Mass Spectrometric Applications for the Quantitative Analysis of Dried Blood Spot and Capillary Micro-Sampling Techniques

Ranbir Singh Mannu

MSc by Research in Chemistry

University of York

Department of Chemistry

February 2014

i) Abstract

A reversed phase UHPLC-MS/MS method for the quantitative analysis of pioglitazone in dried blood spots (DBS) has been used to validate two new novel techniques to analyse sample concentrations that lie above a particular calibration range. The first of the two techniques is mass spectrometer signal dilution (MSSD) which consists of lowering the signal which reaches the detector, which is achieved by lowering the collision energy applied in the collision cell. The second technique designated isotope signal ratio monitoring (ISRM) looks at $[M+2]^{+1}$ ions (caused by natural occurring isotopes) for samples above the limit of quantification. For both techniques the requirements for reanalysis of above range samples can be eliminated.

A reversed phase UHPLC-MS/MS method for the quantitative analysis of a phosphorothioate oligonucleotide in human plasma capillary micro-sampling samples has been developed and validated, to demonstrate the compatibility of capillary micro-sampling (CMS) with an analytically challenging class of compounds.

Contents

List of tables

List of figures

List of abbreviations

DBS- Dried blood spot

CMS- Capillary micro-sampling

EBF-European bioanalysis forum

mRNA- Messenger RNA

FDA- Food and drug agency

CMV- Cytomegalovirus

LC- Liquid chromatography

LLE- Liquid-liquid extraction

SPE- Solid phase extraction

SCX- Strong cation exchange

WCX- Weak cation exchange

HPLC- High performance liquid chromatography

HETP- Height equivalent to a theoretical plate

Oligo- Oligonucleotide

API- Atmospheric pressure ionisation

ESI- Electrospray ionisation

APCI- Atmospheric pressure chemical ionisation

m/z- Mass/ charge

DC- Direct current

CID- Collision induced dissociation

CAD- Collision activated dissociation

MRM- Multiple reaction monitoring

QC- Quality control

LLOQ- Lower limit of quantification

LoQC- Low quality control

MeQC- Medium quality control

HiQC- High quality control

DiQC- Diluted quality control

MSSD- Mass spectrometer signal dilution

ISRM- Isotope signal ratio monitoring

DP- Declustering potential

CE- Collision energy

CXP- Collision cell exit potential

EP- Entrance potential

HFIP- 1,1,1,3,3,3-hexafluoro-2-propanol

TEA- Trethylammonium

Acknowledgements

I would like to acknowledge my industrial supervisor Dr Phillip Turpin whose support and guidance has helped me in this research and has enabled me to develop as scientist within the bioanalytical arena. I would also like to acknowledge my supervisor Professor Jane Thomas-Oates.

I would like to also acknowledge the help of Dr Matthew Ewles, Jamie Spence, Dr David Firth, Dr Lee Goodwin, Paul Snelling, Sarah Ball and Anthony Freeman Clarke.

I would also like to acknowledge Covance Laboratories for funding this research as well as my attendance at the European Bioanalysis forum 2013.

I would also like to thank all the current and previous members of my team (Ben Bratby, James Michael Duffy and Charles Holder) for their understanding and patience over the past two years.

Finally, I would like to thank my family and beautiful wife for their support, patience and encouragement.

Declaration

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

Ranbir Singh Mannu

Publication

Alternative strategies for on-line mass spectrometer based sample dilution for bioanalytical samples, with particular reference to dried blood spot analysis; Ranbir Singh Mannu, Phillip E. Turpin, Lee Goodwin: *Bioanalysis march 2014 Vol 6 No. 6 page773-784 journal* in March.

Development of a bioanalytical method for the quantification of a phosphorothioated oligonucleotide in human plasma capillary micro-sample using LC-MS/MS: Ranbir Singh Mannu, Phillip E. Turpin: In the process of being submitted for publication in *Bioanalysis.*

Presentation

Development of a bioanalytical method for the quantification of a phosphorothioated oligonucleotide in human plasma capillary micro-sample using LC-MS/MS. Presented at the European Bioanalysis Forum 2013 in Barcelona.

Chapter 1 – Introduction

1.0 Introduction

The modern day drug development process involves many different elements. Bioanalysis is one critical aspect of the drug development process and involves the accurate and quantitative analysis of xenobiotics in biological samples.

The aim of bioanalysis is to provide accurate quantitative measurements in pharmacokinetic, toxicokinetic or bioequivalence studies. Scientific decisions in drug development are often based on the quantitative analysis provided by a bioanalyst. Pharmaceutical companies invest heavily in the drug development process, hoping to find a therapeutic compound that will improve lives and generate a profit. Animal testing is a critical part of drug development and there is now a lot of pressure to ensure that the minimum numbers of animals are used in drug development studies. Much time and money have been invested to reducing the usage of animals.

1.1 Micro-sampling

Micro-sampling has increased in popularity over the last decade. The most prominent advantage of micro-sampling is the reduction in blood sample volume required. The potential to utilize smaller blood volumes is particularly beneficial for pharmacokinetic and toxicokinetic studies. Currently extra animals known as satellite groups have to be dosed to evaluate toxicokintic effects, as main study animals are used for clinical pathology evaluation.

Conventionally, toxicokinetic studies require blood volumes between 100-500 µL to be collected from a single animal at each sampling time point. Given the physiology of rodents, composite sampling (combining samples taken from different animals at different time points to create 1 full profile) is routinely employed, as the total circulating blood volumes are too low. The reduction in the volume of blood required for micro-sampling makes serial sampling from the same animal possible and consequently micro-sampling is ethically more favourable, as fewer animals are required (Spooner *et al* 2009).

Dried blood spot (DBS) and capillary micro-sampling are two micro-sampling techniques that have recently increased in popularity. These two techniques simplify the handing and

bioanalysis of small plasma and blood volumes. Handling and extracting small sample volumes by the convention method (sample placed into a small plastic tube) is not practical because accurately removing a small volume of plasma or blood required for analysis is not always possible.

1.1.1 Dried blood spot

The DBS technique is well established and consists of collecting blood samples onto paper or card and leaving them to dry at room temperature (Guthrie *et al* 1963). A core is taken from the DBS card and placed into an extraction plate, for extraction and subsequent analysis. Historically, DBS samples have been used in screening for phenylketonuria, congenital hypothyroidism, sickle cell disorders and HIV infection (Guthrie *et al* 1963). Due to the ethical, financial, and practical advantages of DBS analysis, this methodology has increased in uptake over the past 5 years. Other advantages include stability; DBS samples are generally stable at room temperature partially due to the fact that enzyme activity is effectively inhibited as a result of removing the aqueous component of the sample, which eliminates the requirement for freezers and temperature controlled shipping (Barfield *et al* 2008). DBS samples also pose less of a biohazard than blood/plasma samples due to the antimicrobial properties of some DBS cards and therefore the use of this technique is also an attractive proposition for clinical studies.

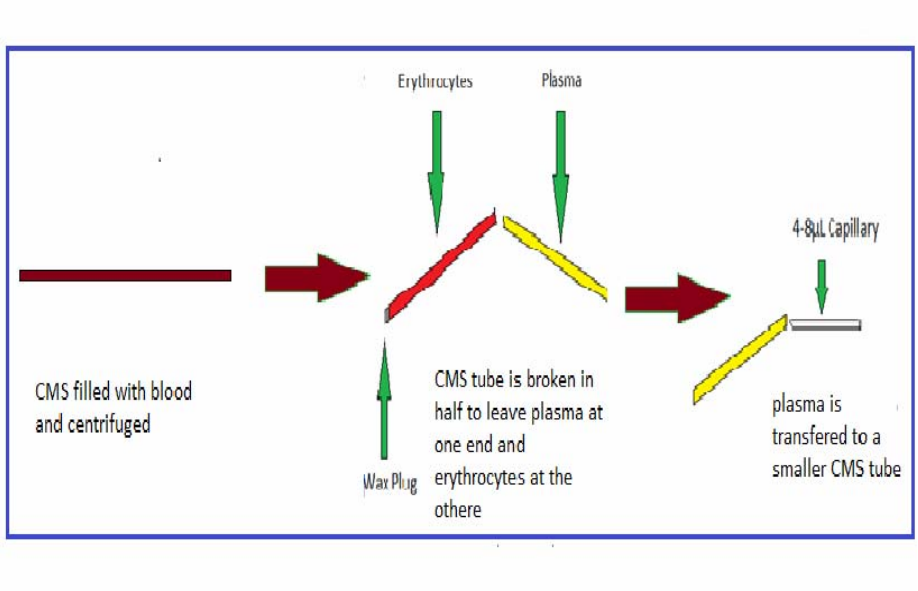
Quite recently, some disadvantages of DBS have been highlighted. The haematocrit levels (the amount of red blood cells present) can vary in blood samples from subject to subject and have been demonstrated to affect the size of the spot which can lead to inaccurate drug concentrations detected and reported. Haematocrit levels have an effect on the experimental recovery of the analyte (Malvagia *et al* 2009). As a result, the majority of micro-sampling interest has now turned to capillary micro-sampling (CMS).

1.1.2 Capillary micro-sampling

CMS is technique used for the collection and handling of biological fluid samples. CMS is not a new technique for handling biological samples but its use in bioanalysis is a new

concept (Jonsson, European Bioanalysis forum (EBF) presentation 2012, provided by Astra Zeneca). Blood is drawn into a glass tube by capillary action. The glass tube can be either transferred to a bio-analytical test site or centrifuged to produce a smaller plasma capillary sample, as shown in figure 1

Figure 1 Method of producing a plasma capillary from a whole blood sample

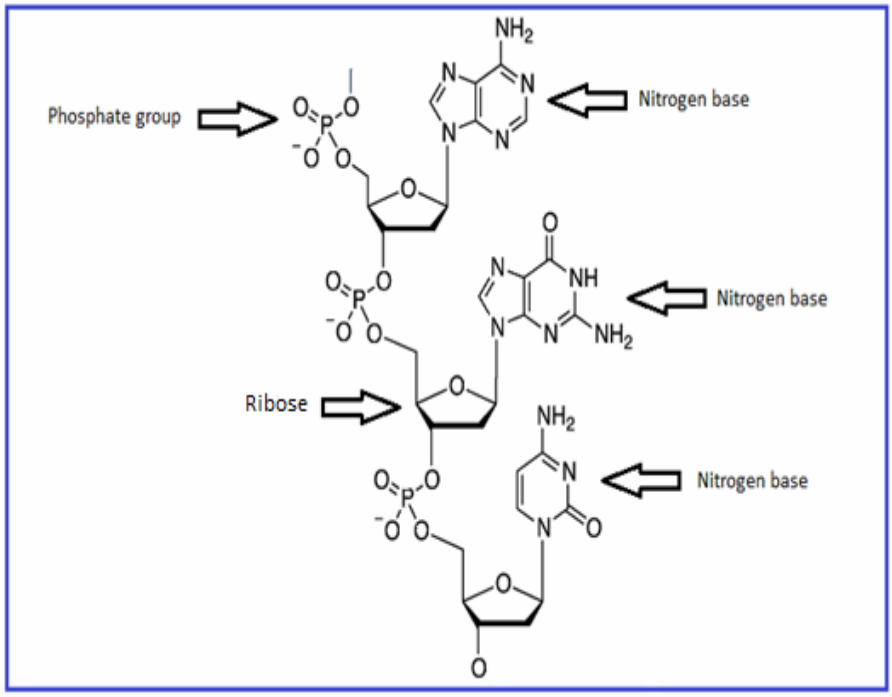


CMS has the same ethical advantages as DBS without the haematocrit level or variable recovery issues. However the robustness of the technique has not been investigated as much as that of DBS and its use in regulated studies has to date been low. CMS also does not have the additional sample stability advantages of DBS and therefore CMS cannot be stored at room temperature, as a result freeze thaw stability will have to be investigated.

1.2 Antisense oligonucleotide

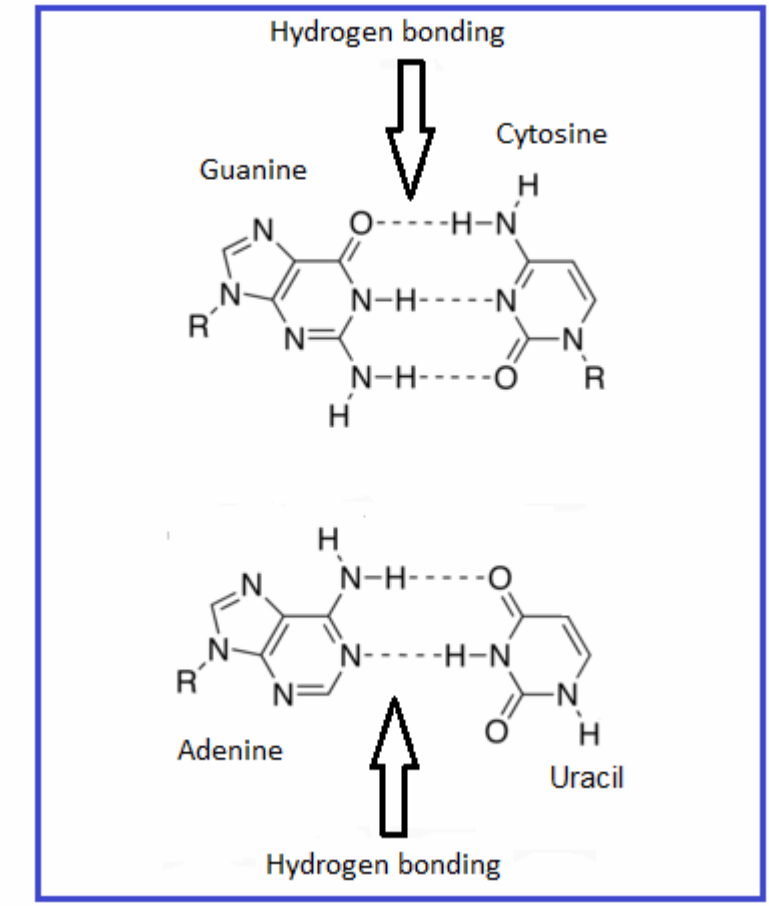
The interest in antisense oligonucleotides has increased significantly since the FDA approved for drug use the first of such molecules, Vitravene, in 1998 (Roehr *et al* 1998). Antisense oligonucleotides are short single stranded RNA or DNA molecules, which typically contain 15-35 nucleotides. A nucleotide is made up of a nitrogen base, ribose (a monosaccharide) and a phosphate group (figure 2), and are joined together by phosphodiester bonds.

Figure 2 Basic structure of anphosphodiester oligonucleotide.



The suffix 'mer' is used to indicate that these molecules are polymers (from the Greek word meros meaning part); an oligonucleotide containing 18 nucleotides may be referred to as an 18mer. Antisense oligonucleotides are designed to prevent or moderate the protein translation of messenger RNA (Sahu *et al* 2007). This is achieved by base-pairing of the antisense oligonucleotide with complementary messenger RNA (mRNA) via the Watson-Crick base pairing (figure 3) and hence physically obstructing translation of the transcript because the complementary sequence can no longer be decoded (Agrawal *et al* 2000).

Figure 3 Watson-Crick base pairing between nitrogen bases

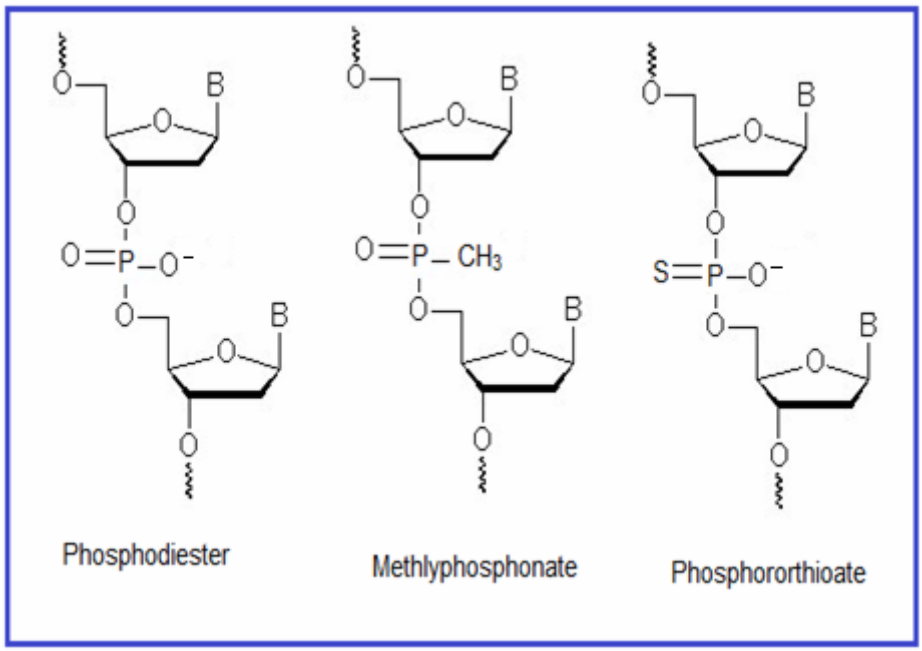


1.2.1 Modified Oligonucleotides

Native oligonucleotides (with phosphodiester bonds) are rapidly degraded by intracellular endonucleases and exonucleases (Wickstrom *et al* 1986). Exonucleases are enzymes that cleave nucleotides from the ends of the molecule; the loss of a few nucleotides can dramatically reduce its selectivity for targeting messenger RNA. Endonucleases are enzymes that cleave phosphodiester bonds. Endonucleases and exonucleases could potentially break down antisence oligonucleotides and therefore various modifications have been tested to improve stability, to enable them to be used as therapeutic agents. The first modification tested by researchers was to replace the non-bridging oxygen of the phosphodiester group with a methyl group to produce methlyphosphonate oligonucleotides (shown in figure 4). Although these have excellent stability, the removal of charge reduces their solubility and cellular uptake (Agrawal *et al* 1981). By replacing the non-bridging oxygen with a sulfur atom (figure 4), the solubility is not affected but the

oligonucleotide's stability is improved (Stein *et al* 1993), so that phosphorothioate oligonucleotides are currently being actively developed as therapeutic molecules.

Figure 4 Different modified oligonucleotide

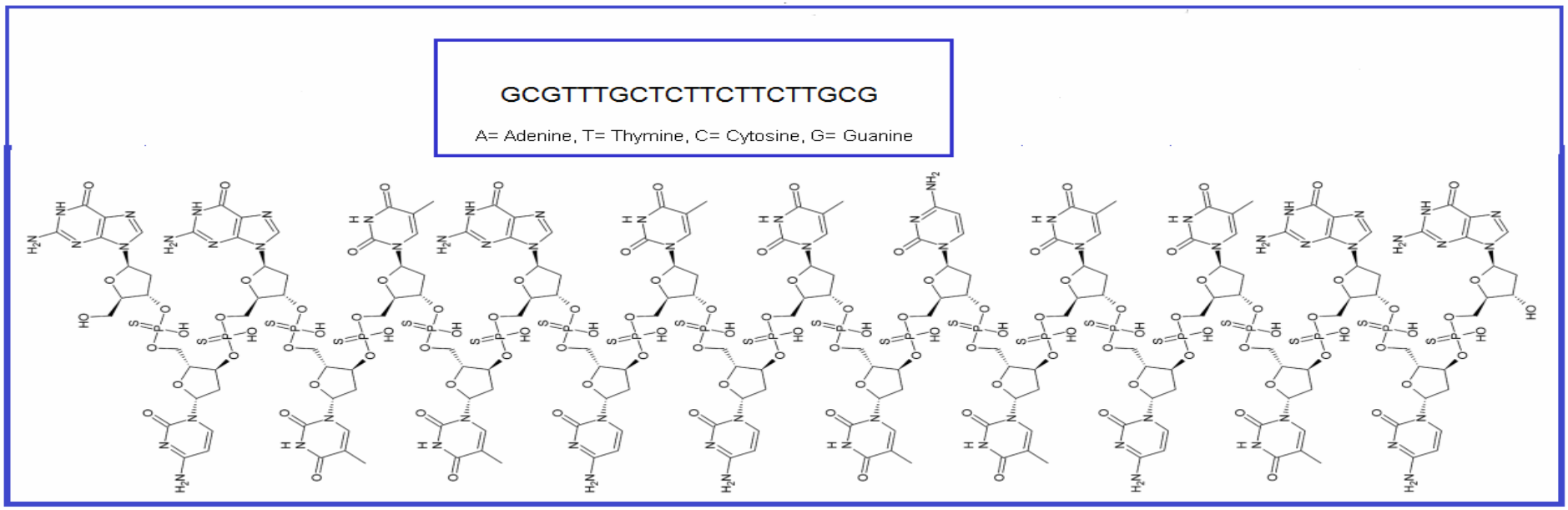


The delivery of oligonucleotides to their site of action is also a challenge. Most oligonucleotides would rapidly degrade in the stomach due to nucleases therefore oral administration would not be feasible. Instead, therapeutic oligonucleotides are administered via IV injection (Crooke *et al* 2008).

1.2.2 Vitravene (also known as Fomivirsen)

Vitravene is a 21 base oligonucleotide containing phosphorothioate linkages (shown in figure 5) and was the first antisence oligonucleotide approved by the FDA. Vitravene is used to treat human cytomegalovirus (CMV) retinitis. CMV retinitis is a viral infection that can lead to blindness if not treated.

