:20, v/v) to each well. This was drawn through the plate under vacuum and the wells allowed to dry. The analyte was eluted into a collection plate using two separate aliquots of 0.4 mL of acetonitrile – 10 mM ammonium acetate (20:80, v/v). 150 μL of ammonium acetate (10 mM) was added to each well and to the wells containing calibration standards, which had not undergone the extraction procedure. The collection plate was sealed and vortex mixed prior to submission for mass spectrometric analysis.

5.2.5 Tandem Mass Spectrometric Detection

A Sciex API 3000 triple quadrupole mass spectrometer was used with an atmospheric pressure chemical ionisation (APCI) interface used in positive ion mode to generate protonated molecular ions $[M+H]^+$. The mass spectrometer was set up in multiple reaction monitoring mode to detect the protonated pseudo-molecular ions at *m/z* 523.0 *via* the first quadrupole filter, with collisionally induced fragmentation (using nitrogen) at Q2 (collision energy 25 eV) and monitoring the product ions *via* Q3 at *m/z* 281.0. The APCI was set at 350°C. Chromatographic peak heights were obtained for the transition *m/z* 523.0→281.0. Linear regression of response (peak height) against concentration was used to generate the calibration function.

5.3 Results and Discussion

5.3.1 Analytical Approach

The first phase of the study was to confirm that the originally observed promotion of ionisation was reproducible. The original sample preparation methodology as listed above had been used to validate an analytical method for the determination of deltamethrin to match and exceed rigorous, internationally recognised standards [122]. In order to confirm the phenomenon that had been seen during this validation, further liver samples, together with muscle (which had seen no apparent promotion of ionisation during the original validation) samples were prepared using this methodology.

Following the premise that surfactants had been responsible for the observed high recoveries in the validated method, it was decided to add varying amounts of different surfactants to the sample extracts to determine whether any difference in response was created. Accordingly a number of extracts of both liver and muscle, spiked to common concentrations, were prepared according to the method listed. To these extracts were added quantities of the four main sub-groups of surfactant – anionic, cationic, zwitterionic and non-ionic, to result in concentrations within the samples that were both below the critical micelle concentration (cmc) and above the cmc. (The principal surfactants expected to be present in liver are the anionic surfactants glycocholic acid and taurocholic acid) [136].

An example from each of the four main groups of surfactant was selected for investigation (i.e. all main types of surfactant rather than just the types expected in liver). They were prepared in ammonium acetate (10 mM) in the concentrations listed, then added to the final extracts detailed in the sample preparation section of the method in a ratio of 1:1, resulting in the final surfactant concentrations as indicated in Table 5.1.

Table 5.1 Concentrations of surfactants investigated for modification of ionisation. [137, 138, 139]

Surfactant	Alternative Name	Type of Surfactant	Critical Micelle Concentration (mM)	Sample Concentrations (mM) (Above CMC)	(Below CMC)
Sodium 1-dodecanesulphonate	SDS	Anionic	7.2	10	1
Benzyldimethyltetradecyl ammonium chloride	Zephiramine	Cationic	0.37	2.5	0.05
N-decyl,-N, N-dimethyl-3-ammonio-1-propane	Zwittergent 3-10	Zwitterionic	25-40	50	0.25
Polyoxyethylene 6 lauryl ether	Dodecylhexaglycol	Anionic	0.087	0.1	0.005

As well as the samples to determine the effect of adding surfactant to the sample extracts, a number of samples were extracted according to the original sample preparation methodology. In addition, control matrix samples were extracted according to the usual sample preparation method and then spiked with deltamethrin to a concentration equivalent to 100% recovery through the extraction.

5.3.2 Statistical Treatment of Data

In the following discussion sections the statistical significance of the relationship between sample sets is discussed. The following formulae [140] were used for these comparisons:

To determine whether the standard deviations of two sample sets are equivalent the F-test was used.

Equation 1: $F = s_1^2/s_2^2$

Where s_1 and s_2 are the standard deviations of the sample sets 1 and 2.

If the standard deviations are not significantly different as determined by equation 1, then the standard deviations are pooled using equation 2 and means are compared using the t-test detailed in equation 3.