Figure 5 Vitravene structure (Roehr *et al* 1998)



The production of proteins which are essential for the productions of infectious CMV is inhibited because Vitravene binds to the complementary sequence of the mRNA (Roehr *et al* 1998).

1.3 Extraction procedure

Sample preparation is required in the majority of bioanalytical methods. Biological samples such as plasma, blood and DBSs cannot easily be analysed using analytical techniques like LC-MS/MS without sample preparation. Matrices such as plasma have a high protein content which would rapidly compromise the performance of an HPLC column and mass spectrometer. The most frequently applied sample extraction techniques are protein precipitation, liquid-liquid extraction and solid phase extraction (Chang *et al* 2005).

1.3.1 Protein precipitation

Protein precipitation is the simplest sample preparation technique used. The objective of this technique is to reduce the solubility of the proteins in a given sample, causing the proteins to form a precipitate that can be removed by filtration or centrifugation. The electrostatic repulsion between protein molecules is at its lowest at the isoelectric point (PI), which is the pH at which any given protein has an equal number of positive and negative charges and so is overall neutral. Therefore less solvent molecules cluster around the proteins, allowing the proteins to aggregate. The main methods of protein precipitation in use involve the use of organic solutions, acids, metal ions or salts (Hagerman *et al* 1978).

Organic solvents are less polar than aqueous solvents. Therefore the addition of organic solvents decreases the dielectric constant of the plasma protein solution. Lowering the polarity of a solution results in less cluster formation around the proteins, causing an increase in the electrostatic interactions between the proteins. Typical solvents used are methanol, acetonitrile and ethanol, at a minimum ratio of 1:3 (plasma: solvent).

Acid reagents such as trichloroacetic acid form insoluble salts with the positively charged amino acid groups on the protein at a pH below their PI. Neutralising the charge on proteins lowers the amount of solvent clusters formed around the proteins leading to precipitation.

Another method is to add a solution of salt. High salt content lowers the availability of water molecules, therefore the proteins become de-hydrated. This allows the hydrophopic parts of the proteins to come together, leading to precipitation.

Metal ions replace protons by binding to the amino groups, which lowers the PI. This lowers the tendency of a polar solvent layer forming around proteins resulting in precipitation.

The precipitated proteins are removed by centrifugation or filtration, leaving the supernatant that can be directly injected onto an LC-MS/MS system or evaporated and then reconstituted in a solvent that is compatible with the liquid chromatography mobile phase.

One advantage of this technique is the ability to automate the extraction procedure on liquid handling robots, enabling high throughput sample preparation (Watts *et al* 2000). The main disadvantage of the technique is the limited sample clean-up which potentially could cause matrix effects such as ion suppression in the mass spectrometer source and add stress to analytical systems such as the pumps (Lagerwerf *et al* 2000). Due to the lack of selectivity protein precipitation provides, a more selective extraction procedure may be required for some assays, particularly where a lower limit of quantification is required. Analytes which bind to proteins will be lost by co-precipitation.

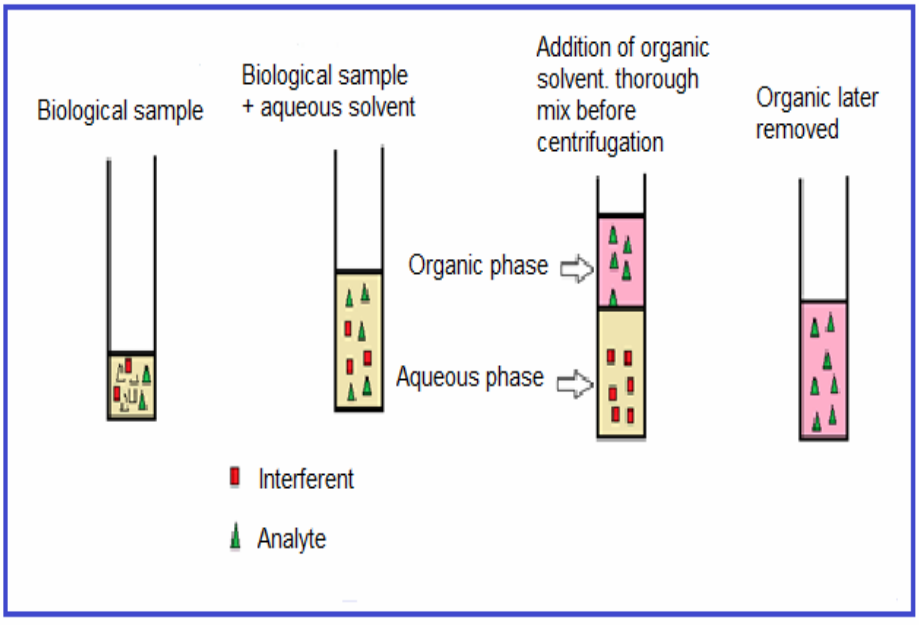
1.3.2 Liquid-liquid extractions

Liquid-liquid extractions (LLEs) are routinely used in bioanalysis because they are fast, simple and easy to automate. LLE is a separation technique which involves the use of two immiscible solvents, one aqueous and the other organic. In bioanalysis, LLE methods involve the addition of aqueous solvent to plasma, urine or blood samples. The pH of the aqueous solution can be adjusted to ensure the analyte is unionised. An immiscible

organic solvent is then added (Loos *et al* 1997). The two solutions are mixed to form an emulsion and then centrifuged to break the emulsion. The analyte is separated from polar interferents because the polar interferents partition into the aqueous phase (shown in figure 6). The polarity of the analyte and the pH determines the phase into which the analyte partitions.

The organic phase can be removed and then either evaporated and reconstituted in more suitable solvent for LC-MS analyses or undergo a further extraction procedure. Methanol or acetonitrile cannot be used in LLE because they have the ability to form hydrogen bonds or have dipole dipole interaction with aqueous solvents and so are miscible with aqueous systems. The lipophilicity, pH of the solvent and the type of solvent used determines the degree of partitioning. Lipophillicanalytes partition into the non-polar solvent (organic phase), whereas polar compounds generally partition into polar solvents (aqueous phase) (Wieling *et al* 1993).

Figure 6 Liquid-liquid extraction procedure.



For acidic analytes, the general approach is to adjust the pH to be two units below the pKa of the analyte to ensure it is not ionized and as a result partitions into the non-polar solvent. For bases, adjusting the pH to two units above the pKa ensures the analyte is not ionised. The solvents used in LLE must be chosen with care; for example, highly lipophilic

analytes extract well into a nonpolar solvent such as hexane. More polar analytes partition well into polar solvents like ethyl acetate. A solvent with a low boiling point is desirable to facilitate evaporation. If the analyte partitions into the organic phase it is wise to choose an organic solvent which has a lower density than water for ease of removal as the upper layer once the emulsion is broken.

1.3.2.1 Phenol-chloroform liquid-liquid extraction.

In 1953 Defner and co first described the use of phenol in extracting proteins from aqueous solutions (Kirby *et al* 1956). Sacchi and co used both chloroform and phenol because this combination is more efficient at denaturing proteins. Sacchi used phenol-chloroform to separate RNA from proteins. Without the addition of chloroform, phenol would retain 10% of the aqueous solvent and therefore 10% of RNA would also be lost to the phenol phase. Chloroform stops phenol from retaining water and thus improving the extraction efficiently (Sacchi *et al* 1987). The addition of isoamyl alcohol prevents foaming and is typical added at a ratio of 1:24 isoamyl alcohol: chloroform v/v.

Phenol has a higher density than either chloroform or water. Therefore the chloroform layer separates the aqueous and phenol layers simplifying the removal of the top aqueous layer.

Oligonucleotide analysis can be challenging because nucleotides may be strongly bound to proteins. Oligonucleotides partition into the upper aqueous phase because of the negatively charged phosphate backbone. Whereas the proteins partition into the lower phenol phase, as the hydrophobic region of the proteins interact with phenol (Zhang *et al* 2007). The aqueous phase can be removed and undergo a secondary extraction procedure.

1.3.3 Solid phase extraction

Solid phase extraction involves the retention of the analyte on a solid sorbent. SPE gained popularity in bioanalyisis in the late 1980s because of its compatibility with polar compounds and zwitterions (Hennion *et al* 1999). The first step in SPE is to pre-treat the sample which could involve the addition of a reagent to adjust the pH, centrifugation to

remove particulates or a dilution step to reduce the viscosity of the sample. Once the sample has been pre-treated, the SPE plate or cartridge is conditioned by passing water-miscible organic solvent (typically methanol or acetonitrile) through the SPE material. This wets the phase creating a suitable environment for retention of the analyte (Rossi *et al* 2000). The next step is to equilibrate the sorbent, which is achieved by passing a solvent through the SPE material with a similar polarity and pH to the treated analyte-containing solution. Once the sorbent has been equilibrated, the pre-treated sample can be loaded onto the SPE material under conditions that result in retention of the analyte. One or more washing steps remove interferences, and are then followed by an elution step which removes the analyte from the sorbent.

Reversed phase and ion exchange are the two main retention mechanisms used in SPE in drug development. Reversed phase SPE involves hydrophobic interactions between the sorbent (typically a C8 or C18 chain bound to silica) and the analyte (Martin *et al* 1998). Non-polar solvents are used to elute the analyte.

The retention mechanism in ion exchange is electrostatic interaction between the analyte and the sorbent (Martin *et al* 1998). For basic compounds, cation exchange is used, while anion exchange is used for acidic compounds. For weakly basic compounds a strong cation exchange (SCX) sorbent is used. The analyte is generally eluted from the sorbent by pH manipulation; the pH of the elution solution is designed to remove the charge from the analyte, and as a result the electrostatic interactions are disrupted. For strong bases, weak cation exchange sorbents (WCX) are used. The pH of the elution solution is designed to neutralise the sorbent thus disrupting the electrostatic interactions.

1.4 High performance liquid chromatography

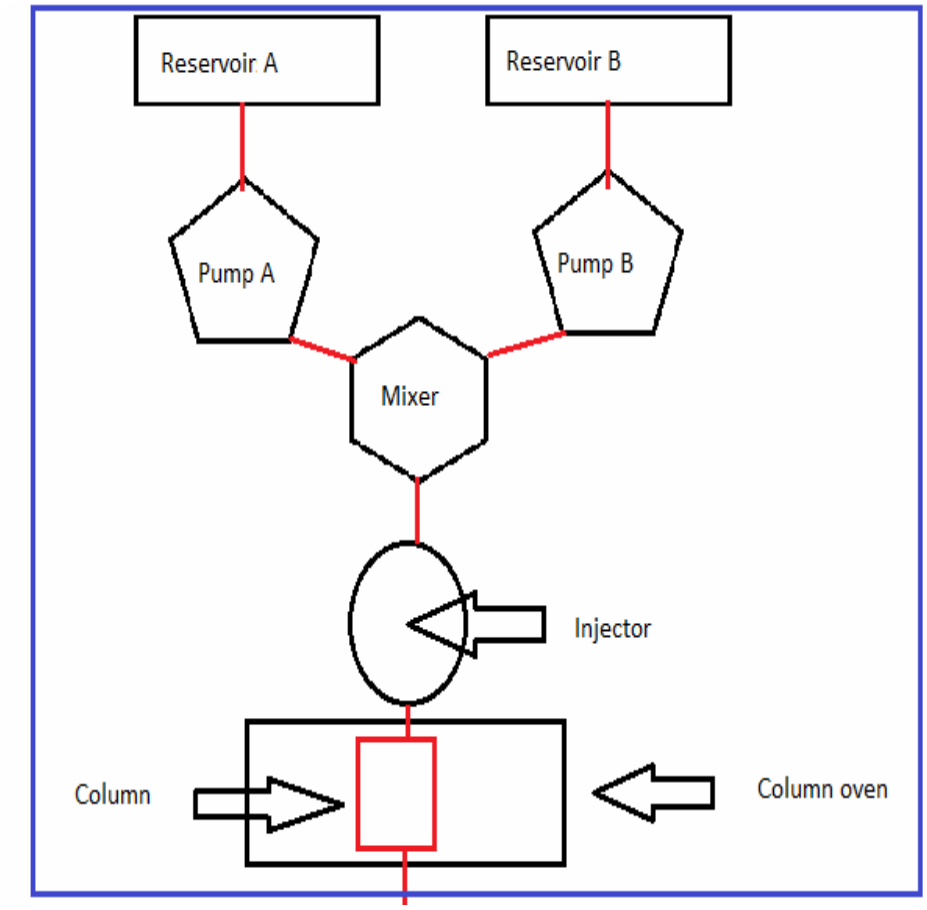
High performance liquid chromatography (HPLC) is a form of liquid chromatography which involves the separation of compounds in solution. Mobile phase containing analytes and impurities passed over an absorbent stationary phase which is typical contained within a stainless steel column. The physical and molecular properties of the analyte and potential interferants will determine their affinity for the mobile phase or stationary phase. The migration of the analyte can only occur if the analyte is dissolved in the mobile phase,

therefore analytes which have a high distribution into the stationary phase will elute later than those which have a higher affinity for the mobile phase (Huber *et al* 1967).

The molecular forces involved in the retention of the analyte in LC are Van der waal, dipole-dipole interactions, hydrogen bonding, dielectric interactions and electrostatic interactions (Brown *et al* 1989).

A typical HPLC system consists of two solvent reservoirs, two high performance pumps, a solvent mixer, an injector to introduce the sample and a column oven (shown in figure 7)

Figure 7 Schematic of an HPLC system (Modified from principles and practice of bioanalysis page 44)



Mobile phase A (typically an aqueous solution) is placed into reservoir A and an organic solution is placed in reservoir B and is typically known as mobile phase B. The pumps ensure that the delivery of solvent is pulseless, avoiding unnecessary variations in detection. The pumps are typically capable of operating at 6000 psi. Mobile phase A and mobile phase B are combined in the solvent mixer. An injector is used to introduce the analyte into the mobile phase flow, which then passes through a stainless steel column containing an absorbent stationary phase. The column is placed within a column oven which heats the column to reduce back pressure. This ensures that no variations of retention times are observed due to variations in ambient temperature. Also increasing the temperature tends to increase the solubility of the analyte in the mobile phases resulting in faster run times. The viscosity of the mobile phases is reduced at higher temperatures resulting in less pressure within the system.

1.4.1 Distribution

The degree of separation of components by LC is dependent on the differences in their equilibrium distribution coefficient (K).

Equation 1 Calculation for equilibrium distribution coefficient (K)

$$K = C_s / C_m$$

Where Cs = concentration of solute in stationary phase
Cm = concentration of solute in mobile phase

Solutes with higher K values will elute from the LC column later than those with a lower K value (Hendriks *et al* 2009).

1.4.2 Retention

The degree to which an analyte is retained on a column is known as retention factor (k'). Equation 2 can be used to determine the k' (provided the flow rate remains unchanged).

Equation 2 Calculation for retention factor (k').

$$k' = (t_R - t_0) / t_0$$

Where t_R is the time from injection to the peak maximum
t_0 is the time the mobile phase takes to pass through the column from injection

Analytes with high k' values have higher retention times.

1.4.3 Selectivity

The separation of various analytes and impurities is vital in quantitative bioanalysis. In order to separate two analytes, their respective k' values must be different. The selectivity factor (α) is a ratio of the respective k' values and be calculated using the formula 3. Ideally the retention factor for an analyte is between one and five.

Equation 3 Calculation for selectivity factor (α)

$$\alpha = k_B/k_A$$

Were B is the compound that elutes last

1.4.4 Theoretical plate theory

Theoretical plate theory suggests that a column is made up of a series of theoretical plates. Within each of these plates the analyte is said to be fully equilibrated between the mobile phase and the stationary phase. The greater the number of theoretical plates (N) the greater resolution between two analytes can be achieved. The smaller the plate height (HETP, Height equivalent to a theoretical plate) the better efficiency is observed. Equation 4 can be used to calculate HETP (Hendriks *et al* 2009).

Equation 4 Calculation for height equivalent to a theoretical plate

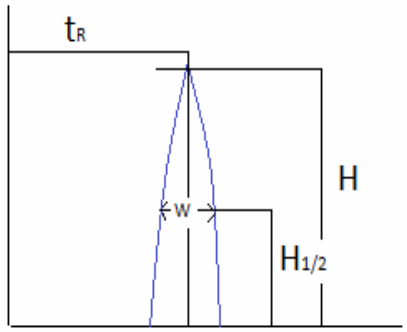
$$HETP = L/N$$

Were L is the column length

The number of theoretical plates can be estimated experimentally by using the equation 5. The number of theoretical plates is used to access the column efficiency.

Equation 5 Calculation for the number of theoretical plates

$$N = 5.54 \left(\frac{t_R}{w_{0.5}} \right)^2$$



where : t_R is the retention time
w is the peak width at half the peak hieght

1.4.5 Rate theory

The rate theory takes into account the time taken for the analyte to fully equilibrate between the mobile phase and the stationary phase. Unlike plate theory which presumes that equilibration occurs instantly, therefore rate theory provides a more accurate model of the inner workings of a column. Rate theory also takes into account factors which affect the band width of the chromatographic peak, such as the eddy diffusion (the analyte will take different routes through the column), longitudinal diffusion (the concentration of the analyte will be greater at the centre of the band than the edges) and resistance to mass transfer (the analyte will take time to equilibrate between the stationary phase and the mobile phase if the velocity of the mobile phase is high and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will have a greater velocity than the analyte in the stationary phase). Van Deemter equation takes these factors into account (shown in equation 7) (Van Deemter *et al* 1956).

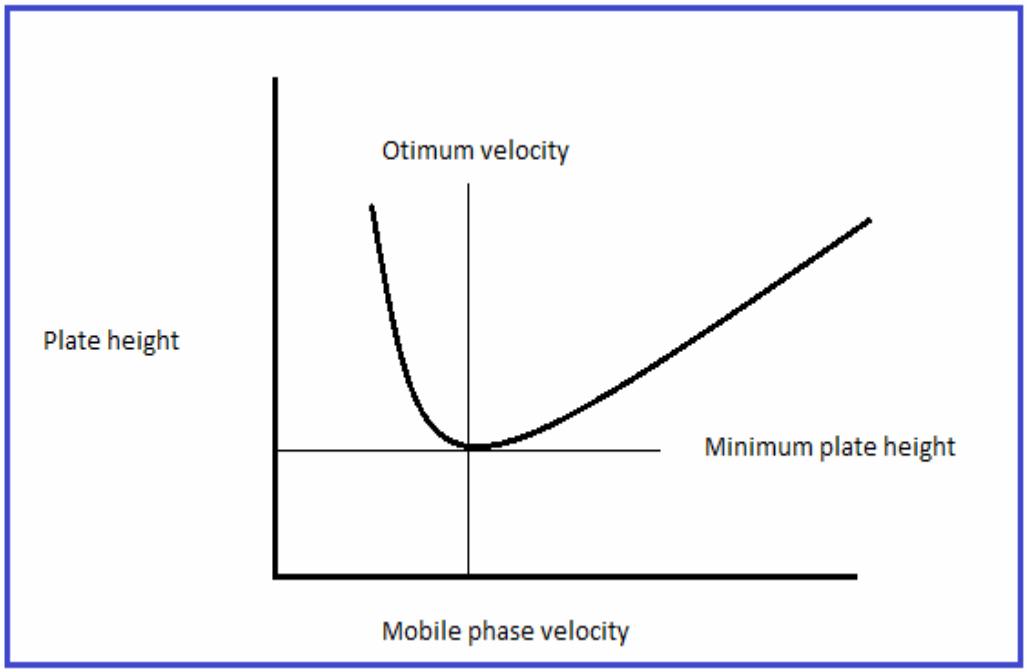
Equation 7 Van Deemter equation for plate height

$$HETP = A + B/u + Cu$$

Where A = Eddy diffsion
B = Longitudinal diffusion
C = Resistance to mass transfer
U = The average mobile phase velocity

The relationship between the plate height and the mobile phase velocity is described by the Van Deemter plot (shown in figure 8). The Van Deemter plot can be used to find the optimal mobile phase velocity.