Equation 2: $s^2=\{(n_1-1)s_1^2+(n_2-1)s_2^2\}/(n_1+n_2-2)$

Equation 3: $t = (x_1-x_2)/s\sqrt{(1/n_1+1/n_2-2)}$

Where t has $n_1 + n_2 - 2$ degrees of freedom.

Where the standard deviations are significantly different, then equation 3 is modified to equation 4 and the number of degrees of freedom is calculated by equation 5;

Equation 4: $t = (x_1-x_2)/\sqrt{(s_1^2/n_1+s_2^2/n_2)}$

Equation 5: $d.f. = \{(s_1^2/n_1 + s_2^2/n_2)^2/[(s_1^2/n_1)^2/(n_1+1) + (s_2^2/n_2)^2/(n_2+1)]\} - 2$

With the result rounded to the nearest whole number.

5.3.3 Promotion of Ionisation in Liver Extracts

Comparison of the quality control samples that underwent the normal extraction procedure with samples of extracted control matrix that were spiked to concentrations equivalent to 100% recovery was made. In this way the absolute recovery of deltamethrin from the quality control samples could be assessed.

5.3.3.1 Muscle

The observed recovery for the muscle samples was 62.1% (Table 5.2), with the spiked recovery samples having a mean of 98.1% of the same nominal concentration. Therefore

the absolute recovery (mean concentration in extracted samples / mean concentration in samples spiked to assume 100% recovery, x 100) was 63.3%. As there was no significant difference between the observed and absolute recoveries, this showed that there was no modification of ionisation of deltamethrin in extracts of muscle tissue, following the applied methodology.

Table 5.2 Nominal recovery of deltamethrin from muscle.

Concentration ($ng.mL^{-1}$)	Control matrix extracts spiked with test substance and internal standard prior to LC-MS/MS		QC sample extracts spiked with internal standard prior to LC-MS/MS	
	Observed concentration ($ng.mL^{-1}$)	Mean (CV%)	Observed concentration ($ng.mL^{-1}$)	Mean (CV%)
100	104	98.1	65.8	62.1
	81.1		60.2	
	99.9		54.6	
	108	10.0	64.2	6.7
	92.3		63.2	
	103		64.7	

5.3.3.2 Liver

The observed recovery for the liver samples was 77.6% (Table 5.3), with the spiked recovery samples having a mean of 118.5% of the same nominal concentration. Therefore, from liver the absolute recovery was 64.1%. These results showed that the recovery of deltamethrin from the method was extremely similar between the two tissues (63.3% compared with 64.1%). However the values in the quality control samples showed widely differing values (62.1 compared with 77.6). This significant increase in response in the liver tissues was a clear example of promotion of ionisation, which was

also clear from observation of the observed concentrations found in the spiked recovery samples (98.1% for muscle compared with 118.5% for liver). As it had been sought to confirm, the presence of some component of the liver extracts resulted in promotion of ionisation of deltamethrin.

Table 5.3 Nominal recovery of deltamethrin from liver.

Concentration ($ng.mL^{-1}$)	Control matrix extracts spiked with test substance and internal standard prior to LC-MS/MS		QC sample extracts spiked with internal standard prior to LC-MS/MS	
	Observed concentration ($ng.mL^{-1}$)	Mean (CV%)	Observed concentration ($ng.mL^{-1}$)	Mean (CV%)
100	150	118.5 15.6	94.7	77.6 16.8
	131		91.6	
	114		71.9	
	104		72.0	
	109		74.5	
	103		60.7	

5.3.4 Addition of surfactant to tissue extracts

Liver and muscle (which had not seen the enhanced sensitivity found with liver) extracts had various amounts of a range of different surfactants added to determine the effect on the observed response for deltamethrin.

5.3.4.1 Muscle

The observed recovery in samples following the addition of the anionic surfactant sodium 1-dodecanesulphonate (SDS) at a concentration of 0.5 mM, which is below its cmc of 7.2 mM, showed a significantly increased response, as measured by a nominal recovery of 70.3%, compared with 62.1% for an untreated quality control sample (Table 5.4)). At a concentration of 10 mM, which is above its cmc, the mean nominal recovery was also significantly increased to 72.6%.