Figure 8 Typical Van Deemter plot



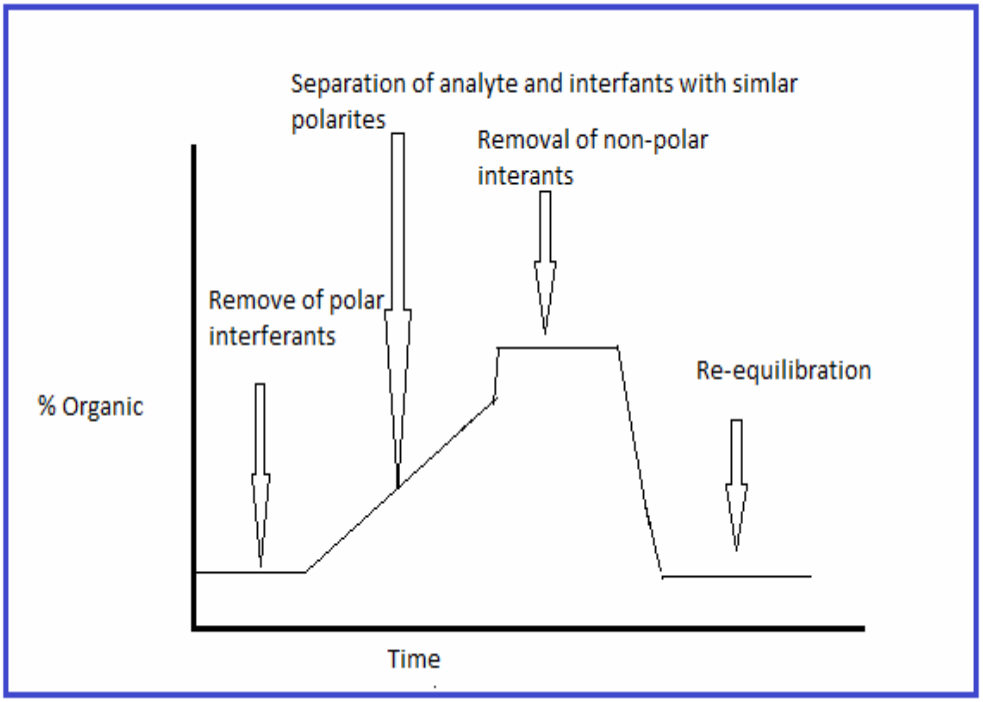
1.4.6 Reversed phase chromatography

Reversed-phase chromatography is a LC separation technique predominantly used in bioanalysis. Reverse-phase stationary phase is typically made up of carbon chains (Typically C8 or C18) on a silica support. Silica is used because it is stable under the high pressure observed in LC and it does not swell or shrink when exposed to organic solvents. In reversed-phase the stationary phase is non-polar and as a result non-polar analytes will interact with the stationary phase more than polar compounds. Polar compounds will have a lower retention time than non-polar compounds. When the mobile phase is predominantly aqueous, polar compounds will have little retention whereas non-polar compounds will be well retained. Increasing the percentage of organic solvent (non-polar solvent) in the mobile phase, the interaction between the mobile phase and non-polar analyte will increase resulting in a decrease in retention.

1.4.7 Isocratic and gradient separation

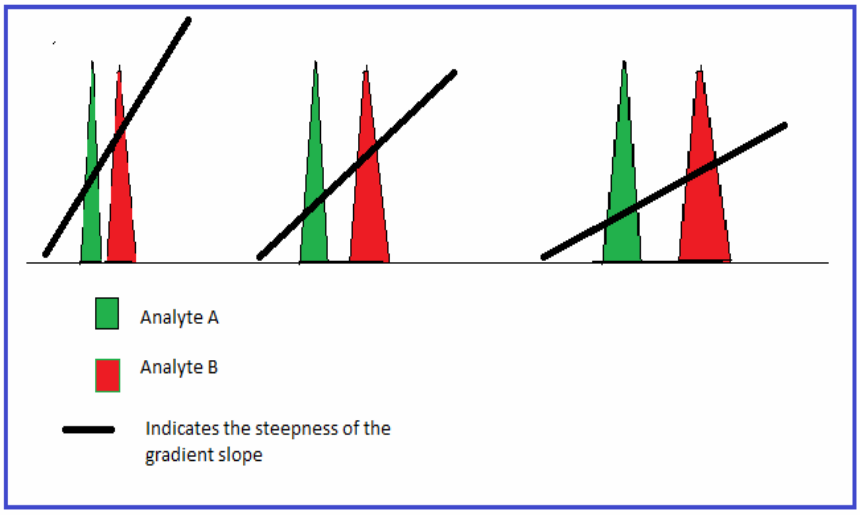
Isocratic separation is when the ratio of aqueous and organic solvent in the mobile phase remains the same throughout the analytical run (Tiler *et al* 2002). No equilibration step between sample injections is required in isocratic separation. Gradient elution is when the ratio of aqueous and organic solvents changes throughout an analytical run. Typically gradient elution starting conditions have a high percentage of aqueous solvent present to remove polar interferants, followed by an increase in organic solvent to separate the analyte from potential interferants with similar polarity. Non-polar interferant are removed by a wash step which involves using high percentage of organic solvent, shown in figure 9.

Figure 9 Typically gradient in LC



Increasing the slope of the gradient will result in the analyte eluting faster. If multiple analytes are present the resolution between analytes will decrease with a steeper slope (shown in Figure 10). A shallower gradient will result in greater resolution but could cause peak broadening. Maintaining a balance between resolution, peak shape and over all run time is vital in quantitative analysis (Horvath *et al* 1967).

Figure 10 the effect of slope steepness on resolution and peak shape



1.4.8 Ion-pair reversed phase chromatography

Ion-pair reversed-phase is the chromatographic separation technique often used for oligonucleotide quantitative analysis (Apffel *et al* 1997). Due to the highly polar and ionic nature of oligonucleotides very little resolution and retention is achieved by standard reverse phase. The addition of a positive ion pairing modifier trethylammonium (TEA) to mobile phases results in the positive charged TEA forming ion-pair with the oligonucleotides (Lin *et al* 2007).

The oligonucleotide affectively becomes a neutral analyte and therefore resolution and retention is achieved in reversed-phase. The main issue with this approach is poor mass spectrometer sensitivity (analytes must be charged for mass spectrometer detection). Increasing the pH is favourable for dissociation of the oligonucleotide-TEA (oligo-TEA) ion pair, therefore 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) is also added to the mobile phase. HFIP lowers the pH of the mobile phase which strengths the oligo-TEA ion pair resulting in retention, however HFIP ready evaporates in the mass spectrometer source, as a result the pH of the remaining solvent increase sufficiently to dissociate the oligo-TEA ion pair (Van Dongen *et al* 2011).

1.5 Mass spectrometry in bioanalysis

Mass spectrometers are a common feature in pharmaceutical and contract research laboratories all around the world. They are the preferred method of quantitation of small and large compounds including oligonucleotides. Mass spectrometry is an analytical technique which involves ionisation of molecules followed by separation according to their mass to charge ratio. The recent improvement in mass spectrometer sensitivity has had a great impact in the bioanalysis world. One good example is the increased interest in micro-sampling which is only possible due to the advances in mass spectrometer sensitively.

A mass spectrometer consists of a sample inlet, ion source, mass analyser, detector and data recorder/ processor. There are a number of technologies that exist for each component. The requirements of the analysis define which components are used.

1.5.1 Ionisation techniques

The use of atmospheric pressure ionisation (API) sources coupled with liquid chromatography systems was first reported by Horning in 1974 (Horning *et al* 1974). API sources are widely used for both qualitative and quantitative analysis in all stages of drug development. Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are two ionisation techniques which operate at atmospheric pressure. ESI is a liquid phase ionisation technique, whereas in ACPI the ionisation occurs in the gas phase.

1.5.1.1 Electrospray Ionisation

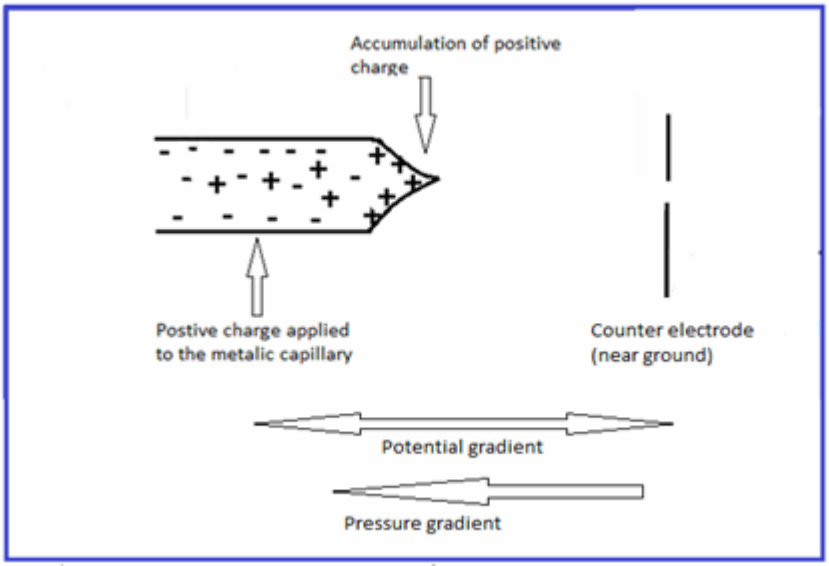
ESI was first described by Dole in the late 1960s (Dole *et al* 1968). However the first ESI source for mass spectrometers was first introduced by Fenn in1985 (Fenn *et al* 1985). Fenn was awarded a share of Nobel prize in Chemistry in 2002 for his work on ESI. There are three major steps in ESI 1: production of charged droplets, 2: solvent evaporation and 3: production of charged ions in the gas phase.

The solvent containing the analyte, often from a liquid chromatography system, is pumped through a stainless steel or fused silica capillary to which a high voltage is applied. The high voltage on the capillary causes an electrochemical reaction.

There is a potential gradient difference within the mass spectrometer source, due to the charged capillary and the counter electrode (typically located 1-2 cm from the capillary tip), as shown in figure 11. There is also a pressure gradient caused by the atmospheric pressure within the source and the vacuum within the mass spectrometer

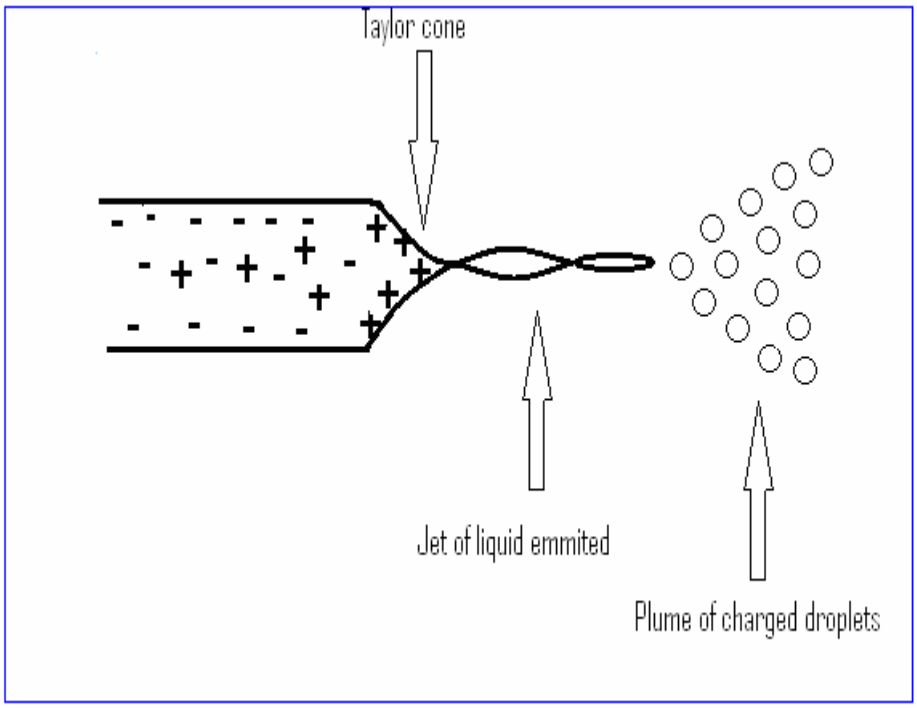
In positive mode the electrons from molecules within the solvent are drawn towards the positive capillary producing positive ions (oxidation of the solvent). The positive ions are repelled by the capillary and accumulate at the capillary tip, as shown in figure 11. The opposite occurs in negative mode (reduction of the solvent) (Fenn *et al* 1984).

Figure 11.Accumulation of positively charged ions at the tip on application of voltage to ESI capillary.



As the positive ions accumulate, the repulsion between the positive ions deforms the liquid at the tip of the capillary. As the force that is exerted on the liquid by the electric field approaches the same force of the surface tension, a convex cone formed. As a threshold voltage is reached the convex cone inverts (Taylor cone) and emits a jet of liquid, resulting in a plume of charged droplets, as shown in figure 12. The Taylor cone was first theoretically described by Sir Geoffrey Taylor in 1964 (Taylor *et al* 1964), well before the invention of ESI.

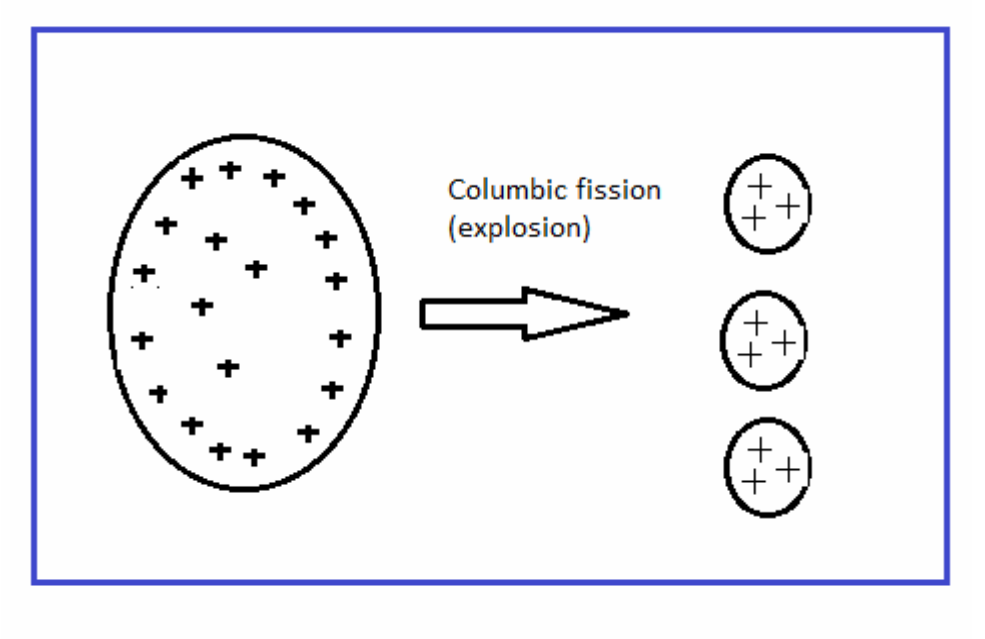
Figure 12 Jet of liquid emitted from the Taylor cone in ESI source



A nebulising gas (typically N_2) is pumped around the outside of the needle to direct the electrospray towards the mass spectrometer; it also assists in desolvation of the charged droplets (Zhou *et al* 2001).

The density of charged ions within the droplets increases as the solvent is evaporated. When the electrostatic repulsion of the charged ions is greater than the surface tension of the solvent (as the Rayleigh limit is exceeded), columbic fission (explosion) occurs. The smaller droplets produced on this explosion have 2% of the mass and 15% of the charge of the original droplet. Therefore they have a much higher charge to volume ratio, as shown in figure 13 (Tu *et al* 2005).

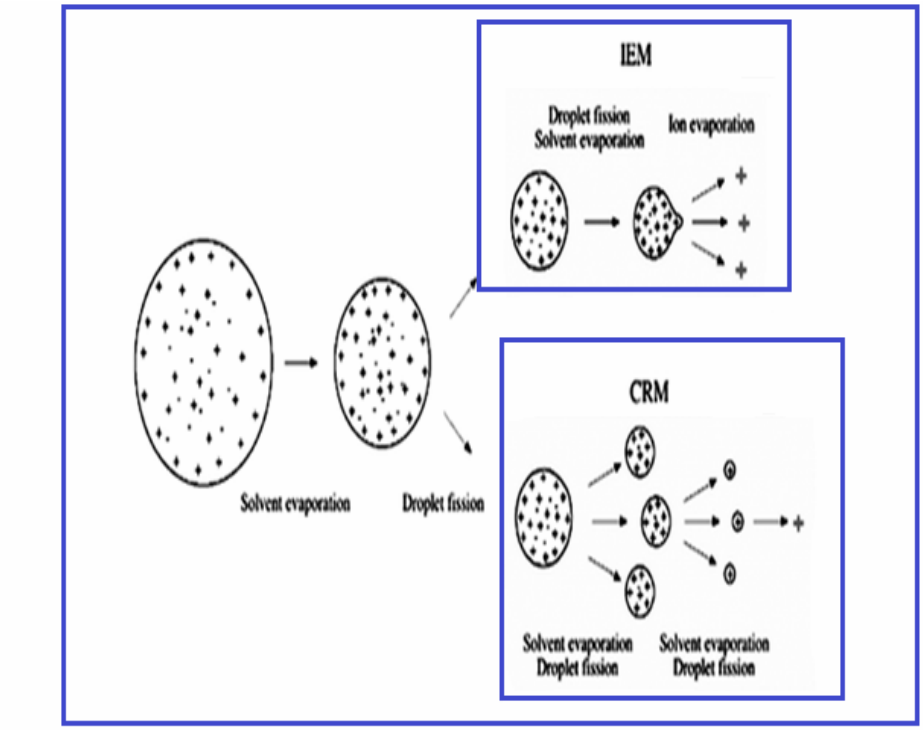
Figure 13 Columbic fission of charged droplets in ESI source.



There are two theories behind the electrospray production of charged ions in the gas phase from these charged droplets: the charged residue model and the ion evaporation model. In the charge residue model it is postulated that there are multiple fission steps until only single ions remain, which are draw in to the mass spectrometer via the counter electrode, as shown in figure 14 (Dole *et al* 1968).

In the ion evaporation model it is proposed that once the droplets reaches a radius smaller than 10 nm, the surface field strength becomes large enough to overcome solvation forces. At this point ions are ejected from the surface of the droplets into the gas phase, as shown in figure 14 (Thomson *et al* 1979).

Figure 14 Ion evaporation model and charged residue model of ESI ion production.



The majority of ESI sources used in modern mass spectrometers have introduced a nebulizer gas (typically nitrogen) to assist in generating the droplets. Pneumatically-assisted electrospray ionisation can handle higher flow rates than traditional ESI sources. As a result they have become very popular in high throughput bioanalysis.

It is important to consider the effects of the mobile phases on ESI. Polar solvents such as methanol, acetonitrile and water are very susceptible to electrochemical reaction and therefore are a good choice for liquid chromatography mobile phases when LC is to be coupled with ESI. Organic solvents such as methanol and acetonitrile have a lower surface tension and boiling point than water, and as a result the desolvation and solvent evaporation (especially when high flow rates are used) will be more efficient. The addition of acid or base to the mobile phase can assist in the production of positive or negative ions, although this will have an impact on chromatography.

ESI can produce either singly or multiply charged ions and therefore ESI can be used as an ionisation technique in the analysis of large molecules such peptides, oligonucleotides

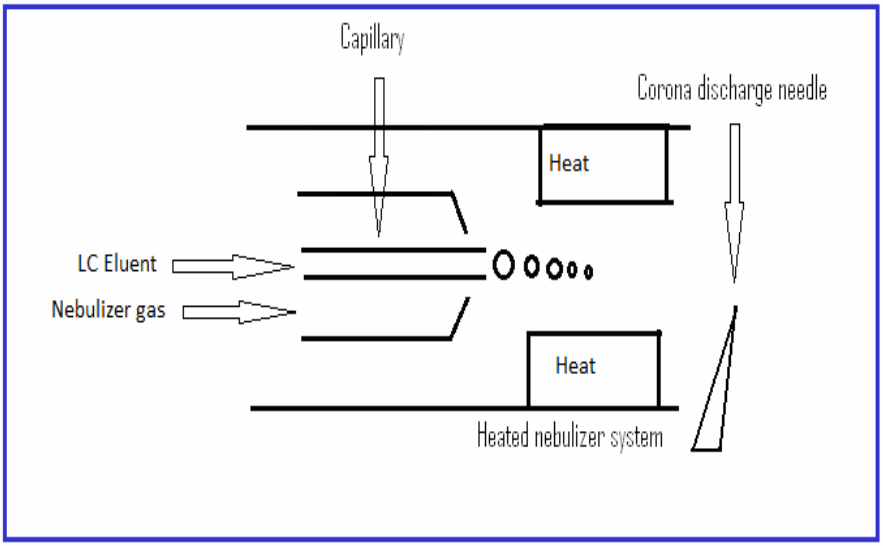
and proteins (Fenn *et al* 1989). The mass to charge ratio (*m/z*) of a singly charged large molecule such as an oligonucleotide would fall outside the *m/z* limits of a mass spectrometer.

1.5.1.2 Atmospheric pressure chemical ionisation

Atmospheric pressure chemical ionisation (APCI) is a gas phase ionisation process, which consists of a capillary (used to introduce the liquid from a LC system), a heated nebulization system and a high voltage corona discharge needle, as shown in figure 5. The corona discharge was first used in mass spectrometry ionisation in 1975 by Horning (Horning *et al* 1975).

The HPLC eluent is passed through the capillary into the nebulizer, where a nebulizer gas (typical nitrogen) is used to create a spray of neutral droplets. The nebulizer gas forces the droplets through a heated tube and as a result the solvent in the droplets is evaporated leaving solvent and analyte molecules. The nebulizer gas forces the solvent and analyte molecules into the region of the corona discharge. The nebulizer gas also assists in stabilising the corona discharge region.

Figure 15 Atmospheric pressure chemical ionisation source



Chemical ionisation is the method of ionisation in APCI. The electrons emitted by the corona discharge needle initiate the chemical reaction process. The nebulizer gas (nitrogen) is ionised by electron ionisation which initiates the chemical reactions.