The observed recovery in samples following the addition of the cationic surfactant benzyldimethyltetradecylammonium chloride (Zephiramine) at a concentration of 0.05 mM, which is below its cmc, showed an increased response, as measured by a nominal recovery of 69.1% (compared with 62.1%). However although this appeared on viewing to be a significant increase it was not considered to be significantly different when accepted statistical treatment (Section 5.3.2) was applied (Table 5.5). At a concentration of 2.5 mM, which is above its cmc, the mean nominal recovery was significantly reduced to only 22.6%. This large reduction in response suggests that there has been suppression of ionisation, either as a result of the containment of deltamethrin within the micelles formed, or as a result of competition at the surface of the droplets being ionised.

The observed recovery in samples following the addition of the zwitterionic surfactant N-decyl-N, N-dimethyl-3-ammonio-1-propane (Zwittergent 3-10) at a concentration of

0.5 mM, which is below its cmc, showed a small, but statistically insignificant increase in response, as measured by a nominal recovery of 65.2% (compared with 62.1%). At a concentration of 50 mM, which is above its cmc, there was a significant reduction in mean nominal recovery to 55.8%.

The observed recovery in samples following the addition of the non-ionic surfactant polyoxyethylene 6 lauryl ether ($C_{12}E_6$) at a concentration of 0.001 mM, which is below its cmc, showed a statistically significant increase in response, as measured by a nominal recovery of 71.5% (compared with 62.1%). At a concentration of 0.01 mM, which is above its cmc, there was a small, but statistically insignificant, reduction in mean nominal recovery to 58.3%.

Table 5.4 Recovery of deltamethrin from muscle following the addition of surfactant at concentrations above and below the critical micelle concentration.

Sample Treatment	Nominal Recovery (%)	Mean Nominal Recovery (%) (SD)	Sample Treatment	Nominal Recovery (%)	Mean Nominal Recovery (%) (SD)
QC 100 ng/mL	65.8 60.2 54.6 64.2 63.2 64.7	62.1 (4.15)	QC 100 ng/mL	65.8 60.2 54.6 64.2 63.2 64.7	62.1 (4.15)
1 mM SDS	70.2 74.4 75.3 65.8 69.8 66.4	70.3 (3.94)	20 mM SDS	61.9 71.6 61.7 82.9 73.0 84.4	72.6 (9.80)
0.1 mM Zephiramine	58.9 69.0 59.1 78.9 69.4 79.3	69.1 (8.99)	5 mM Zephiramine	22.8 23.6 21.1 23.9 22.6 21.4	22.6 (1.13)
0.5 mM Zwittergent 3-10	65.2 67.6 66.7 61.4 67.3 62.8	65.2 (2.55)	5 mM Zwittergent 3-10	58.8 53.7 51.9 56.7 58.8 54.9	55.8 (2.80)
0.001 mM $C_{12}E_6$	63.5 78.8 63.2 79.3 72.0 72.1	71.5 (7.04)	0.01 mM $C_{12}E_6$	53.5 62.1 53.5 63.9 57.6 59.4	58.3 (4.33)

Table 5.5 Statistical Treatment of Deltamethrin Extracted from Muscle Data. Where calculated t value exceeds the critical value (i.e. shows a statistically significant difference) the t values are in bold.

Sample Sets for Comparison	Mean	SD	F-Test	Critical Value for a one-tailed test (P=0.05)	Are SDs Equal?	Calculated t	Degrees of Freedom	Critical Value, P=0.05	Critical Value, P=0.025	Critical Value, P=0.01
Muscle vs Liver	62.1 77.6	4.15 13.0	9.85	5.05	No	2.78	6	**1.94**	**2.45**	3.14
Muscle with SDS added below CMC	62.1 70.3	4.15 3.94	0.90	5.05	Yes	3.55	10	**1.81**	**2.23**	**2.76**
Muscle with SDS added above CMC	62.1 72.6	4.15 9.80	5.58	5.05	No	2.41	7	**1.89**	**2.36**	3.00
Muscle with Zephiramine added below CMC	62.1 69.1	4.15 8.99	4.70	5.05	Yes	1.73	10	1.81	2.23	2.76
Muscle with Zephiramine added above CMC	62.1 22.6	4.15 1.13	13.2	5.05	No	22.4	8	**1.86**	**2.31**	**2.90**
Muscle with Zwittergent 3-10 added below CMC	62.1 65.2	4.15 2.55	2.65	5.05	Yes	1.58	10	1.81	2.23	2.76
Muscle with Zwittergent 3-10 added above CMC	62.1 55.8	4.15 2.80	2.21	5.05	Yes	3.12	10	**1.81**	**2.23**	**2.76**
Muscle with $C_{12}E_6$ added below CMC	62.1 71.5	4.15 7.04	2.88	5.05	Yes	2.81	10	**1.81**	**2.23**	**2.76**
Muscle with $C_{12}E_6$ added above CMC	62.1 58.3	4.15 4.33	1.09	5.05	Yes	1.57	10	1.81	2.23	2.76

5.3.4.2 Liver

The observed recovery in samples following the addition of the anionic surfactant SDS at a concentration of 0.5 mM, which is below its cmc of 7.2 mM, resulted in no significant change in mean response, as measured by a nominal recovery of 76.0%, compared with 77.6% for an untreated quality control sample (Table 5.6). At a concentration of 10 mM, which is above its cmc, there was a significant reduction in mean nominal recovery to 61.7% (Table 5.7).

The observed recovery in samples following the addition of the cationic surfactant Zephiramine at a concentration of 0.05 mM, which is below its cmc, showed a small, but statistically insignificant, reduction in mean response, as measured by a nominal recovery of 69.7% (compared with 77.6%). At a concentration of 2.5 mM, which is above its cmc, the mean nominal recovery was significantly reduced to only 43.9%.

The observed recovery in samples following the addition of the zwitterionic surfactant Zwittergent 3-10 at a concentration of 0.5 mM, which is below its cmc, showed a small, but statistically insignificant, increase in mean response, as measured by a nominal recovery of 82.7% (compared with 77.6%). At a concentration of 50 mM, which is above its cmc, the mean nominal recovery was significantly reduced to only 48.7%.

The observed recovery in samples following the addition of the non-ionic surfactant polyoxyethylene 6 lauryl ether ($C_{12}E_6$) at a concentration of 0.001 mM, which is below its cmc, showed a small, but statistically insignificant, increase in mean response, as

measured by a nominal recovery of 81.4% (compared with 77.6%). At a concentration of 0.01 mM, which is above its cmc, the mean nominal recovery was significantly reduced to 58.6%.

The significant increases in mean nominal recovery in muscle following addition of SDS, either above or below the cmc, supported the premise that the higher nominal recoveries obtained for liver tissue was as a result of the endogenous surfactants. There were no significant differences in mean nominal recovery when any of the surfactant types were added below their cmc. This, combined with the fact that there were significant reductions in mean nominal recovery in liver for all surfactants added above their cmc, suggested that the concentration of surfactant required to create the promotion of ionisation effect was near the optimum in the liver tissues following the stated extraction. The reason for the significant increase in response when a non-ionic surfactant was added at a concentration below its cmc to muscle was likely to be that the non-ionic surfactant was also reducing the droplet size of the mobile phase reaching the mass spectrometer and was thereby improving response.

Table 5.6 Recovery of deltamethrin from liver following the addition of surfactant at concentrations above and below the critical micelle concentration.