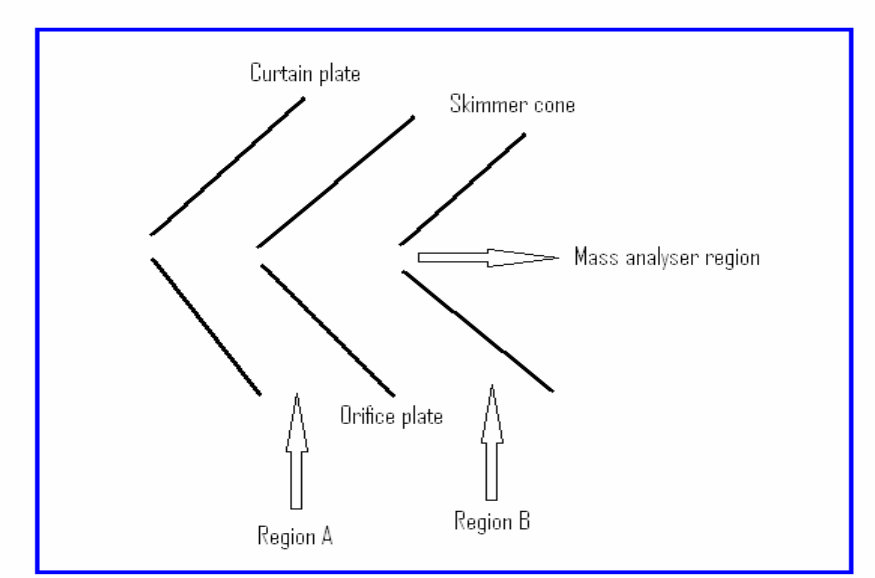
In positive mode, the ionised nitrogen molecules collide with water or mobile phase molecules resulting in charge transfer. Further collisions produce different charged molecules which ultimately collide with the analyte molecules resulting in proton transfer producing analyte ions.

ACPI is not suitable for very polar compounds, thermally labile analytes, or compounds which are typically charged in solution (proteins or oligonucleotides). Thermally labile compounds may decompose in the heated nebuliser. Multiply charged ions are not produced in APCI.

1.5.2 Movement of ions from atmospheric pressure to a vacuum

The ions produced in the API source are drawn into the mass spectrometer by the potential and pressure difference within the source. Within an AB SCIEX API 4000 or 5000 mass spectromters. The ions first pass through a curtain plate into region A shown in figure 16. This region contains a curtain gas (typically nitrogen) which is used to stop solvent entering the mass spectrometer. It also assists in declustering analyte ions from solvent molecules or ions. Region A is still at atmospheric pressure. The ions and the remaining solvent molecules are drawn into region B because of the difference in pressure. In region B a pump is used to remove solvent molecules. The remaining ions are drawn into the mass analyser region by the potential difference between the orifice plate and the skimmer cone.
(http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry.pdf).

Figure 16 Mass spectrometer-AP ionisation source interface schematic.



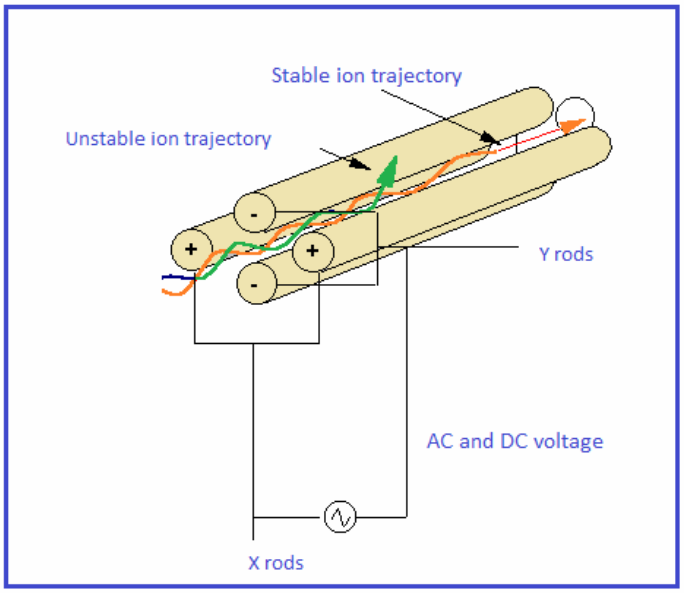
1.5.3 Mass analysers

Mass analysers are used to separate ions based on their mass to charge ratio (*m/z*). They can be used to separate ions produced in the ion source or collision cell. Quadrupole mass analysers are predominantly used in quantitative bioanalysis, due to their selectivity as mass filters.

1.5.3.1 Quadrupole mass analysers

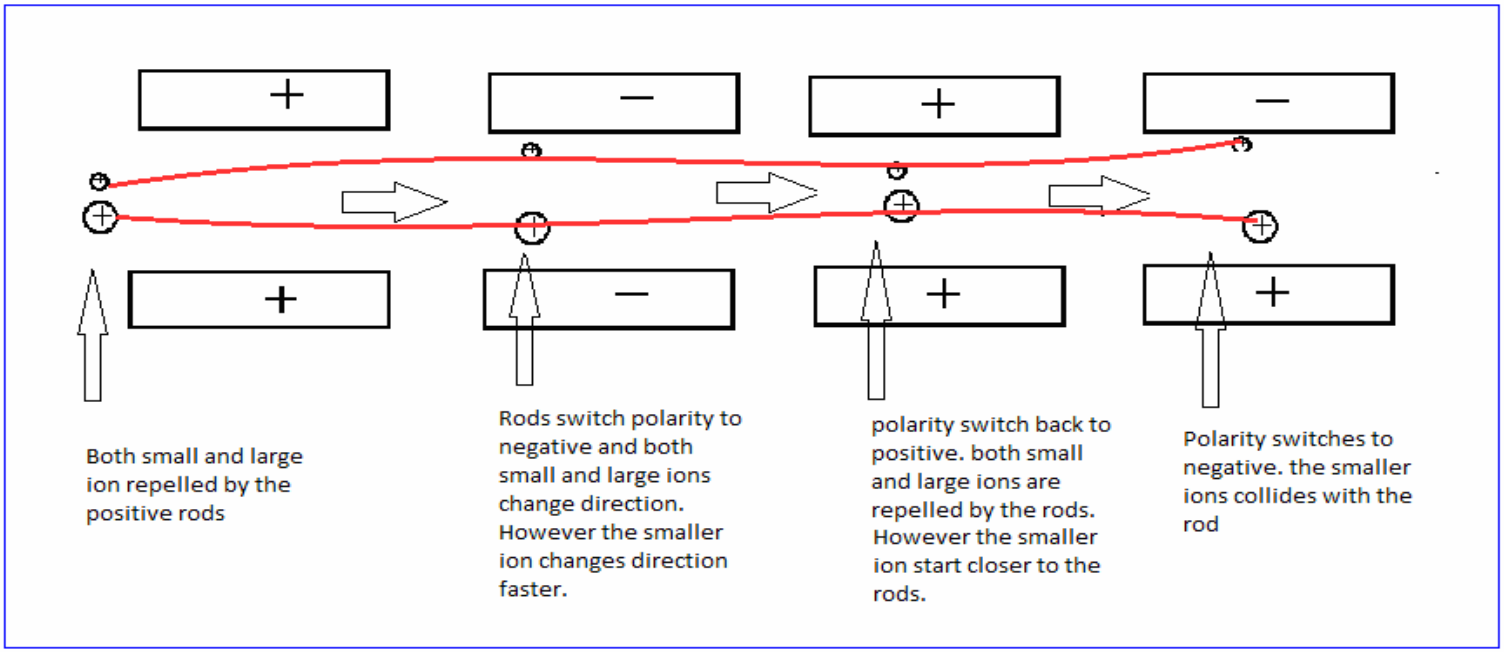
Paul and colleagues developed the quadrupole mass analyser in parallel with the quadrupole ion trap (Paul *et al* 1953). Paul was awarded the Nobel prize for physics in 1989 for his work. Quadrupole mass analysers (also known as mass filters) consist of four electrically conducting parallel rods. The rods are arranged symmetrically and ideally the cross sectional shape is hyperbolic, helping to achieve a hyperbolic field. The rods have a fixed direct current and an altering radio frequency voltage applied. Diagonally opposing rods (the X rods make one pair and the Y rods another) are electrically connected, shown in figure 17.

Figure 17 Quadrupole mass analyser schematic.



Due to the alternating RF voltage, the polarity of the rod switches over time, however the overall polarity of the rod remains the same as the polarity of the DC voltage for a greater length of time. If we consider the X rods only, positive ions are repelled by the rods when the overall polarity of the rods is positive and attracted to the rods as the polarity switches to negative. Small ions have the ability to change direction more quickly than large ions, ultimately resulting in the small ions colliding with the rods during their negatively charged phase (shown in figure 18). Therefore the X rods can be described as a low mass filter, while the opposite holds for the Y rods, which act as a high mass filter.

Figure 18. Pair of rods acting as a low mass filter



In 1868 Mathieu was able to describe regions of stability and instability after his investigation into the mathematics of vibrating stretched skins (Mathieu *et al* 1868). The motion of ions within a quadrupole can be described by the solutions to the Mathieu equations. By using the Mathieu equations, the voltage required for a stable oscillating trajectory through the quadrupole can be achieved for ions with a given *m/z*. By the combined effect of the two pairs of rods, only ions with a certain *m/z* ratio will have a stable trajectory through the whole quadrupole for a given RF/DC voltage.

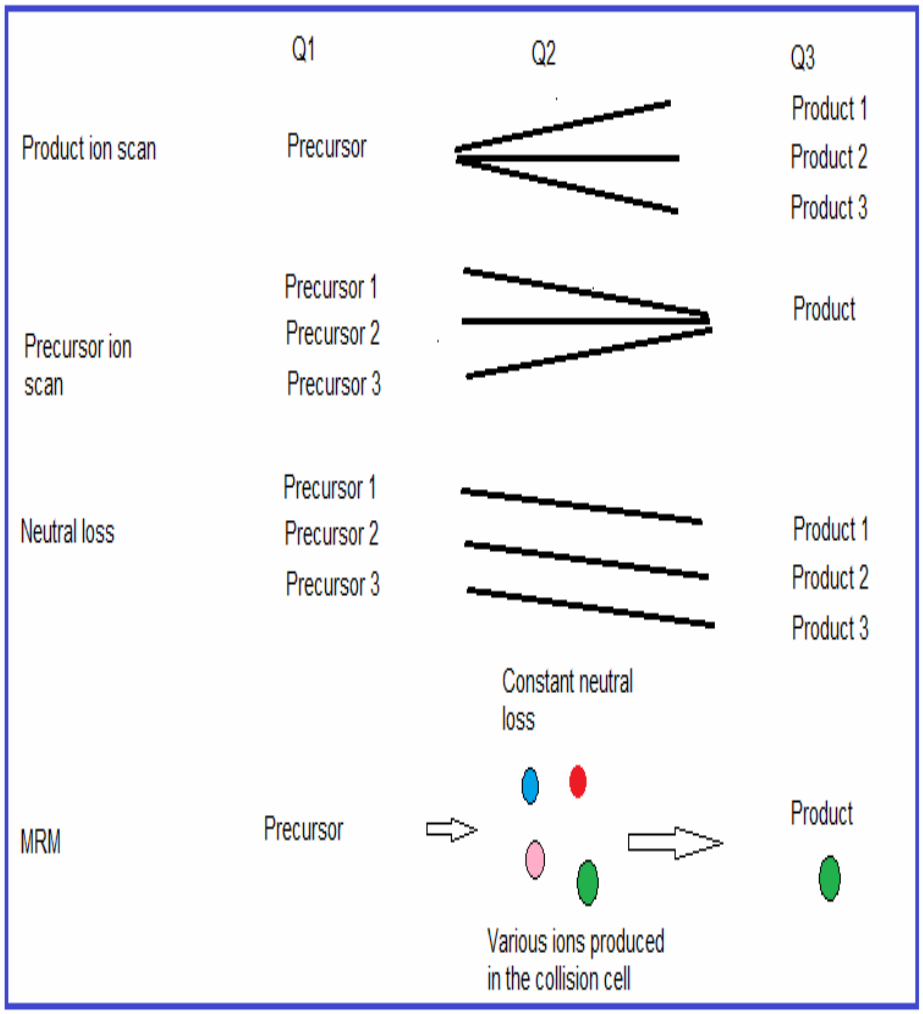
Quadrupoles are used as a scanning mass analyser and as a result have a poor duty cycle. Duty cycle is the fraction of time that a particular m/z is monitored versus the total time spent on monitoring the *m/z* range.

1.5.3.2 Triple quadrupole mass spectrometry

The first triple quadrupole mass spectrometer was developed in the late 1970s by Yost (Yost *et al* 1978). A triple quadrupole mass spectrometer typically consists of two mass filters Q1, Q3 and a collision cell. The collision cell is a quadrupole to which only RF voltage is applied. The ions leaving Q1 are accelerated into the collision cell which contains a neutral or inert gas typically nitrogen or argon (known as the collision induced dissociation (CID) or collision activated dissociation (CAD) gas). The collisions with the CAD gas results in fragmentation of the ion.

There are various scan modes that can be performed with a triple quadrupole mass spectrometer. These include product ion scan, precursor ion loss, neutral loss and multiple reaction monitoring (MRM) (shown in figure 19).

Figure 19 Different scan modes in tandem mass spectrometry



In product ion scan mode, Q1 only transmits ions at one *m/z* into the collision cell, where they are fragmented. Q3 scans for all the products produced (shown in figure 19).

In precursor ion scan mode all the ions generated in the source are transmitted into the collision cell, where they are fragmented to produce various product ions. Q3 transmits a selected product ion. Neutral loss involves Q1 and Q3 scanning for fixed mass differences between them (shown in figure 19).

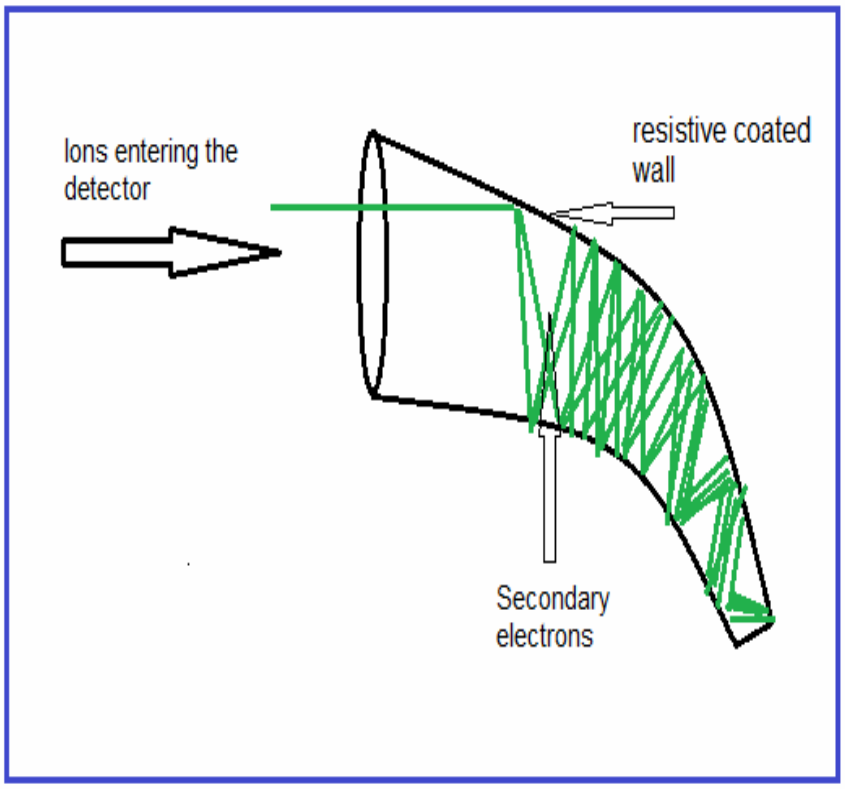
In MRM mode, Q1 is set to transmit ions with only one *m/z* into the collision cell, where various product ions are formed due to fragmentation caused by collisions between ions and collision gas. Q3 is set to transmit product ions with only one *m/z* into the detector.

1.5.4 Detectors

There are a variety of devices that can be used to detect the ions leaving the mass analyser, such as electron multipliers and faraday cups. Electron multipliers are the most commonly used detector in bioanalysis due to their low response time, sensitivity and accuracy.

Electron multipliers in the majority of current instruments are of the continuous dynode type. The ion beam from Q3 enters the detector and hits the resistive coated wall. As a result of the collision, secondary electrons are released. The secondary electrons move deeper into the horn (shown in figure 20). As these secondary electrons impact the wall, further secondary electrons are released, multiplying the amount of electrons by a factor of 2, this process is repeated until the electrons reach the preamplifier.

Figure 20 Electron multiplier horn diagram.



1.6 Validation

Before an analytical method can be used on a regulated study the method must first be validated. The foremost objective of method validation is to demonstrate the reliability of the method for the quantification of an analyte in a given biological matrix. The method must be rigorously tested to ensure that it generates accurate, precise and reliable data. The fundamental parameters for method validation are accuracy, precision, selectivity, sensitivity, recovery, reproducibility and stability (Guidance for industry: bioanalytical method validation, FDA 2001).

1.6.1 Internal standards

Internal standards are compounds which account for variations in sample extraction, chromatographic separation and ionisation efficiencies of individual samples. Either a structural analogue or a stable isotope labelled internal standard can be used. The internal standard response for a given sample (Sample X) is compared to the mean internal standard response of all the samples injected onto the LC-MS/MS system. If the internal standard response of sample X is 10 % lower than the mean response, then sample X has lost 10% of the internal standard and therefore also lost 10 % of analyte at some point during the analysis. As a result the analyte concentration is automatically corrected by 10%. Therefore any variations during analysis have been compensated for (Shah *et al* 2000).

A stable isotope labelled internal standards is the most effective because they have extremely similar extraction recovery, response and chromatographic retention time. Stable isotopes used are ^{13}C, ^{15}N and Deuterium. It is essential that the stable isotope labelled internal standard contains sufficient isotopes to increase the overall mass above that of the natural mass distribution of the analyte, otherwise the analyte could contribute to the internal standard mass spectrometer response.

1.6.2 Calibration curve

Known concentrations of an analyte are spiked into aliquots of the sample matrix to produce calibration standards. The calibration standards are then subjected to the same sample preparation and extraction procedure as the test samples. The detector response for the analyte is divided by the detector response of the internal standard to produce an area ratio. A calibration curve is then generated by plotting the area ratio against the concentrations of the analyte in the calibration standards. The calibration curve is used to determine the concentration of analyte in quality control samples containing defined analyte concentrations and the test samples (unknown analyte concentration). The range of the calibration curve is determined by the expected analyte concentrations in the study samples. The lower limit of quantification (LLOQ) should have a detector response five times greater than the background signal to noise ratio (Shah *et al* 2000).

1.6.3 Quality control samples

Quality control samples (QCs) are produced by adding known amounts of analyte into the sample matrix and they are used to assess the performance of a method. QCs are stored and handled in the same manner as the test samples. A calibration curve is only deemed acceptable if the QC concentrations determined using the calibration curve are within +/- 15% of the analyte concentration in the QCs (+/- 20 % at the LLOQ) (Shah *et al* 2000).

1.6.4 Selectivity

A bioanalytical method must be able to quantify the analyte in a sample which contains many other components from which the analyte must be distinguishable. The method validation must demonstrate that the substance being quantified is indeed the analyte and not a peak originating from the control matrix. This is achieved by analysing blank matrix from at least six different sources, for matrix-derived interference at the retention time of the analyte and its internal standard (Shah *et al* 2000).

1.6.5 Accuracy

The closeness of the mean test result obtained by a method on analysis of the QCs to the concentration of the analyte in those QCs allows the accuracy of an analytical method to be defined. Accuracy is determined by analysing replicates of quality control samples at three concentrations levels within the calibration range and also at the lowest limit of quantification (LLOQ) .The mean concentration determined for the analyte in the quality control samples should be within +/- 15% of the nominal values for analyte concentration in these samples (20 % for the LLOQ QC) (Shah *et al* 2000).

1.6.6 Precision

Precision of an analytical method is the variation in concentrations determined for replicate QCs. Multiple QCs aliquots are taken from a single homogeneous aliquot and individually measured. The precision test is repeated for all QC concentration levels and the coefficient of variation [(standard deviation / mean concentration)*100] should not exceed +/- 15% (20% for LLOQ QC) (Shah *et al* 2000).

1.6.7 Dilution

Successful dilution of samples with concentrations above the upper limit of quantification must be demonstrated during the validation. Dilution QCs prepared at a concentration higher than the upper limit of quantification (generally fivefold) are diluted with blank matrix prior to addition of internal standard, so that the response falls with the calibration range. The accuracy and precision of the dilution process should be within +/- 15% nominal value (Shah *et al* 2000).

1.6.8 Sensitivity

Sensitivity is the lowest concentration that can be measured with acceptable accuracy, reproducibility and precision. The measured response should be at least five times greater than the background noise (Shah *et al* 2000).

1.6.9 Recovery

Recovery is measured by comparing the detector response of an extracted QC sample with the detector response of an extracted control matrix sample into which the analyte has subsequently been spiked to yield the same analyte concentration as in the QC, using a solution of pure standard. The recovery test is done at low, medium and high analyte concentrations and should be precise and reproducible across the concentration range (Shah *et al* 2000).

1.6.10 Stability

The stability of the analyte in primary, diluted working stock solutions, and matrix must be evaluated to ensure that the data generated using the method are valid. Stability experiments test room temperature stability, bench-top stability, freeze/thaw stability and stock solution stability. Freeze/thaw experiments are conducted to mimic the handling of test samples. Freeze/thaw stability is assessed after a minimum of three freeze/thaw cycles. Freeze thaw stability samples are initially stored frozen for a period of 24h before being thawed at room temperature for a minimum 1 hour period. The QC samples are then returned to the freezer for 12 hours. Bench-top stability experiments with frozen QC samples are conducted over the length of time that the samples will be at room temperature during the test sample analyte extraction procedure. Long-term stability whilst frozen experiments are also conducted to cover the sample storage duration between sample generation and subsequent analysis (Shah *et al* 2000).

1.7 Aims

The overall aim of this research was to increase the popularity and use of micro-sampling within the bioanalysis arena. At the start of this research in 2011 DBS analysis was increasing in popularity and therefore the first part of this MSc research is focused on DBS analysis. The aim of the research was to develop techniques which limit the requirements for reanalysis of samples, which fall outside the calibration range for any given method.

Concerns surrounding DBS analysis were brought to light midway through the project (discussed in section 1.1). Therefore my research was broadened to also include CMS. The aim of this research was to develop a bioanalytical method for the quantification of a phosphorothioated oligonucleotide in human plasma capillary micro-samples using LC-MS/MS and in doing so demonstrate the compatibility of capillary micro-sampling (CMS) with a challenging class of compounds.

Chapter 2:

Alternative strategies for on-line mass spectrometer based sample dilution for bioanalytical samples, with particular reference to dried blood spot analysis

2.1Introduction

Over the past 10 years there have been rapid advances in automated sample extraction used in the analysis of bioanalytical samples. Instruments such as the Flexus®, Tomtec Quadra ® and Hamilton Star® robots are now a regular feature in laboratories around the world (Watt *et al* 2000). With the use of such robots it is possible to automate the majority of the sample analysis preparation procedure, but in the case of dried blood spot (DBS) sample dilution is impossible due to the fact that the samples are solid rather than liquid. Therefore solvent is first added to the DBS to remove the analyte and the solvent containing the analyte is then diluted. Given that it is not always possible to predict which samples will require diluting, reanalysis of plasma or DBS samples is generally required for a portion of the samples, with associated cost implications to the pharmaceutical industry; in addition, the limited sample size may hinder the ability to perform a repeat analysis. Samples which have a higher concentration than the calibration range must currently be diluted, re-extracted (where extracted mean the sample has been processed to produce extracts, which are ready to be injected on to a LC-MS/MS system) and reanalysed. The original sample extracts often cannot be diluted and re-injected because of stability and evaporation issues).

In the case of dried blood spot samples, there is no simple automated technique for sample dilution. In the past, DBS analysis could be labour intensive, as each sample would require manual punching which is a laborious and repetitive process. The introduction of DBS punching instruments and newly developed on-line extraction systems has alleviated this issue (Ganz *et al* 2012). Combining punching instruments with liquid handling systems enables the entire sample extraction process to be fully automated (Mess *et al* 2012). However there is currently no process by which repeat extraction of samples which are above the limit of quantification during initial analysis can be fully avoided or drastically reduced. The AB Sciex “SignalFinderTM”algorithm can compensate for detector saturation at high concentrations, which can help increase the quantitative

linear range, resulting in fewer samples that require diluting (Absciex website. http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-Multiquant.pdf). Signal finder algorithm uses the unsaturated portion (the portion of the peak not effected by detector saturation) of the peak to extrapolate the actual response.

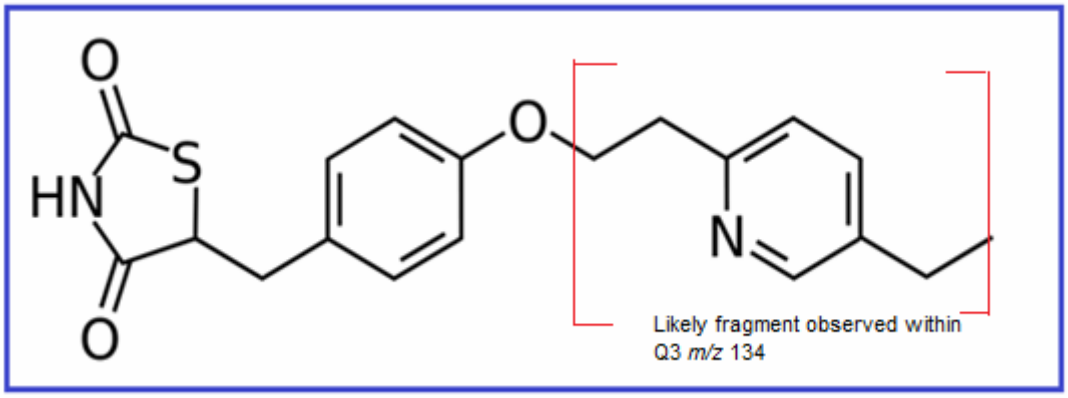
2.1.2 Current method of analysing DBS-derived sample which fall outside the calibration range

One current method of diluting DBS-derived samples consists of punching a disk from a sample spot and from a spot of a matrix blank (control blood), extracting the disks using a solvent containing an appropriate concentration of internal standard, then diluting the supernatant from the sample disk with that from the matrix blank disk. An alternative method of dilution for DBS samples is 'internal standard tracked' dilution (Liu *et al* 2011). This method consists of the addition of a 'dilution factor–adjusted' internal standard solution to the sample requiring dilution. For a tenfold dilution, an internal standard solution ten times higher in concentration than that added to undiluted samples is added to the sample requiring a tenfold dilution; the sample is then diluted 10 fold with matrix blank supernatant. The dilution factor adjusted internal standard tracks the dilution process, as the final internal standard concentration should match the internal standard concentration added to undiluted samples.

2.1.3 Pioglitazone

Pioglitazone is used to treat type 2 diabetes mellitus and was the tenth best-selling drug in the US in 2008. As a result, Pioglitazone has been previously used to demonstrate successful quantitative analysis from DBS samples (Turpin *et al* 2008). Pioglitazone (structure in figure 21) contains atoms which have naturally occurring isotopes. This could be used in any mass spectrometer based signal reduction techniques.

Figure 21 Pioglitazone structure.



2.1.4 Aim

The aim of this research is to develop and demonstrate the use of alternative techniques for the analysis of sample that fall outside the calibration/linear concentration range. An LC-MS/MS assay for the quantitative analysis of pioglitazone in DBS samples over the calibration range of 10-10000 ng/mL has been chosen as a model assay to generate limited validation data for the alternative techniques and to compare this data to those generated using a more conventional sample dilution procedure. As the aim of the research is to demonstrate the successful analysis of sample which falls outside the calibration/linear concentration range, it is not necessary to fully validate the method because not all the experiments conducted in a full validation are required to demonstrate the successful use of any new techniques developed (for example stability, matrix effects and recovery experiments are not required). Therefore a partial/limited validation will be conducted. The compatibility of the new techniques developed with plasma sample analysis will also be investigated.

2.2 Experimental

2.2.1 Chemicals and reagents

HPLC grade methanol, 2-propanol and acetone were obtained from Rathburn Chemicals (Walkerburn, UK). HPLC grade acetonitrile was obtained from Fisher Scientific (Loughborough, UK). Isotopically labelled internal standard [$^2H_4$]-pioglitazone and unlabelled pioglitazone were obtained from Toronto Research Chemicals (Toronto, Canada). Ultra-pure water was produced in-house using a Millipore Super-Q™ osmosis system. All other chemicals were supplied by Sigma-Aldrich (Poole, UK). Human blood was obtained fresh in-house.

2.2.2 Equipment

DMPK B® blood spot cards and desiccant sachets were supplied by GE Healthcare Life Sciences. The Flexus® liquid handling system was supplied by Anachem. The BSD600 DBS card punching instrument was supplied by BSD Robotics and 96-well sample plates were obtained from Corning.

2.2.3 Analysis by UHPLC-MS/MS

A Waters Acquity UPLC system attached to a Sciex API-4000 mass spectrometer made up the UHPLC-MS/MS system (using Sciex Analyst 1.5.1 software). After acquisition, the UHPLC-MS/MS data were processed using Sciex Analyst 1.5.1 software. UHPL chromatographic and MS conditions are detailed in Tables 1-3. Mobile phase A was 0.1% formic acid in 10 mM ammonium acetate (aq) and Mobile phase B was 0.1% formic acid in methanol. The analytical column was a Waters Acquity BEH C18, 50 x 2.1 mm, 1.7 µm and the column oven temperature was nominally set to 40°C. Auto-sampler weak wash solvent was 0.1% formic acid in 10 mM ammonium acetate: methanol (80:20 v/v). The auto-sampler strong wash solvent was methanol: acetone: water: trifluoroacetic acid (50: 40: 10: 0.1 v/v/v/v).

Table 1 Liquid chromatography conditions

Gradient settings:			
Time (min)	A (%)	B (%)	Flow rate (mL/min)
0.0	70	30	0.6
0.2	70	30	0.6
1.5	50	50	0.6
2.1	50	50	0.6
2.2	2	98	0.8
4.5	2	98	0.8
4.6	70	30	0.6

An API 4000 mass spectrometer using an atmospheric pressure chemical ionisation APCI source was used in positive ion mode to quantify Pioglitazone. Pioglitazone was infused at 500 ng/mL, at a flow rate of 20 µL/min, tables 2 and 3 list the mass spectrometer parameters and the various MS/MS transitions used, respectively.

Table 2 Mass spectrometer parameters

Collision gas setting (CAD)	9
Curtain gas setting (CUR)	40
Ion source gas 1 (GS1)	35
Nebuliser Current (NC)	3.0 µA
Temperature (TEM)	500°C
Pause time	5 ms
Collision gas	Nitrogen

Table 3 Mass spectrometer transitions for Pioglitazone and internal standard

Compound name	*m/z* of ions monitored	Dwell time (ms)	Declustering potential setting (v)	Collision energy setting (v)
Pioglitazone	357.1 → 134.1	100	75	37
[2H4] -Pioglitazone	361.2 → 134.1	100	75	37
Pioglitazone C13 A	358.1 → 134.1	100	75	37
Pioglitazone C13 B	358.1 → 135.1	100	75	37

Figure 21 indicates the potential fragment observed (m/z of 134.1). The expected intensity of the 358.1-134.1 and 358.1-135.1 transitions would be similar, given that the 134.1 fragment contains 9 out the 19 carbons available. The observed intensity for both transitions was very similar.

2.2.4. Limited DBS validation and analysis procedures

2.2.4.1 Dried blood spot analysis

Partial validation to FDA guidelines was carried out for the DBS method. This included determination of assay selectivity, determination of precision and accuracy on three occasions, and determination of several dilution factors. Stock solutions of pioglitazone (1 mg/mL) and [$^{2}H_{4}$]-pioglitazone (0.1 mg/mL) internal standard were prepared in methanol. Before use, all the solutions were brought to room temperature after storage at nominal 5°C.

Calibration standards were prepared by diluting the stock solutions into appropriate concentration working solutions with methanol, then spiking into blank whole human blood to be used to generate a single calibration line with standards at 10, 20, 50, 200, 1000, 2000, 4500, 8500 and 10000 ng/mL. It was ensured that the volume of the working solution used for spiking remained less than 5% of the total standard volume. A second stock solution from an independent weighing was used to prepare quality control (QC) samples, (which will be used to assess the calibration line accuracy throughout the validation) by diluting the stock solution into appropriate concentration working solutions with methanol. The diluted working solutions were then spiked into blank whole human blood to give quality control samples at 10 (LLOQ, lowest limit of quantification is lowest calibration standard), 30 (LoQC level, three times the concentration of the LLOQ), 500 (MeQC level, the geometric mean of the calibration range), 8000 (HiQC level, 80% of the upper limit of quantification, highest calibration standard concentration) and 50000 (DiQC level, 5 times higher than the highest calibration standard concentration) ng/mL. LLOQ, LoQC, MeQC, HiQC and DiQC are known as QC levels (Section 1.6). For DBS analyses, 20 µL of calibration standards and QC samples were spotted onto DMPK B ® Elute cards. The spotted cards were left for at least 2 hours to dry at room temperature and then analysed or stored at room temperature in a polypropylene bag containing a desiccant sachet.

To analyse the DBSs, a 3.2 mm diameter disk was punched from the centre of the dried blood spot into a 2 mL 96 well plate using a BSD600 instrument. Analyte extraction was performed by adding 200 µL of methanol containing 50 ng/mL of the internal standard ([$^2H_4$]-pioglitazone). After vortex mixing for approximately 30 minutes the plate was then centrifuged at 3000 g for 5 minutes. A 100 µL aliquot of supernatant was transferred to a 1.2 mL 96-well plate using a Flexus robot. 100 µL of 10 mM ammonium acetate/ formic acid (100: 0.1 v/v) was added to the 1.2 mL 96-well plate and the plate vortex mixed. 10 µL was injected on the UHPLC-MS/MS system.

The DiQC is at a concentration above the calibration range and so is normally diluted into the range of the calibration line for analysis. The dilution of unknown samples in a particular analytical experiment is verified by the successful dilution of the DiQC. In this research, the DiQC will be used to assess the successful use of alternative techniques for the analysis of sample that fall outside the calibration/linear concentration range.

2.2.4.2 Plasma sample analysis

Calibration standards were prepared fresh on the day of analysis by diluting stock solutions into appropriate concentration working solutions with methanol, then spiking into blank whole human plasma to give the following calibration standards at 10, 20, 50, 200, 1000, 2000, 4500, 8500 and 10000 ng/mL. It was ensured that the volume of the working solution used for spiking remained less than 5% of the total standard volume. QC samples were prepared from a second stock solution by diluting the stock solution into appropriate concentration working solutions with methanol, then spiking into blank whole human plasma to give QCs at 10, 30, 5000, 8000 and 50000 ng/mL.

A 50 µL aliquot of plasma was pipetted into a 2 mL 96 well plate and 200 µL of methanol containing 50 ng/mL internal standard ([^{2}H4]-pioglitazone) was added. The plate was vortex mixed for approximately 30 minutes then centrifuged at 3000g for 5 minutes. A 100 µL aliquot of supernatant was transferred to a clean 1.2 mL 96-well plate using a Flexus robot and 200 µL of 10 mM ammonium acetate/ formic acid (100:0.1 v/v) plus 100 µL of

methanol was added to each well and the plate vortex mixed. A 10 µL volume was then injected onto the UHPLC-MS/MS system.

2.2.5 Dilution Evaluation

2.2.5.1 Conventional dilution procedure

In both DBS and plasma extractions, QC solutions that were outside the calibration range were diluted using a traditional method to enable comparison of the performance of the new signal 'dilution' procedures to be made with the traditional technique. For the DBS samples this was done by punching a disk from a DiQC blood spot and from a control matrix blood spot, extracting as detailed above with an appropriate concentration of internal standard present in both, and then diluting 20 µL of supernatant from the QC sample with 180 µL of supernatant from the control matrix, to generate a 10-fold dilution. For the plasma sample analysis validation, 20 µL of the DiQC sample was added to 180 µL of blank plasma, vortex mixed and then a 50 µL aliquot taken for analysis.

2.2.6 Proposed alternative dilution techniques

Mass Spectrometer Signal Dilution (MSSD) and Isotope Signal Ratio Monitoring (ISRM) are two new mass spectrometer signal reduction techniques developed. Both techniques require no repeat extraction of samples that fall outside the calibration/linear concentration range. Given that LC-MS/MS is already the favoured method for analysing bioanalytical samples, these two new MS/MS based signal dilution techniques can be applied with existing standard equipment.

2.2.6.2Mass spectrometer signal dilution procedure.

MSSD consists of lowering the collision energy settings for samples that fall outside the calibration/linear concentration range. Lowering the collision energy brings the product ion signal for an over range sample into the calibration range, by altering the collision energy for the analyte only**.** Calibration standards and quality control samples (LLOQ, Lo, Me, Hi) were injected onto the LC-MS/MS system using an optimum 'collision energy' setting of 37 V. Six replicates of DiQCs and HiQCs were also analysed using three different

'collision energy' settings of 20.6 V, 22 V and 23 V to generate varying signal responses. The difference in the signal response for the HiQC at optimum and lowered collision energy setting, is used to generate a correction factor (using equation 8). The correction factor is then applied to the DiQC.

Equation 8 Correction factor calculation for MSSD

$$\text{Correction factor} = \frac{\text{Average HiQC area ratio (optimum CE)}}{\text{Average HiQC area ratio (lowered CE)}}$$

2.2.6.3 Extended calibration curve procedure

A calibration line with a concentration range of 0.5-100,000 ng/mL (200,000 fold increase in concentration over the range) was prepared in methanol and the collision energy was lowered for the internal standard and analyte (the collision energy is lowered for both internal standard and analtye in this experiment only) as follows:

0.5 to 1000 ng/mL analysed using a collision energy setting V of 37 for internal standard and analyte.

2000 to 10000 ng/mL analysed using a collision energy setting V of 26 for internal standard and analyte.

20000 to 100000 ng/mL analysed using a collision energy setting V of 22 for internal standard and analyte.

The above reduction in collision energy setting was based on figure 22 (on the following page).

2.2.6.4 Isotope signal ratio monitoring (ISRM).

The ISRM dilution technique consists of monitoring the ^{13}C isotopic peak (or other suitable isotope) for over range samples only. The ISRM approach is appropriate for the majority of therapeutic compounds because they contain atoms which have naturally occurring isotopes. Calibration and quality control samples (LLOQ, Lo, Me, Hi) were analysed using the LC-MS/MS system and the Pioglitazone content monitored using the *m/z* 357.2 to 134.1 transition (^{12}C). Six replicates of DiQCs and HiQCs were also analysed using the *m/z* 358.2 to 134.1 and *m/z* 358.2 to 135.1 transitions (^{13}C).The difference in the signal response for the ^{12}C and ^{13}C transition for the HiQC is used to generate a correction factor (using equation 9). The correction factor is then applied to the DiQC.

Equation 9 Correction factor calculation for ISRM

$$\text{Correction factor} = \frac{\text{Average HiQC area ratio (C12 Transition)}}{\text{Average HiQC area ratio (C13 Transition)}}$$

2.2.6.5 Experiements to be conducted in the partial validation

On three separate occasions, the following experiments will be conducted : accuracy and precision, selectively (see section 1.6) and the successful analysis of the DiQC samples using MSSD, ISRM and convential dilution process methodologies. The same calibration line will be used to assess MSSD, ISRM and convential dilution process. Each occasion is known as a batch or analytical run. For the accuracy and precision experiments six replicates of the LoQC, MeQC and HiQC will be analysed. Selectivety experiments consists of the analysis of matrix blank (matrix blank contains no analyte), reagent blank (water is added in the place of a DBS sample) and matrix blank containing internal standard samples.

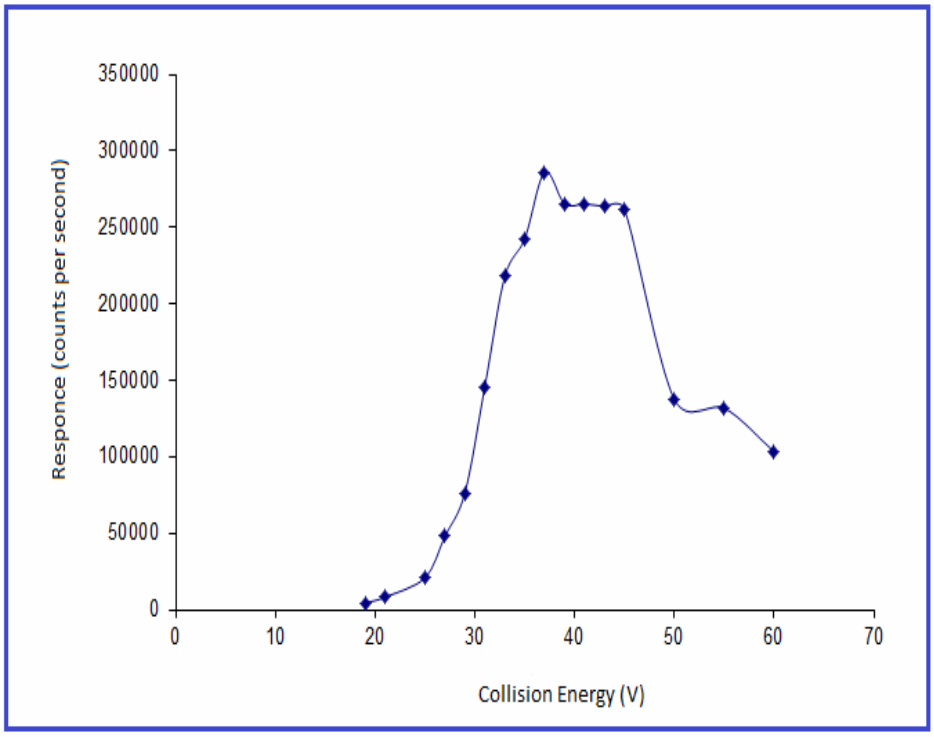
2.3. Results and Discussion

The objective of this investigation was to develop and perform a limited validation for two new techniques that get around the need for physical dilution of precious and difficult to dilute samples, to analyse sample concentrations that lie above the calibration range, without the need to re-extract the samples. An LC-MS/MS method for the quantitative analysis of pioglitazone in DBSs was selected to investigate the two new techniques of MSSD and ISRM, which lower the signal that reaches the detector for chosen samples.