Sample Treatment	Nominal Recovery (%)	Mean Nominal Recovery (%) (SD)	Sample Treatment	Nominal Recovery (%)	Mean Nominal Recovery (%)
QC 100 ng/mL	94.7 91.6 71.9 72.0 74.5 60.7	77.6 (13.0)	QC 100 ng/mL	94.7 91.6 71.9 72.0 74.5 60.7	77.6 (13.0)
1 mM SDS	81.7 77.6 69.8 75.7 83.4 67.5	76.0 (6.33)	20 mM SDS	62.4 61.9 60.0 61.9 62.8 60.9	61.7 (1.03)
0.1 mM Zephiramine	69.2 69.1 68.7 70.6 69.5 70.8	69.7 (0.855)	5 mM Zephiramine	44.5 41.8 43.7 42.0 46.3 45.3	43.9 (1.80)
0.5 mM Zwittergent 3-10	85.0 73.1 72.4 89.5 86.2 89.9	82.7 (7.92)	5 mM Zwittergent 3-10	46.8 50.6 43.1 50.7 16.8 13.7	37.0 (17.1)
0.001 mM $C_{12}E_6$	67.9 90.4 87.5 68.9 84.0 89.8	81.4 (10.3)	0.01 mM $C_{12}E_6$	62.3 57.6 62.9 58.1 55.0 55.9	58.6 (3.28)

Table 5.7 Statistical Treatment of Deltamethrin Extracted from Liver Data. Where calculated t value exceeds the critical value (i.e. shows a statistically significant difference) the t values are in bold.

Sample Sets for Comparison	Mean	SD	F-Test	Critical Value for a one-tailed test (P=0.05)	Are SDs Equal?	Calculated t	Degrees of Freedom	Critical Value, P=0.05	Critical Value, P=0.025	Critical Value, P=0.01
Liver with SDS added below CMC	77.6 76.0	13.0 6.33	4.23	5.05	Yes	0.27	10	1.81	2.23	2.76
Liver with SDS added above CMC	77.6 61.7	13.0 1.03	154	5.05	No	2.98	5	**2.02**	**2.57**	3.36
Liver with Zephiramine added below CMC	77.6 69.7	13.0 0.855	242	5.05	No	1.49	5	2.02	2.57	3.36
Liver with Zephiramine added above CMC	77.6 43.9	13.0 1.80	53.0	5.05	No	6.28	5	**2.02**	**2.57**	**3.36**
Liver with Zwittergent 3-10 added below CMC	77.6 82.7	13.0 7.92	2.70	5.05	Yes	0.82	10	1.81	2.23	2.76
Liver with Zwittergent 3-10 added above CMC	77.6 37.0	13.0 17.1	1.73	5.05	Yes	4.63	10	**1.81**	**2.23**	**2.76**
Liver with C12E6 added below CMC	77.6 81.4	13.0 10.3	1.59	5.05	Yes	0.56	10	1.81	2.23	2.76
Liver with C12E6 added above CMC	77.6 58.6	13.0 3.28	15.7	5.05	No	3.47	6	**1.94**	**2.45**	**3.14**

5.3.5 Addition of surfactant to pure standards

Solutions containing deltamethrin at a concentration equivalent to that found in sample extracts, dissolved in the same solvent mixture as those extracts, were prepared. Sub-sets of samples had each of the surfactants previously investigated added at concentrations both above and below the relevant cmc. These were injected on the same chromatographic system as the sample extracts. The aim of this experiment was to see if the addition of surfactant could be considered as a method of increasing sensitivity in samples of any nature, i.e. as a generic means of increasing sensitivity.

Samples containing both anionic and cationic surfactants below their cmc (Table 5.8) showed statistically significant increases in response of 16% and 15% respectively. Samples containing zwitterionic and non-ionic surfactants below their cmc showed small, but statistically insignificant increases in response of 2% and 5% respectively. Samples containing SDS (anionic surfactant) above its cmc showed a significant increase in response of 20% (Table 5.9); whereas samples containing all other types of surfactant, above their cmc, showed no significant change in response to untreated samples (Table 5.10).

Table 5.8 Addition of surfactants at concentrations below their CMC to pure standards.

Surfactant	Final Surfactant Concentration in Sample (mM)	Nominal Recovery (%)	Mean Nominal Recovery (%)	Absolute Recovery (%)
No surfactant present	n/a	100.7 102.2 105.7 88.4 92.1 92.8	97.0	100.0
Sodium 1-dodecanesulphonate (SDS)	10 mM	110.0 114.4 115.7 110.1 109.6 110.6	111.7	115.2
Benzyldimethyltetradecylammonium chloride (Zephiramine)	2.5 mM	110.5 117.5 111.6 111.2 114.4 109.9	112.5	116.0
N-decyl-N, N-dimethyl-3-ammonio-1-propane (Zwittergent 3-10)	50 mM	103.6 102.9 102.6 94.6 95.0 98.1	99.5	102.6
Polyoxyethylene 6 lauryl ether ($C_{12}E_6$)	0.1 mM	103.9 107.7 102.3 101.0 98.4 98.4	102.0	105.1

Table 5.9 Addition of surfactants at concentrations above their CMC to pure standards.

Surfactant	Final Surfactant Concentration in Sample (mM)	Nominal Recovery (%)	Mean Nominal Recovery (%)	Absolute Recovery (%)
No surfactant present	n/a	100.7 102.2 105.7 88.4 92.1 92.8	97.0	100.0
Sodium 1-dodecanesulphonate (SDS)	10 mM	109.7 119.2 115.8 116.0 118.6 120.0	116.6	120.2
Benzyldimethyltetradecylammonium chloride (Zephiramine)	2.5 mM	109.2 89.2 85.5 109.6 92.7 85.2	95.2	98.2
N-decyl-N, N-dimethyl-3-ammonio-1-propane (Zwittergent 3-10)	50 mM	99.3 95.8 94.7 98.8 94.4 97.7	96.8	99.8
Polyoxyethylene 6 lauryl ether ($C_{12}E_6$)	0.1 mM	94.1 98.2 100.6 95.4 99.6 103.4	98.6	101.6

Table 5.10 Statistical treatment of pure standard data.

Sample Sets for Comparison	Mean	SD	F-Test	Critical Value for a one-tailed test (P=0.05)	Are SDs Equal?	Calculated t	Degrees of Freedom	Critical Value, P=0.05	Critical Value, P=0.025	Critical Value, P=0.01
Pure standard with SDS added below CMC	97.0 111.7	6.81 2.62	6.72	5.05	No	4.93	7	**1.89**	**2.36**	**3.00**
Pure standard with SDS added above CMC	97.0 116.6	6.81 3.77	3.27	5.05	Yes	6.18	10	**1.81**	**2.23**	**2.76**
Pure standard with Zephiramine added below CMC	97.0 112.5	6.81 2.89	5.52	5.05	No	5.13	7	**1.89**	**2.36**	**3.00**
Pure standard with Zephiramine added above CMC	97.0 95.2	6.81 11.3	2.75	5.05	Yes	0.34	10	1.81	2.23	2.76
Pure standard with Zwittergent 3-10 added below CMC	97.0 99.5	6.81 4.10	2.76	5.05	Yes	0.77	10	1.81	2.23	2.76
Pure standard with Zwittergent 3-10 added above CMC	97.0 96.8	6.81 2.11	10.3	5.05	No	0.07	6	1.94	2.45	3.14
Pure standard with $C_{12}E_6$ added below CMC	97.0 102.0	6.81 3.55	3.68	5.05	Yes	1.60	10	1.81	2.23	2.76
Pure standard with $C_{12}E_6$ added above CMC	97.0 98.6	6.81 3.43	3.93	5.05	No	0.51	10	1.81	2.23	2.76

N.B. Where the calculated t value exceeds the critical value (showing statistically significant differences) the t values are in bold

5.4 Conclusions

The addition of both anionic and cationic surfactants to samples containing deltamethrin causes an increased response that may be considered to be promotion of ionisation, in a reproducible and robust manner. This increase in response was seen both in samples of extracted tissue and in pure standard solutions; therefore the emulsification of fats and oils by the surfactants was not the only mechanism by which the enhancement was achieved. The presence of surfactants in the samples investigated appears to have affected the formation of droplets by the mobile phase, thereby ultimately enhancing the detected signal.