2.3.1. Mass spectrometer signal dilution

The advantage of the MSSD method over the ISRM signal reduction ('dilution') method is that it can be applied to any compound for which varying the collision energy setting varies the response produced, and so can generate a large range of signal attenuations that can be treated as virtual dilution factors. The effect of altering the collision energy setting on the mass spectrometer response for pioglitazone is shown in Figure 22.Signal attenuations ranging from 13.6 to 42.0 fold were achieved. Given that a calibration curve is created by using analyte/internal standard peak area ratios, by lowering the collision energy for analyte only, the analyte/IS peak area ratios for samples which are above the limit of quantification are lowered, so that they fall within the calibration range. A unique virtual dilution (correction) factor was determined by monitoring the HiQCs (HiQCs were chosen because there analyte concentration lie at the top end of the calibration curve), once with the collision energy set at the optimum value, as it was when measuring the calibration standards and a second time at reduced collision energy, as for the samples which would typically require diluting. The calculated correction factor for (calculated by using equation 8) is then applied to the DiQCs and above range study samples to generate concentration values.

Figure 22.Effect of collision cell 'collision energy' setting on detector response for pioglitazone.



In a validation all three batches (analytical runs) must demonstrate acceptable accuracy and precision for the method to be acceptable for use. The intra- and inter-assay precision and accuracy data are presented in Tables 4 and 5. The mean calculated concentration, accuracy (%) and precision (RDS %) are detailed for all three batches (mean intra assay data is also shown). As table 4 shows all QC levels were within the pre-defined 15% limits (described in section 1.6.2). Therefore the analytical method can be used to assess MSSD technique. The DiQC was diluted 10 fold using the traditional method (data presented in table 4). The mean inter-assay calculated concentration of the DiQC is with 3% of nominal value (nominal value is the actual concentration of analyte added to plasma to prepare the DiQC). The mean intra- and inter-assay data for DiQC analysed using MSSD technique (also shown in table 4) are within 15% of nominal value).

Table 4. Intra- and inter-assay precision and accuracy data for QC containing pioglitazone in human dried blood spots and comparison of a traditional dilution technique withMSSD using a variable collision energy setting

Intra-assay	QC 10 ng/mL (LLOQ QC)	QC 30 ng/mL (LoQC)	QC 500 ng/mL (MeQC)	QC 8000 ng/mL (HiQC)	QC 50000 ng/mL (DiQC) Traditional method diluted 10-fold with extracted blank human blood spots	QC 50000 ng/mL (DiQC) MSSD CE = 20.6 V 42.0-fold dilution	QC 50000 ng/mL (DiQC) MSSD CE = 22 V 19.9-fold dilution	QC 50000 ng/mL (DiQC) MSSD CE = 23 V 13.6-fold dilution
Batch 1								
Mean (ng/mL)	9.82	28.9	478	7300	47100	56700		56000
Accuracy (%)	98.2	96.3	95.6	91.3	94.2	113.4		112.0
RSD (%)	11.5	6.8	6.7	5.8	3.8	6.2		7.3
n	6	6	6	6	6	6		6
Batch 2								
Mean (ng/mL)	9.18	28.1	478	7900	49100	55400	54000	54200
Accuracy (%)	91.8	93.7	95.6	98.8	98.2	110.8	108.0	108.4
RSD (%)	7.1	3.5	4.7	5.6	2.8	3.8	5.1	7.3
n	6	5	6	6	6	6	6	6
Batch 3								
Mean (ng/mL)	9.35	26.4	462	7760	49600	56200	56300	56200
Accuracy (%)	93.5	88.0	92.4	97.0	99.2	112.4	112.6	112.4
RSD (%)	7.7	2.4	6.1	5.0	6.6	4.3	2.8	3.2
n	6	6	6	6	6	6	6	6
Inter-assay								
Mean (ng/mL)	9.45	27.8	473	7660	48600	56100	55200	55500
Accuracy (%)	94.5	92.7	94.6	95.8	97.2	112.2	110.4	111.0
RSD (%)	9.0	6.0	5.7	6.2	5.0	4.7	4.4	6.1
n	18	17	18	18	18	18	12	18

DiQC: Diluted QC; QC: Quality control; LLOQ: Lower limit of quantification QC; LoQC: LowQC; MeQC: Medium QC; HiQC: High QC; RSD: Relative standard deviation; CE: Collision energy.

Table 5.Inter-assay precision (RSD) and accuracy for calibration standards containing pioglitazone in human DBSs prepared when comparing the traditional dilution technique to that of MSSD and ISRM

	concentrations (ng/mL)								
	Calibration standard concentration (ng/mL)								
	10	20	50	200	1000	2000	4500	8500	10000
Mean (ng/mL)	9.92	20.0	52.5	190	1020	1940	4120	8320	10100
Standard deviation (n-1)	0.186	1.19	2.78	6.06	52.1	69.0	243	154	343
RSD (%)	1.9	6.0	5.3	3.2	5.1	3.6	5.9	1.9	3.4
Accuracy (%)	99.2	100.0	105.0	95.0	102.0	97.0	91.6	97.9	101.0
n	3	3	3	3	3	3	3	3	3
Quadratic fit ($y = ax^2 + bx + c$)									

2.3.1.2 Extending the calibration curve

Given that the quality of data generated is dependent on the quality of the calibration curve that is used in any given assay, a linear regression with either a 1/x or $1/x^2$ weighting is the most commonly applied model used for generating a calibration curve in small molecule bioanalysis, with a typical calibration range of 500 to 1000 fold increase in concentration. Saturation of the mass spectrometer detector is a limiting factor when it comes to the range of calibration that can be used on a given assay. Lowering the collision energy for both analyte and internal standard enables a calibration range of 0.5 to 100,000 ng/mL (200,000 fold range) to be used with a linear response (Figure 23). Figure 24 shows the results obtained from the same calibration standards, without altering the collision energy setting; a limited linear calibration range of 0.5- 1000 ng/mL (2,000 fold range) was achieved.

Figure 23 A 200,000-fold (linear $1/x^2$) calibration curve for pioglitazone (0.5 to 100000 ng/mL) demonstrating that lowering the collision energy for both pioglitazone and internal standard can extend the linearity of the calibration range achieved

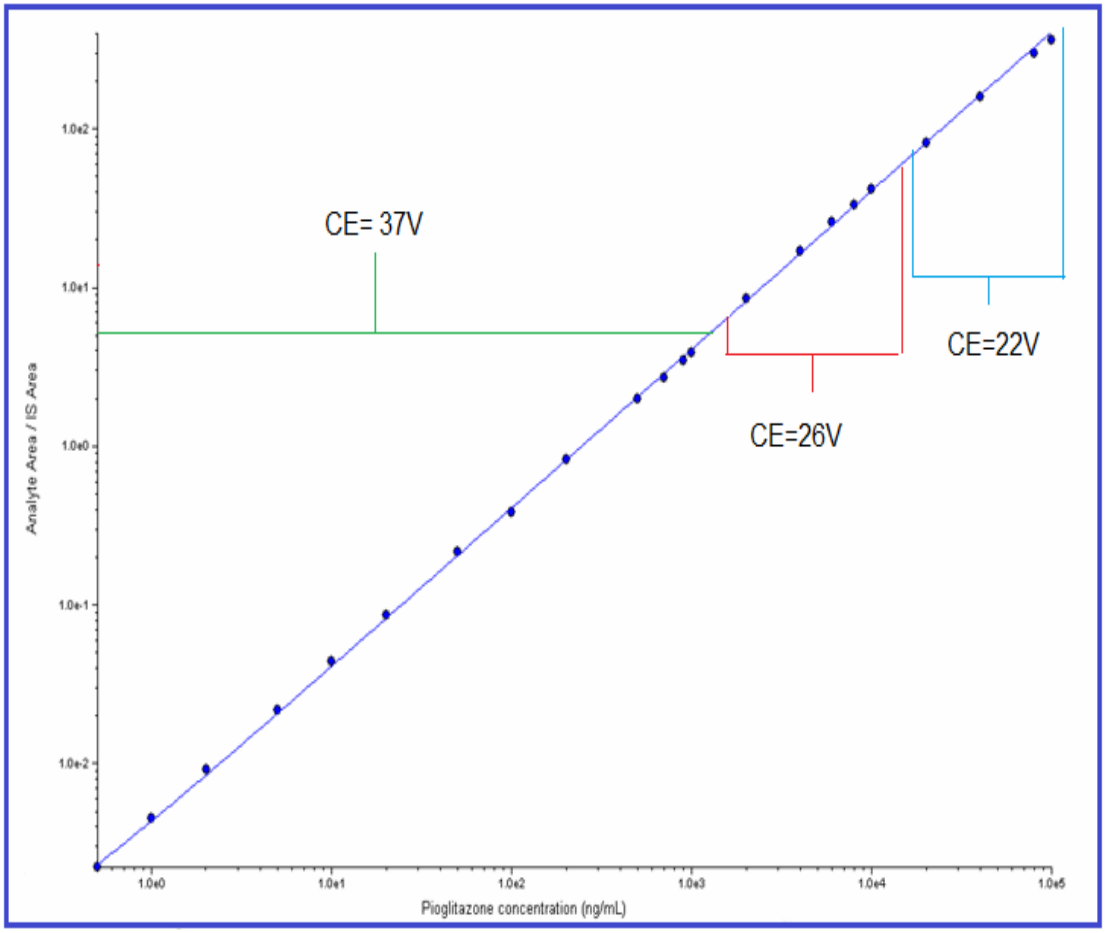
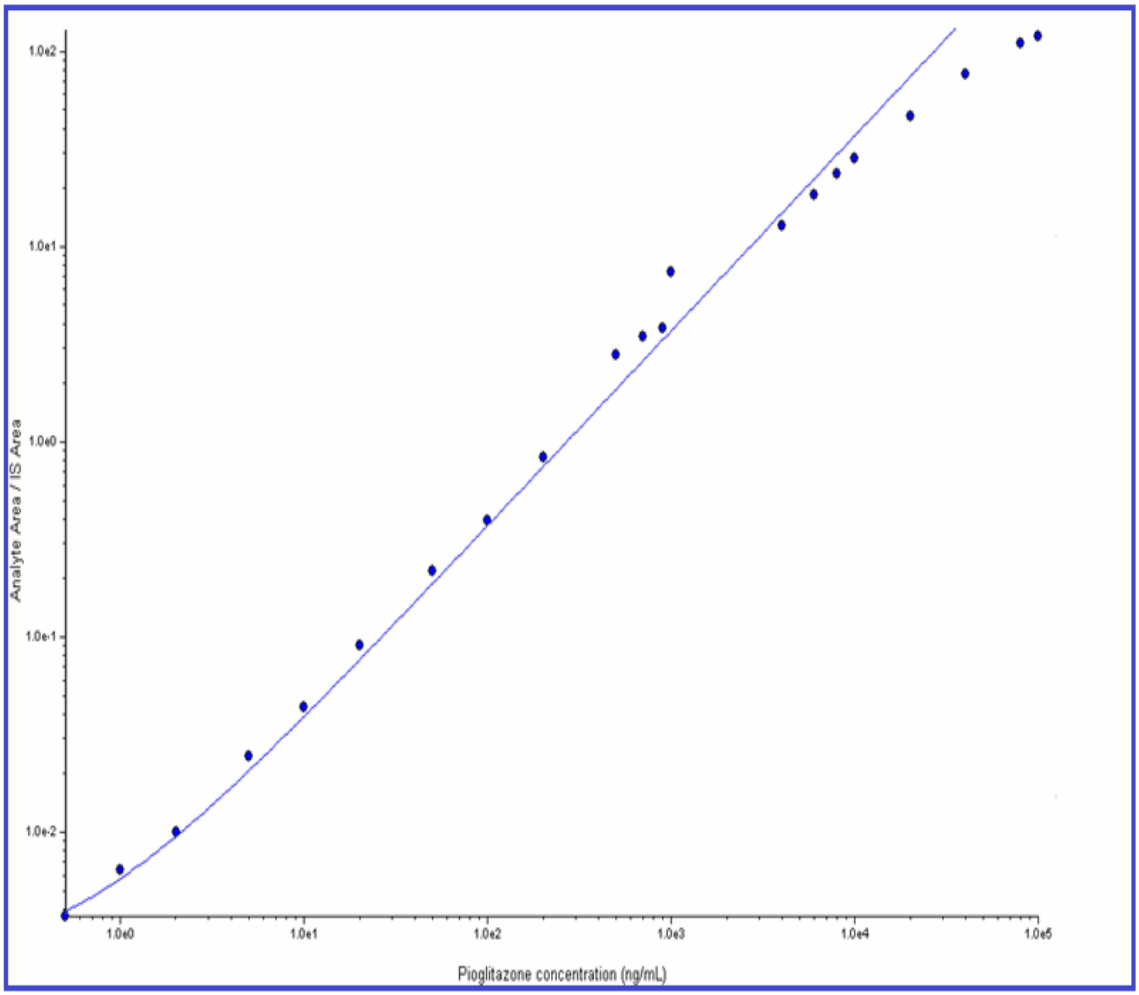


Figure 24. A 200,000-fold (linear $1/x^2$) calibration curve for pioglitazone (0.5-100000 ng/mL), demonstrating that using a singlecollision energy setting for pioglitazone and internal standard results in a limited linear calibration range



2.3.2 Isotope signal ratio monitoring

This technique can be applied to any compound with naturally occurring isotopes. Pioglitazone (Figure 21) has a 22.5% relative abundance of naturally occurring isotopes with a mass one dalton above that of the precursor [Table 6]. The greater the relative abundance of naturally occurring isotopes a compound has, the lower the virtual dilution factor that results, when using the signal one dalton above the precursor ion. However higher dilution factors can be achieved by monitoring a signal two daltons above the precursor ion, caused by multiple isotopes.

Table 6 Isotope abundance in pioglitazone

(www.sisweb.com/mstools/isotope.htm.)

	% abundance of M+1 isotope	Number of atoms in Pioglitazone	Relative intensity of M+1 signal (% Isotope abundance * Number of atoms in pioglitazone)
N	0.3613	2	0.7226
S	0.7893	1	0.7893
C	1.0816	19	20.5499
O	0.0401	3	0.1203
H	0.0160	20	0.320
Total			22.50

The intra- and inter-assay precision and accuracy data are presented in Tables 5 and 7. The mean calculated concentration, accuracy (%) and precision (RDS %) are detailed for all three batches (mean inter assay data is also shown). As table 4 shows all QC levels were within the pre-defined 15% limits. Therefore the analytical method can be used to assess ISRM technique. In all three validation batches the DiQC passed in accordance to international recognised acceptance criteria, with ISRM and the conventional method of diluting samples giving results which were within 15% of the nominal concentration.

Plasma data is represented in table 8. The DiQC analysed using ISRM technique are within 8% of nominal value.

Table 7. Intra- and inter-assay precision and accuracy data for QC containing Pioglitazone in human dried blood spots and comparison of the traditional dilution technique to that oflsotope Signals Ratio Monitoring

Intra-assay	QC 10 ng/mL (LLOQ QC)	QC 30 ng/mL (LoQC)	QC 500 ng/mL (MeQC)	QC 8000 ng/mL (HiQC)	QC 50000 ng/mL (DiQC) Traditional method diluted 10-fold with extracted blank human blood spots	QC 50000 ng/mL (DiQC) ISRM 358.1 → 134.1 Average 7.35-Fold dilution	QC 50000 ng/mL (DiQC) ISRM 358.11 → 135.1 Average 7.92-Fold dilution
Batch 1							
Mean (ng/mL)	9.82	28.9	478	7300	47100	47500	47300
Accuracy (%)	98.2	96.3	95.6	91.3	94.2	95.0	94.6
RSD (%)	11.5	6.8	6.7	5.8	3.8	6.7	6.4
n	6	6	6	6	6	6	6
Batch 2							
Mean (ng/mL)	9.18	28.1	478	7900	49100	50100	50200
Accuracy (%)	91.8	93.7	95.6	98.8	98.2	100.2	100.4
RSD (%)	7.1	3.5	4.7	5.6	2.8	4.3	4.4
n	6	5	6	6	6	6	6
Batch 3							
Mean (ng/mL)	9.35	26.4	462	7760	49600	48900	49100
Accuracy (%)	93.5	88.0	92.4	97.0	99.2	97.8	98.2
RSD (%)	7.7	2.4	6.1	5.0	6.6	3.1	3.4
n	6	6	6	6	6	6	6
Inter-assay							
Mean (ng/mL)	9.45	27.8	473	7660	48600	48800	48900
Accuracy (%)	94.5	92.7	94.6	95.8	97.2	97.6	97.7
RSD (%)	9.0	6.0	5.7	6.2	5.0	4.7	4.7
n	18	17	18	18	18	18	18

DiQC: Diluted QC; QC: Quality control; LLOQ: Lower limit of quantification QC; LoQC: Low QC; MeQC: Medium QC; HiQC: High QC; RSD: Relative standard deviation

Table 8.Intra-assay precision and accuracy data for QC levelsdata for pioglitazone in human plasma (Isotope Signals Ratio Monitoring).

Intra-assay	QC 30 ng/mL (LoQC)	QC 500 ng/mL (MeQC)	QC 8000 ng/mL (HiQC)	QC 50000 ng/mL (DiQC) ISRM 358.1 → 134.1 7.69-Fold dilution	QC 50000 ng/mL (DiQC) ISRM 358.11 → 135.1 8.35-Fold dilution
Mean (ng/mL)	30.8	5230	8950	53800	54200
Accuracy (%)	102.7	104.6	111.9	107.6	108.4
RSD (%)	6.3	4.6	6.7	3.7	3.4
n	6	6	6	6	6

2.3.3 Response for DBS partial validation.

A representative chromatogram is presented in Figure 25 demonstrating an excellent signal to noise ratio at the LLOQ and lack of significant interference at the retention time of Pioglitazone shown in Figure 27. The internal standard peak shape is represented in Figure 26. The analyte response (peak height) at the LLOQ was greater than five times the blank blood spot response. The response of the detector to Pioglitazone was quadratic over the concentration range 10 to 10000 ng/mL with a weighted $1/x^2$ quadratic regression applied to the data.

Figure 25. Representative UHPLC-MS/MS selective reaction monitoring chromatogram of Pioglitazone spiked into a human DBS sample at the LLOQ (10 ng/mL)

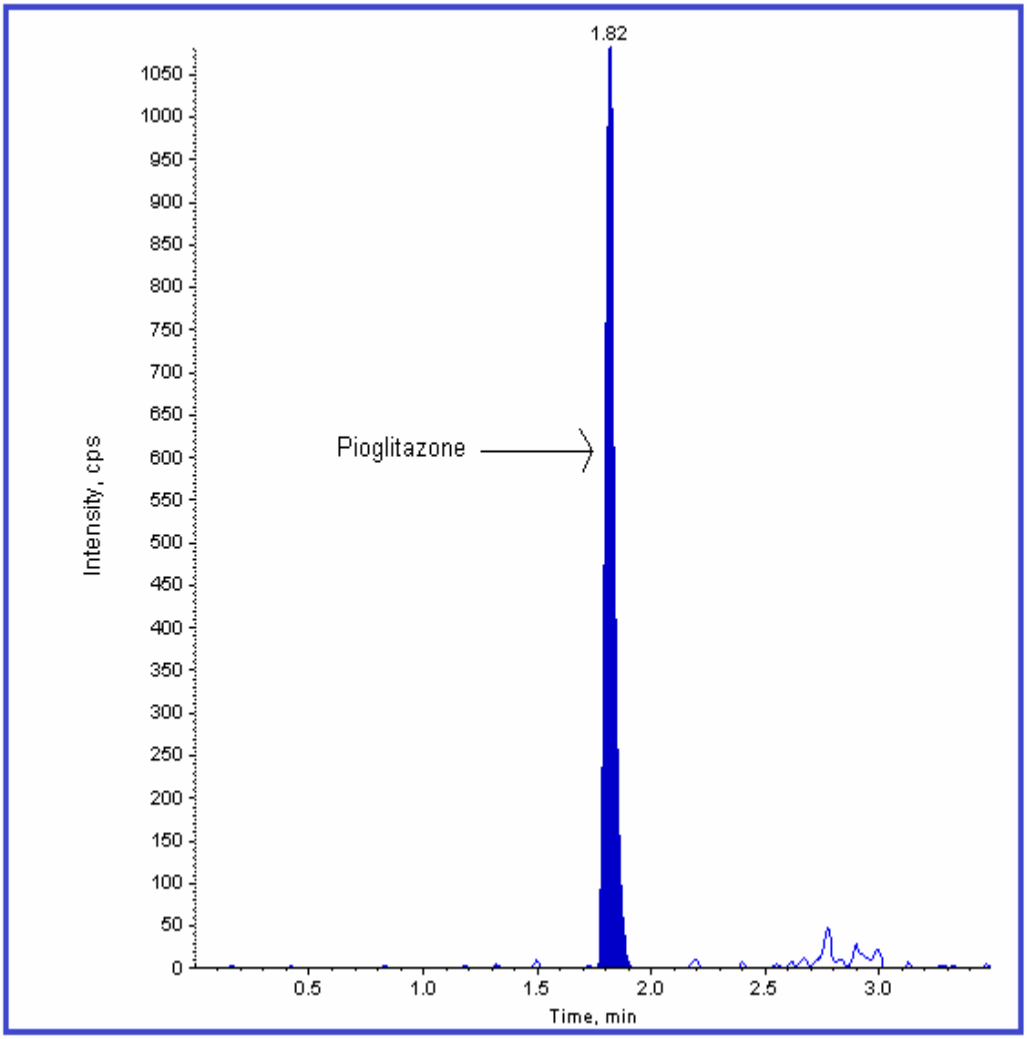


Figure 26. Representative UHPLC-MS/MS selective reaction monitoring chromatogram of a human DBS sample spiked with the internal standard ([$^2H_4$]-pioglitazone)

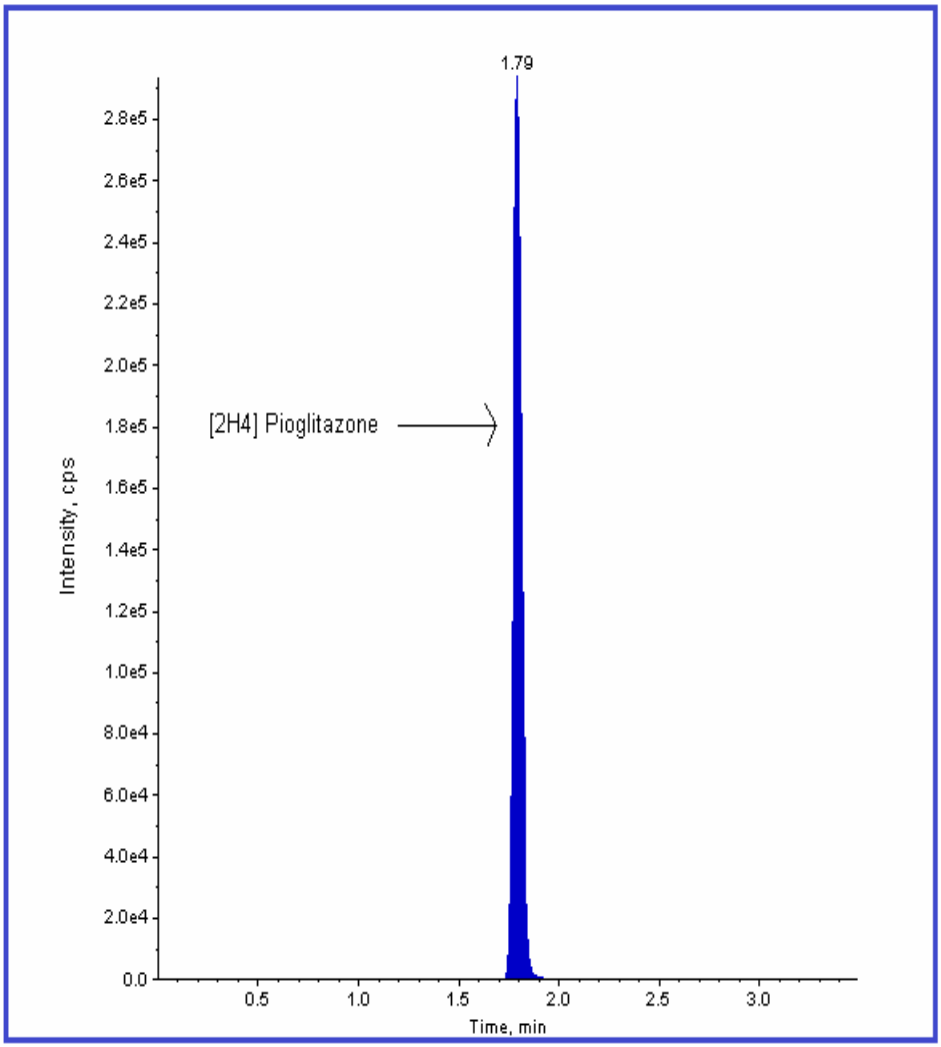
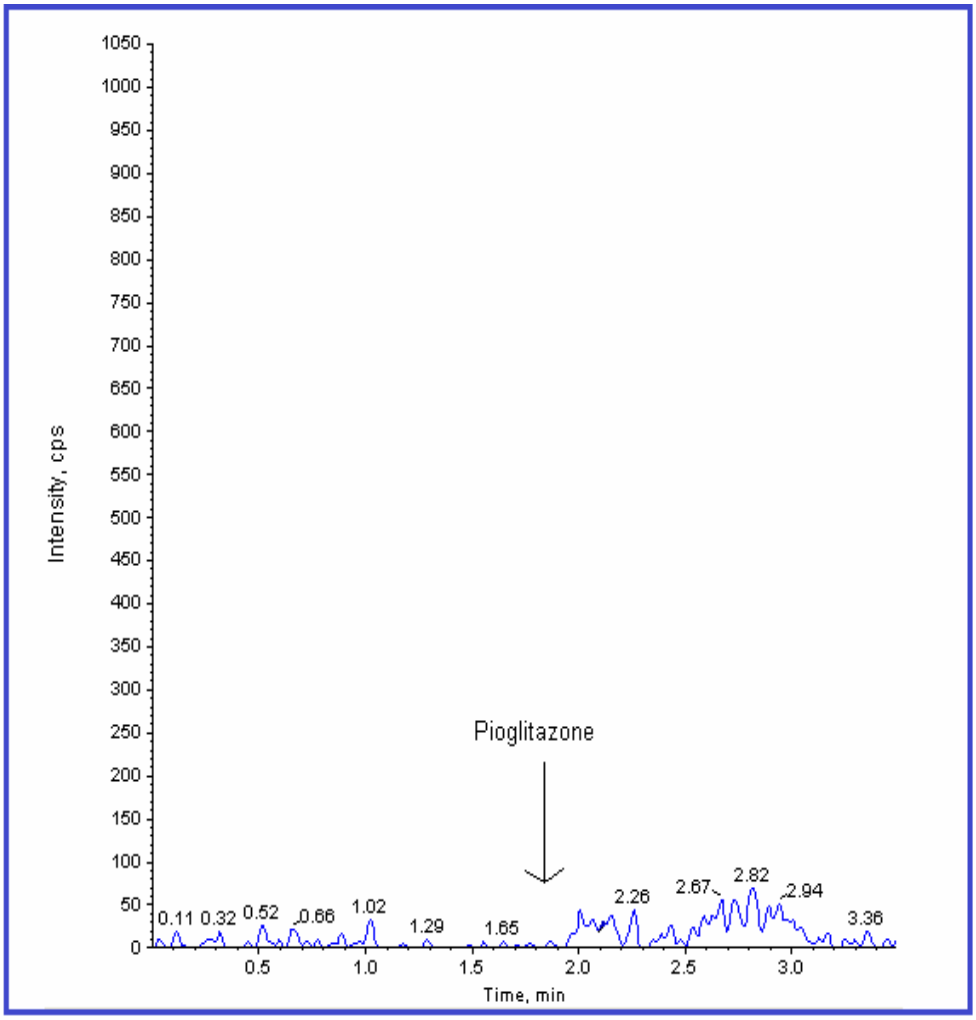


Figure 27. Representative UHPLC-MS/MS selective reaction monitoring chromatogram of a blank human DBS sample



2.3.4 Selectivity for DBS partial validation

There were no significant interfering peaks at the retention time of either Pioglitazone or the internal standard response) detected in reagent blanks, matrix blanks or the highest concentration calibration standard (without internal standard) in the retention window of Pioglitazone and its internal standard respectively. This showed that no carryover from the LC system or contamination during the sample extraction was observed and therefore the data generated had not been compromised.

2.4. Conclusions

Two new mass spectrometer-based sample dilution concepts were successfully developed and partially validated on dried blood spot samples for the analysis of Pioglitazone. Both Mass Spectrometer Signal Dilution and Isotopic Signal Ratio Monitoring dilution techniques have shown comparable results to the traditional method of sample dilution. In each case, sample analysis time and cost attributed to reanalysis of samples with analyte concentrations above the calibration range was significantly decreased. Either of the methods can be implemented in laboratories around the world with minimal additional cost implications. The two techniques have been demonstrated to be compatible with DBS and plasma analysis. Given the level of interest in and development of supporting technology for automated DBS analyses, these two new virtual dilution techniques may help to alleviate the issues involved with manually diluting DBS samples. The newly developed dilution methods have the potential to simplify the analysis of the majority of bioanalytical samples for which previously a physical dilution of the sample was required to bring analytes within the calibration range of an assay used for quantification.

A potential limitation of the new methodologies is that for highly concentrated samples the assay in use might encounter mass spectrometer ionisation saturation, and as a result only sample concentrations below that of the DiQC should be accepted. However, ionisation saturation for the pioglitazone assay described here was not observed but is a

factor to take into account when implementing the new methodologies. Some manual data processing was required for the new techniques, but this could be eliminated by future changes to mass spectrometer instrument software.

Another limitation of a large dynamic range or in this case analyte concentrations above the upper limit of quantification is auto-sampler carry over. However, with the improvements in liquid chromatograph systems and the addition of extra blanks within the analytical run, carry-over can be reduced; whether these additional precautions are needed should be investigated during validation.

Chapter 3:

Development of a bioanalytical method for the quantification of a phosphorothioated oligonucleotide in human plasma capillary micro-sample using LC-MS/MS

3.0 Introduction

The aim of this research was to develop a bioanalytical method for the quantification of a phosphorothioate oligonucleotide in human plasma samples made available following capillary micro-sampling (CMS), using LC-MS/MS, and in doing so demonstrate the compatibility of CMS with an analytically challenging and novel class of compounds. There has to date been no method published which demonstrates the successful analysis of oligonucleotide CMS samples by LC-MS/MS.

3.1 Method development challenges

The method development challenges faced when developing CMS or oligonucleotide methods have been discussed in chapter 1. However, additional challenges arise when combining this particular class of compound with this sampling technique. To enable adequate monitoring of exposure to dosed oligonucleotides during clinical trials, the routinely desired lower limit of quantification (LLOQ) is low ng/mL levels. This is typically achieved by processing 50-200 µL of plasma sample, whereas CMS would only provide 4-20 µL. Additionally, oligonucleotides, like many polar or charged molecules, are prone to adsorb to glass and therefore adsorption to the glass capillary during sample collection and storage could be an issue. Using silanised glass would alleviate this problem because the interactions between the polar oligonucleotides and the glass surface is reduced. Silanised glass is produced by treating a hydrophilic surface with a reactive silane (dimethyldichlorosilane), this converts the hydrophilic silanols (found on the glass surface) into lipophilic alkylsiloxane moieties. Not all glassware used during quantitative analysis is available in silanised form.

3.1.2 Test oligonucleotides

Two oligonucleotides with phosphorothioate linkages have been chosen as test reference materials, due to that fact the phosphorothioate oligonucleotide therapeutics are currently the most studied and tested oligonucleotides. The oligonucleotide containing 18 nucleotides was used as the analyte and the oligonucleotide containing 15 nucleotides

was used as the internal standard. The sequence and masses of the chosen oligonucleotides are shown in figure 28.

Figure 28 Analyte and internal standard sequence

3.2 Experimental

3.2.1 Chemicals and reagents

The reference materials were custom synthesised for the work by Biomers (Ulm,Germany). Methanol and 2-propanol of HPLC grade were obtained from Rathburn Chemicals (Walkerburn, UK).Acetonitrile of HPLC grade was obtained from Fisher Scientific (Loughborough, UK).Ultra-pure grade water was produced in-house using a Millipore Super-Q™ osmosis system (Bedford, USA). All other chemicals were supplied by Sigma-Aldrich (Poole, UK). Human blood was obtained fresh from in-house volunteers, with full written consent. 10 mg HLB SPE plates were obtained from Waters.

3.2.2 Equipment

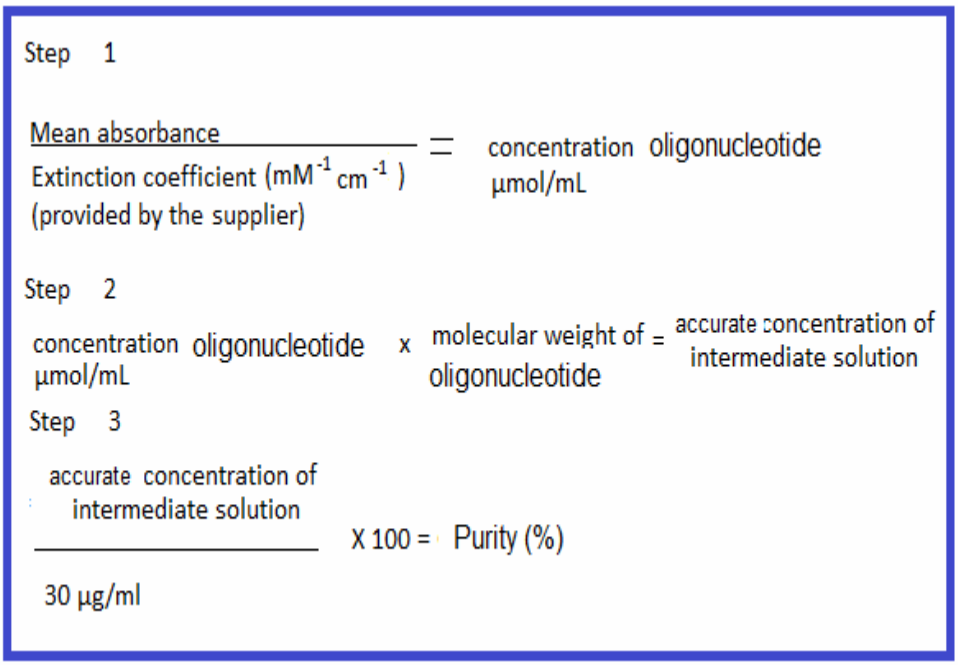
CoStar 96-well sample plates were obtained from Corning. Centrifuge (Rotanta 460R model) was supplied by Hettich. Glass capillaries (20 µL, 2.9 cm, part number 19.447, lot 1511538) were obtained from Vitex. The API4000 MS/MS system was obtained from AB Sciex (Warrington, uk). The Acquity® autosampler, binary solvent manager, column and column oven were obtained from Waters.

3.2.3 Oligonucleotide solution preparation

The water content of oligonucleotide reference standards may be particularly high and can increase during storage or with repeated use. Therefore it is necessary to determine the oligonucleotides stock solution concentration experimentally.

Separate primary-stock solutions of the analyte and internal standard were prepared by dissolving a weighed portion of the oligonucleotide reference material in water: acetonitrile (90:10 v/v) to a concentration of 500 µg/mL (assuming that the reference material supplied is 100% pure). From these primary-stock solutions, intermediate solutions were prepared at 30 µg/mL in the same solvent. Triplicate absorbance readings at 260 nm using a UV spectrophotometer were performed for both analyte and internal standard intermediate solutions. The mean absorbance reading was used to determine the accurate concentration of the intermediate solution (demonstrated in calculation 1) and therefore the accurate primary stock solution concentration could be calculated.

Calculation 1 Calculation for percentage purity of oligonucleotides



Using the calculation above it was determined that the analyte primary-stock concentration was 457.5 µg/mL and the internal standard concentration was 476.5 µg/mL.

3.2.4 Direct infusion into the mass spectrometer

A solution containing each oligonucleotide at 50 µg/mL in water/ methanol/ HFIP/ TEA (70/ 30/ 1/ 0.1 v/v/v/v) was infused directly into a flow of mobile phase matching the starting chromatographic mobile phase and flow rate (Figure 29). This is an unusually high analyte infusion solution concentration for an MS/MS infusion solution but was necessary due to the adsorption losses of a large amount of the analyte and internal standard to the glass infusion syringe.

Tables 9 and 10 show the optimised transitions for the analyte and internal standard. The different charged states were identified by a full scan of precursor ion and the product ions were identified by using MRM mode. Not all product ions observed were fully optimised (the most abundant product ions for all observed charge states were fully optimised).

Table 9 Mass spectrometer transitions and source and mass analyser parameters for the analyte oligonucleotide

		Analyte				
Charge state	Precursor *m/z*	Product ion *m/z*	DP Setting (V)	CE setting (V)	CXP setting (V)	EP setting (V)
$[M-6H]^{-6}$	968.7	94.9	-130	-100	-20	-10
		134.0	-130	-60	-20	-10
		318.9	-130	-33	-20	-10
*** $[M-7H]^{-7}$**	**830.2**	**94.9**	**-150**	**-135**	**-20**	**-10**
		134.0	-150	-70	-20	-10
		319.0	-150	-53	-20	-10
$[M-8H]^{-8}$	726.3	94.9	-135	-125	-11	-10
		134.0	-135	-85	-11	-10
		319.0	-135	-40	-11	-10
$[M-9H]^{-9}$	645.45	94.9	-125	-110	-11	-10
		134.1	-125	-72	-11	-10

DP = Declustering potential, CE = collision energy, CXP =Collision cell exit potential, EP = Entrance potential

***Transition used in analysis**

Table 10 Mass spectrometer transitions and parameters for the internal standard

		Internal standard				
Charge state	Precursor *m/z*	Product ion *m/z*	DP setting (V)	CE setting (V)	CXP setting (V)	EP setting (V)
$[M-6H]^{6-}$	804.4	94.9	-150	-130	-20	-10
		319.0	-150	-47	-20	-10
		344.0	-150	-50	-20	-10
$[M-7H]^{7-}$	**689.3**	**94.9**	**-125**	**-140**	**-25**	**-10**
		319.0	-125	-40	-25	-10
		344.0	-125	-40	-25	-10

DP = Declustering potential, CE = collision energy, CXP =Collision cell exit potential , EP = Entrance potential

***Transition used in analysis**

3.2.5 Analysis by UHPLC-MS/MS

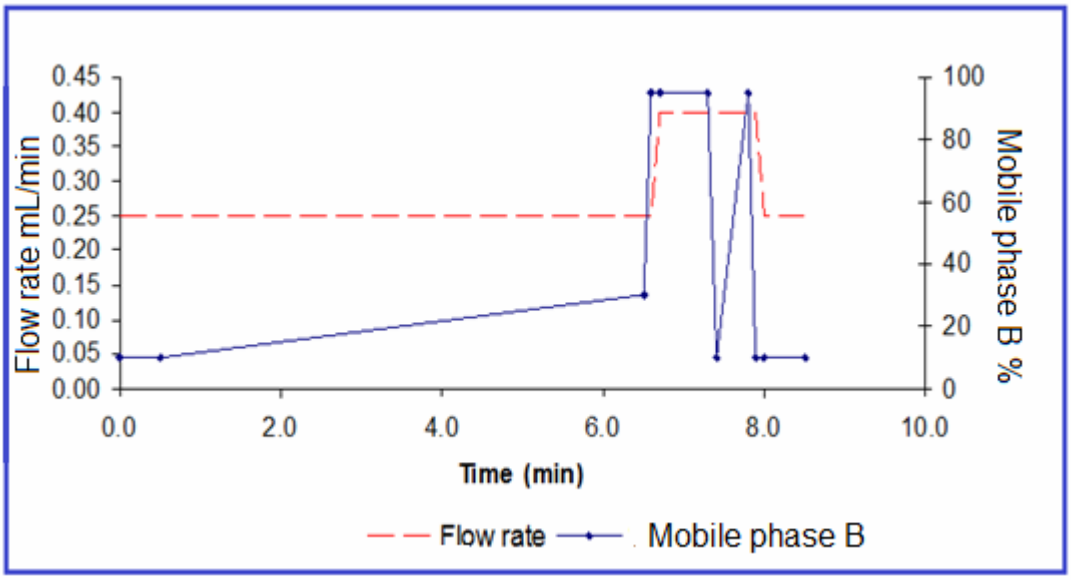
An Acquity UPLC system attached to an AB-Sciex API-4000 mass spectrometer comprised the LC-MS/MS system. After acquisition, the UHPLC-MS/MS data were processed using Analyst 1.5.1 version software. The chromatographic conditions are also shown in Table 11 and Figure 29.

Table 11 LC setup details

Column	Acquity BEH C18, 100 x 2.1 mm, 1.7 µm
Mobile phase A	Water/ HFIP/ TEA (100/ 1/ 0.1 v/v/v)
Mobile phase B	Methanol/ HFIP/ TEA (100/ 1/ 0.1 v/v/v)
Column temperature	50 º C
Injection volume	35 µL

The percentage of mobile phase B (organic solvent) was increased linearly from 10% to 30% over 6 minutes with an LC flow rate of 0.25 mL/min (figure 29). The percentage of organic solvent was then rapidly increased to 98% and the flow rate increased to 0.4 mL/min to remove non-polar impurities from the LC column (for example heavily retained lipids). Due to observed column carryover of the analyte between injections during method development, an additional rapid increase in the percentage of organic was introduced after the column flush which eliminated this effect. The measured pH of mobile phase A was 8.5, mobile phase B was 9.2.

Figure 29 LC gradient conditions



3.2.6 Quality control and calibration standard

Calibration standards were prepared by diluting the stock solutions into appropriate concentration working solutions with methanol: water (10:90 v/v), then spiking into blank human plasma to give a calibration series with standards at 5, 10, 25, 50, 100, 500, 1000, 1500, 2250 and 2500 ng/mL. The volume of the working solution used for spiking remained less than 5% of the total standard volume. A second, independently prepared stock solution was used to prepare QC (quality control) samples, by diluting the stock solution into appropriate concentration working solutions with methanol: water (10:90 v/v). The diluted working solutions were then spiked into blank human plasma to give QC samples at 5 ng/mL (LLOQ), 15 ng/mL (LoQC), 175 ng/mL (MeQC), 2000 ng/mL (HiQC) and 10000 (DiQC) ng/mL (The DiQC is 5 times higher in concentration then the highest calibration standard, therefore a dilution of this sample is required). QC samples were drawn into a 20 µL glass capillary; each capillary was placed into a 1.5 mL plastic microfuge tube (Eppendorf, Stevenage, UK) which was stored at -20 °C. CMS calibration standards were prepared fresh on the day of analysis.