Although the increased response may not appear great, i.e. was less than an order of magnitude, it was nevertheless of suitable significance that it may be considered as a way of improving sensitivity when problems achieving a required limit of detection occur. With similar methods to this, where the purpose is to determine that the analyte residue level in tissues is below a certain concentration, the typical dynamic range of an assay is 4-fold, (e.g. 1-4 $ng.mL^{-1}$); therefore increases in sensitivity of 20% or more may be extremely significant in achieving the required lower limit of quantification. The data reported refers to deltamethrin only; however the data represents an important contribution with respect to the overall need of greater understanding of factors affecting ionisation. Such small increases in the linear dynamic range would be less significant in a typical method for the analysis of drugs in biological fluids (or tissues) where concentration profiles are required. However, with optimisation, perhaps there is scope

for producing greater enhancement in a controlled fashion. Further it might be considered that the addition of surfactant might result in greater robustness of the method, by reducing the dependency on the variable amount of natural surfactant that survives the sample preparation (solid phase extraction) process.

Following this investigation, there are a number of further investigations of this phenomenon that suggest themselves. Initially the same technique could be adopted to determine the impact on a wider range of analytes, in order to determine whether this has the potential to be available as a generic technique for the promotion of ionisation. This is likely to be the case with tissue samples as used in the initial investigation, but may not be so applicable for all samples. The medium and longer term effects of the use of the surfactants on the mass spectrometer performance should also be considered.

Further investigations to determine the optimum surfactant concentration or range of surfactant concentrations using several different analytes could enable a standard methodology to be prepared, either for all analytes, or for various types of analyte. Alternatively if greater enhancement was to be found with certain classes of analyte, this might shed further light on the nature of the phenomenon.

Interestingly, it is accepted wisdom that MEKC-MS (micellar electrokinetic chromatography, where separation is based on differential partitioning between a pseudo-stationary micellar phase and an aqueous mobile phase) can’t be done because of the presence of micelles. Further investigations are required in order to resolve the apparent

contradiction in this investigation to accepted thinking on the effect of micelles on MS analysis.

Chapter 6

Conclusions and Further Work

Principally, even before the outset of this research programme, as a result of the introduction of the current generation of atmospheric pressure ionisation interfaces, liquid chromatography coupled with tandem mass spectrometric detection had become the analytical method of choice for the determination of pharmaceutical compounds and their metabolites. Despite this there had remained a number of problems associated with developing reliable and robust assays for a number of combinations of pharmaceutical compound and sample matrix. However, during the course of the research programme some light was shed on some of the problems and some useful solutions emerged.

Failure to remove large numbers of potentially interfering species during the sample preparation process can lead to competition in the ionisation process, thereby leading to reduced signal for the analytes of interest, or to a reduction of robustness of the analytical method.

The specificity of multiple reaction monitoring in tandem mass spectrometers may mask the coelution of species present in biological samples, which can cause matrix effects.

Good chromatographic separation from potentially interfering species is essential in order to avoid matrix-related suppression (or less commonly promotion) of ionisation.

In order to provide an optimum chromatographic separation, it may be necessary to utilise mobile phases that are not ideally compatible with current LC-MS/MS instruments and that therefore provide inadequate sensitivity for the compound under investigation. In this case it may be possible to reduce or eliminate problems associated with the choice of mobile phase by the use of some artefact, such as post-column modification of mobile phase, in order to enhance the ionisation to allow the required limits of detection to be achieved. This can be effective to varying degrees depending on the chemical nature of the compound being analysed, but as illustrated in the work discussed in Chapter 3 it can be relatively easy to increase sensitivity by up to an order of magnitude for many classes of compound. Optimisation of this technique was successfully used to reach the required lower limit of quantification which would otherwise not have been possible, without a complete overhaul of the methodology available, with the prohibitively problematic accompanying issues of time and materials that this might have resulted in.

If an ideal chromatographic separation from matrix components is not possible, due to the similarity of the interfering species with the target analytes, then alternative strategies need to be employed, such as changing the mode of ionisation [141]. The mode of ionisation, i.e. the choice of IonSpray or APCI, may not be significant for some analytes that have sufficient sensitivity for both modes of ionisation. However, as illustrated in

Chapter 2, it can be critical to the development of methods for some analytes, even when sensitivity in both modes seems acceptable.

With regards to the sample preparation stage, the chromatographic separation and the mobile phase selected, there are also practical considerations to take into account, where "dirty" samples cause blockages within the extremely small orifice, thereby causing a gradual loss in sensitivity in the mass spectrometer. As well as changing sensitivity a build up of sample residue can cause a complete blockage of the interface tubing, thereby stopping all analyte ions from entering the mass spectrometer. This therefore becomes a particular consideration when dealing with small sample volumes, which make repeat injection difficult, or even impossible.

Ultimately all of the above problems are associated with the desire to provide optimum conditions for the analytes of interest, to the exclusion of any potential interfering species. A combination of the sample preparation methodology, the mode of ionisation and artefacts such as post-column modification of mobile phase can be used to enhance the signal for the analyte under investigation.

The examples described in this thesis illustrate that even with a well-defined technique there are a number of approaches to improving sensitivity and robustness of methods.

In summary, as evidenced by the studies in this research programme, there are no absolute rules to dealing with analytical problems with API-MS. However, an

understanding of the causes of most of the problems, and a choice of strategy to deal with problems when they arise, is required to enable an analyst to develop suitable methodologies for the determination of pharmaceuticals and biologicals in bioanalytical samples.

There are several main areas of work for further investigation resulting from the data discussed in this thesis. For example, post-column modification of mobile phases in order to enhance signals should be optimised for a range of analytes. This should provide information to confirm which types of compound are most readily susceptible to this kind of sensitivity enhancement as well as providing an indication of the potential sensitivity benefits that can arise as a result of modifying the mobile phase before the injected sample enters the mass spectrometer source region. There are two main ways to perform this – either to use the methodology discussed in Chapter 4, or to involve the use of column switching, where the analytes are loaded onto a fully retaining column, and are then eluted using a mobile phase more suited to API-MS interfaces.

At present the methodology described in Chapter 4 has not yet been commonly reported [142] although modification of mobile phase for example changing from principally acetonitrile to methanol has been shown to provide potentially large increases in sensitivity (depending on the analyte) [143]. The only “commonly” used post-column modification of mobile phase is the so-called “TFA fix” [144]. In this, mobile phases containing trifluoroacetic acid (which is known to suppress the signal of polar compounds by forming ion pairs with them) are modified post-column with a weaker

acid. A modification of this has been used by addition of the modifying agent pre-column with some success [145]. Other examples include addition of a basic solution to alter the mobile phase pH [146] which has been shown to improve sensitivity by over an order of magnitude for some analytes. However, despite the limited modifications reported above, the use of post-column modification of highly aqueous mobile phases to enhance sensitivity has not yet been reported. (Generally it seems preferable to completely change the chromatography, etc. in order to improve sensitivity, though as discussed it is not always possible, particularly for some chiral separations).

The phenomenon of promotion of ionisation using surfactants provides a huge amount of potential for further investigation. This could consist of a number of separate investigations. The first is to utilise a far wider range of surfactant types and concentrations to determine additional information about the ability to enhance analyte response as a result of the addition of surfactants. The surfactant type, even within a major surfactant class, could be critical to the effect that has been observed and discussed. A possibly more significant investigation would be to perform similar experiments to those discussed in this thesis on a range of compound types. This could initially take the form of investigations into the effect of surfactant on the response of pure standards of the appropriate compounds. This would provide an indication of whether the compound itself had enhancement/suppression as a result of the presence of surfactants. Once this was established further investigations into different sample matrices would be required (to determine the effect of the surfactant on other potentially interfering species within a complex sample matrix). Finally the impact of different

sample preparation techniques with the presence of added surfactant could also be investigated.

At present there has been no reported work on this phenomenon. Most work investigating surfactants is in the environmental analysis sector where the surfactant is the target analyte or group of analytes [147], although investigation of surfactant levels in liver tissues has been reported [148]. In no case has the suggestion been made that the presence of surfactants can be used to enhance sensitivity.

There will always be scope for improvement in any technology. However, with the benefit of some of the techniques available for improvement in sensitivity and specificity discussed in this programme, together with other developments such as a number of new interfaces such as those that combine APCI with electrospray [62] the practice of LC-MS for drug bioanalysis and other applications is becoming much more reliable and well understood.

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