3.2.7 CMS plasma analysis extraction procedure

20µL of internal standard solution (500 ng/mL) was added to all plastic microfugetubes (which already contain glass capillaries with either QC or calibration standards in them) with the exception of capillaries which contain matrix and water blank (blank refers to a sample which contains no analyte). 500 µL of water: 30% NH_4OH solution (95:5 v/v) was then added to each microfuge tube (to wash the sample out of the capillary). The microfuge tubetubes were gently mixed for 10 min (to ensure the plasma and washout solution has fully equilibrated with the microfuge tube). 100 µL of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v) was then added to all microfuge tubes. The microfuge tubes were gently mixed for a further 20 min and then centrifuged at 3000 g for 5 min (nominal 5 °C). A 600 µL aliquot was taken from the aqueous layer and placed into a 2 mL 96 well plate. 600 µL of water: HFIP: TEA (100: 2: 0.2, v/v/v) was added to each well and the entire well plate was vortex mixed for 10 min.

An HLB 10 mg 96 well SPE plate was primed with 1 mL of acetonitrile followed by 1 mL of water: HFIP: TEA (100: 1: 0.1, v/v/v). The entire sample was transferred to the SPE plate. The SPE plate was then washed with water: HFIP: TEA (100: 1: 0.1, v/v/v). 600 µL of acetonitrile: water: TEA (60: 40:1, v/v/v) was used to elute the samples into a 96 well plate. The contents of 96 well plate was then evaporated to dryness under a stream of nitrogen (approximately 2 hours). 150 µL of methanol: water: HFIP: TEA (10: 90: 2: 0.2, v/v/v/v) was added to re-dissolve the sample. The 96 well plate was then capped, vortex mixed for 2 min and centrifuged at 3000 g for 5 min (nominal 5 °C) and then 35 µL injected on the LC-MS/MS system.

3.2.8 Experiments conducted in validation

The following aspects were determined during method validation: accuracy and precision, selectively , sensitivity, freeze thaw stability, 24 hour room temperature stability, matrix effects, dilution process and recovery (section 1.6).

For the accuracy and precision experiments six replicates of the LoQC, MeQC and HiQC were analysed on three separate occasions (each occasion is known as a batch or an analytical run). Selectivity was assessed by the analysis of matrix blank (glass capillary containing blank plasma, no internal standard will be added to this sample), reagent blank (glass capillary containing water) and matrix blank containing internal standard (only blank sample to which internal standard is added) and six blank matrix individual samples for interference. Freeze-thaw stability was assessed by storing MeQC frozen for a period of 24 h before thawing at room temperature for a minimum 1 hour period. The MeQC samples were then returned to the freezer for 12 hours, before repeating the process twice more. Room temperature stability was assessed by analysising MeQC samples which have been stored at room temperature for a period of 24 hours.

Extraction recovery was assessed by comparing the detector response of an extracted QC sample with the detector response of an extracted control matrix sample into which the analyte has subsequently been added to yield the same analyte concentration as in the QC, using a solution of pure standard. The recovery test was done at LoQC, MeQC and HiQC analyte concentrations.

The dilution of CMS samples was assessed. 500 µL of water: NH_4OH (30% solution) (95:5 v/v) was added to tube 1 (which contains DiQC plasma sample in a glass capillary) and tube 2 (which contains blank plasma in a glass capillary). The microfuge tubes was gently mixed by shaking sideways for 10 min. Tenfold dilution was achieved by adding 10 µL out of tube 1 to 90 µL of the contents of tube 2.

Matrix effects can be defined as the variation in the amount of analyte detected in the presence of plasma produced from different donors. The different components found in plasma can interfere with ionisation in the mass spectrometer source resulting in analyte suppression or enhancement. Therefore the FDA guidelines for bioanalytical studies (Shah *et al* 2000) require the assessment of matrix effects.

Matrix effects were assessed by extracting (extracting means the sample has gone through the sample extraction procedure and is ready to be injected onto the LC-MS/MS. Described in section 3.2.7) six different individual lots of matrix and three water samples. Once fully extracted a pure standard solution (at the same concentration as an extracted LoQC level and internal standard, assuming 100% recovery) was added to all samples. These samples were then injected on a LC-MS/MS system. LoQC were prepared in six different individual lots of matrix. These LoQC samples will be extracted and analysed (also to test for matrix effects).

Recovery was assessed by extracting blank matrix (n=3) and then adding analyte at the same concentration as an extracted LoQC level and internal standard, assuming 100% recovery. The mean area of these samples is compared to the mean peak area of the extracted MeQC.

3.3 Results

3.3.1 Method development

The first stage in developing an analytical method is to optimise the mass spectrometer for the detection of the analyte and internal standard. Once this is achieved LC separation is developed, followed by development of the extraction procedure.

3.3.1.1 Mass spectrometer Infusion

The internal standard and analyte mass spectrometer parameters were originally determined by direct infusion (10 µL/ min) into the mass spectrometer. However this approach did not provide adequate sensitivity for pure standards. Therefore both analyte and internal standards were re-infused under LC flow. This approach allows one to fully optimise the mass spectrometer parameters under the conditions which best mimic a true analytical run. The response for both compounds dramatically decreased after the first minute of infusion, due to absorption to the glass infusion syringe. Therefore the concentration of the infusion solution was increased by tenfold to compensate for the absorption.

The same product ions were observed for the analyte and internal standard (not all product ions observed were fully optimised). Although the base sequences are different for the analyte and internal standard, as oligonucleotides, they contain the same bases and linkages. The product ion at *m/z* 94.9 is a phosphoric acid fragment, *m/z* 134.1 corresponds to a fragment of adenine, *m/z* 319 to thymidine fragment phosphate and *m/z* 344 to guanosine phosphate fragment (Zhang *et al* 2007). Multiple charge states were observed for analyte and internal standard (figure 30). For each charge state multiple peaks were observed, caused by naturally occurring isotope and salt adducts (figure 31). The *m/z* 830.2 – 94.9 transitions provided the best sensitivity for the analyte. However the internal standard $[M\text{-}6H]^{6-}$plus a salt/ metal ion adduct also had a precursor ion at *m/z* 830 and given that the same product ions were observed, an additional peak caused by the internal standard was present in the analyte chromatography (due to the amount of adducts observed this phenomenon was observed for all charge states). Therefore adequate LC separation was essential. All phosphorophioate oligonucleotides would have the same Q3 fragment ions because they contain the same base and linkages (but different sequence), therefore any method monitoring more than one oligonucleotide would likely encounter the problem described above. All transitions monitored during method development (listed in table 10) for the internal standard cause an additional peak in the analyte trace.

Figure 30 Q1 full scan of a 50 µg/mL infusion solution containing the analyte.

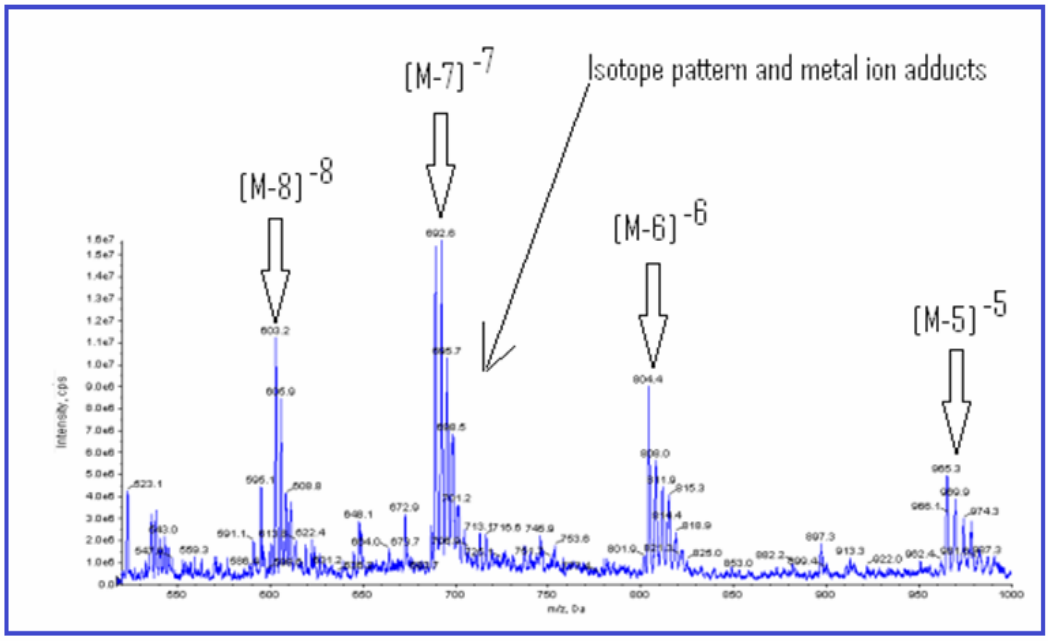
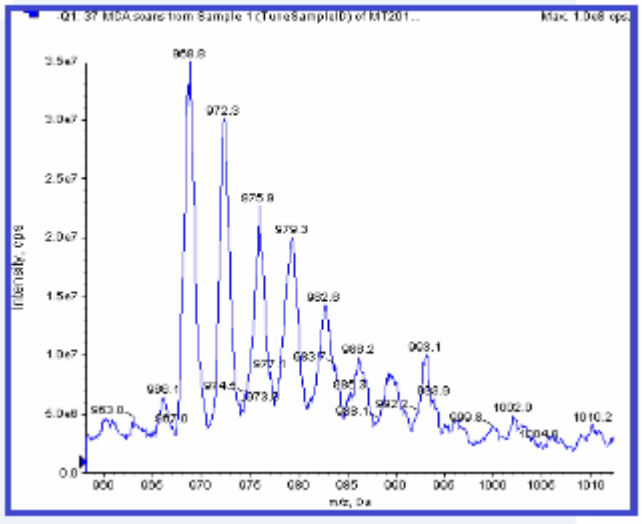


Figure 31 Q1 scan showing Isotope pattern and salt or metal ion adducts.



3.3.1.2 LC separation

Various analytical columns of various chemistries and lengths were tested during the method development stage. Table 12 lists the various columns tested and their effects on separation of the analyte and internal standard. However these did not significantly alter the achievable separation between the analyte and internal standard, with Acquity BEH C18, 100 x 2.1 mm, 1.7 µm the only column achieving slight separation.

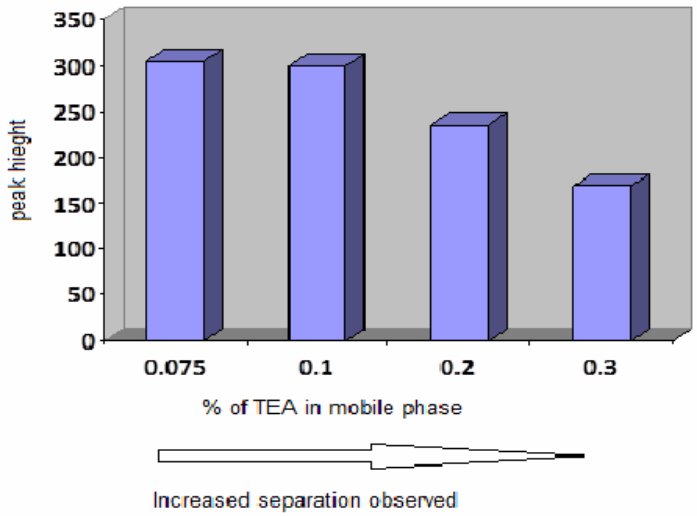
Table 12 Different column effects on separation between analyte and internal standard

Column	Effect on separation
Acquity BEH C8, 50 x 2.1 mm, 1.7 µm	No separation achieved
Acquity BEH C18, 50 x 2.1 mm, 1.7 µm	No separation achieved
Acquity BEH C8, 100 x 2.1 mm, 1.7 µm	No separation achieved
Acquity BEH C18, 100 x 2.1 mm, 1.7 µm	Slight separation achieved.

The main factors which affected separation were flow rate, run time and the concentration of TEA and HFIP in the mobile phase. The addition of a positive ion pairing modifier trethylammonium (TEA) to mobile phases results in the positive charged TEA forming ion-pair with the oligonucleotides (Lin *et al* 2007).

The oligonucleotide affectively becomes a neutral analyte and therefore resolution and retention is achieved in reversed-phase. The main issue with this approach is poor mass spectrometer sensitivity (analytes must be charged for mass spectrometer detection). Increasing the pH is favourable for dissociation of the oligonucleotide-TEA (oligo-TEA) ion pair, therefore 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) is also added to the mobile phase. HFIP lowers the pH of the mobile phase which strengths the oligo-TEA ion pair resulting in retention, however HFIP ready evaporates in the mass spectrometer source, as a result the pH of the remaining solvent increase sufficiently to dissociate the oligo-TEA ion pair (Van Dongen *et al* 2011). Increasing the concentration of TEA decreased the sensitivity (figure 32) with 0.1 % TEA providing the best separation versus sensitivity. Therefore more emphasis was placed on altering the flow rate and elution time to achieve the desired separation between the internal standard and analyte. An increase in the elution time was achieved by increasing the gradient run time run (section 1.4), however a significant increase in the length of the gradient time resulted in poor peak shape for both analyte and internal standard (peak broadning was observed). The optimum gradient time versus peak shape was 6.5 min with a flow rate of 0.25 mL/ min, as increasing the flow rate further resulted in a decrease in separation.

Figure 32 Graph showing the effects of increasing TEA concentration on peak height



Auto sampler or column carryover during development was assessed by injecting a matrix blank (plasma matrix containing no analyte) after the highest calibration standard. The observed carryover was originally 30% of the lowest calibration standard. To resolve the carryover it was essential to identify the cause of the carryover (column or auto sampler). The cause of the carryover was identified by injecting the highest calibration standard, then changing the column before injecting a matrix blank. No carryover was observed therefore the carryover was a result of analyte sticking to the column. This issue was resolved by adding an additional rapid decrease and then increase in the amount of organic solvent (see figure 29) to flush the column.

3.3.2 Extraction procedure

It was decided that first developing a conventional plasma extraction method which could then be modified to incorporate CMS would be the simplest approach. The first stage in the development of the extraction procedure was a simple solid phase extraction using a HLB SPE plate. However, poor sensitivity (Lowest limit of quantification was 5 ng/mL) was observed. One reason for this was believed to be the oligonucleotides binding to proteins. Therefore a phenol-choroform liquid-liquid extraction (to separate the analyte and internal standard from proteins, section 1.40) followed by solid phase extraction was tested. This approach increased the achieved sensitively (0.5 ng/mL) and used a plasma volume of 200 µL.

Modifying the plasma method to incorporate CMS exploited the fact that the first step in the phenol chloroform extraction was the addition of 5% ammonia in water which was also determined to be a good washout solution for the capillaries because 5% ammonia in water was observed to not precipitate the proteins within the glass tube and oligonucleotides are soluble in aqueous solution, this was a suitable first step. Therefore, the only significant modification made to the convention plasma extraction methodology was the introduction of a 20 minute mixing step to ensure that the contents of the capillary had fully equilibrated into the washout solution. The plasma volume used in the CMS methodology was 10 fold lower than the conventional methodology; as a result the achieved lower limit of quantification was 5 ng/mL.

3.3.3 Validation results

A developed analytical method must be validated prior to use on known samples. The following experiments are requirements for a full validation; accuracy and precision, selectively , sensitivity, freeze thaw stability, 24 hour room temperature stability, matrix effects, dilution process and recovery.

For the accuracy and precision experiments six replicates of the LLoQC, LoQC, MeQC and HiQC were analysed on three separate occasions. Intra- and inter-assay precision and accuracy data are presented in table 5. All QC (LoQC, MeQC, HiQC) have a mean accuracy within 15% of their nominal values (nominal value is the concentration of analyte added to plasma). The mean precision (RSD) is less than 15% for LLOQ, LoQC, MeQC and HiQC.

The successful dilution of samples must be demonstrated in the validation. For this method a tenfold dilution was assessed. Table 5 also shows accuracy and precision data for the DiQC (which was diluted tenfold). The mean accuracy of the DiQC is within 4% of nominal value and the precision is 3.6%.

Table 13 QC intra- and inter-assay precision and accuracy data

Intra-assay	QC 5ng/mL (LLOQ QC)	QC 15 ng/mL (LoQC)	QC 175 ng/mL (MeQC)	QC 2000 ng/mL (HiQC)	QC 10000 ng/mL (DiQC)
Batch 1					
*Mean (ng/mL)	5.76	14.0	184	1970	10400
Accuracy (%)	115.2	93.3	105.1	98.5	104.0
RSD (%)	11.3	11.1	4.5	2.7	3.6
n	6	6	6	6	6
Batch 2					
*Mean (ng/mL)	5.86	14.7	199	1900	
Accuracy (%)	117.2	98.0	113.7	95.0	
RSD (%)	4.7	6.2	4.4	6.5	
n	6	6	6	6	
Batch 3					
*Mean (ng/mL)	5.26	14.0	192	1980	
Accuracy (%)	105.2	93.3	109.7	99.0	
RSD (%)	6.7	5.6	2.8	1.5	
n	6	6	6	6	
Inter-assay					
Mean (ng/mL)	5.62	14.3	192	1950	
Accuracy (%)	112.4	95.3	109.7	97.5	
RSD (%)	9.0	7.9	5.0	4.3	
n	18	18	18	18	

DiQC: Diluted QC; QC: Quality control; LLOQ: Lower limit of quantification QC; LoQC: LowQC; MeQC: Medium QC; HiQC: High QC; RSD: Relative standard deviation;.
*Mean of the calculated concentration

3.3.3.1 Response

The analyte response (peak height) at the LLOQ was greater than five times the blank matrix response. A representative chromatogram is presented in figure 33 demonstrating an excellent signal to noise ratio at the LLOQ.A lack of significant interference at the retention time of the analyte from a blank human CMS sample is shown in figure 34. The internal standard response is shown in figure 35.

Figure 33. Representative UHPLC-MS/MS selective reaction monitoring chromatogram of a human CMS sample spiked with analyte at the LLOQ (5 ng/mL)

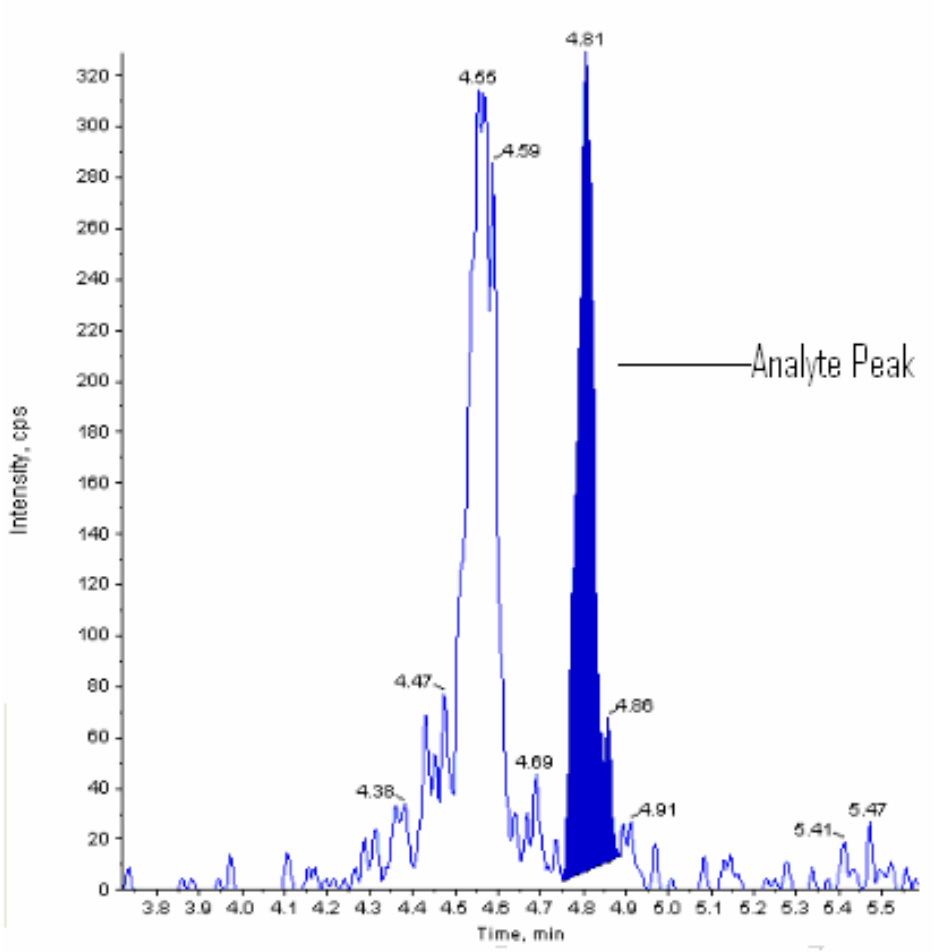


Figure 34. Representative UHPLC-MS/MS selective reaction monitoring chromatogram of a blank human CMS sample

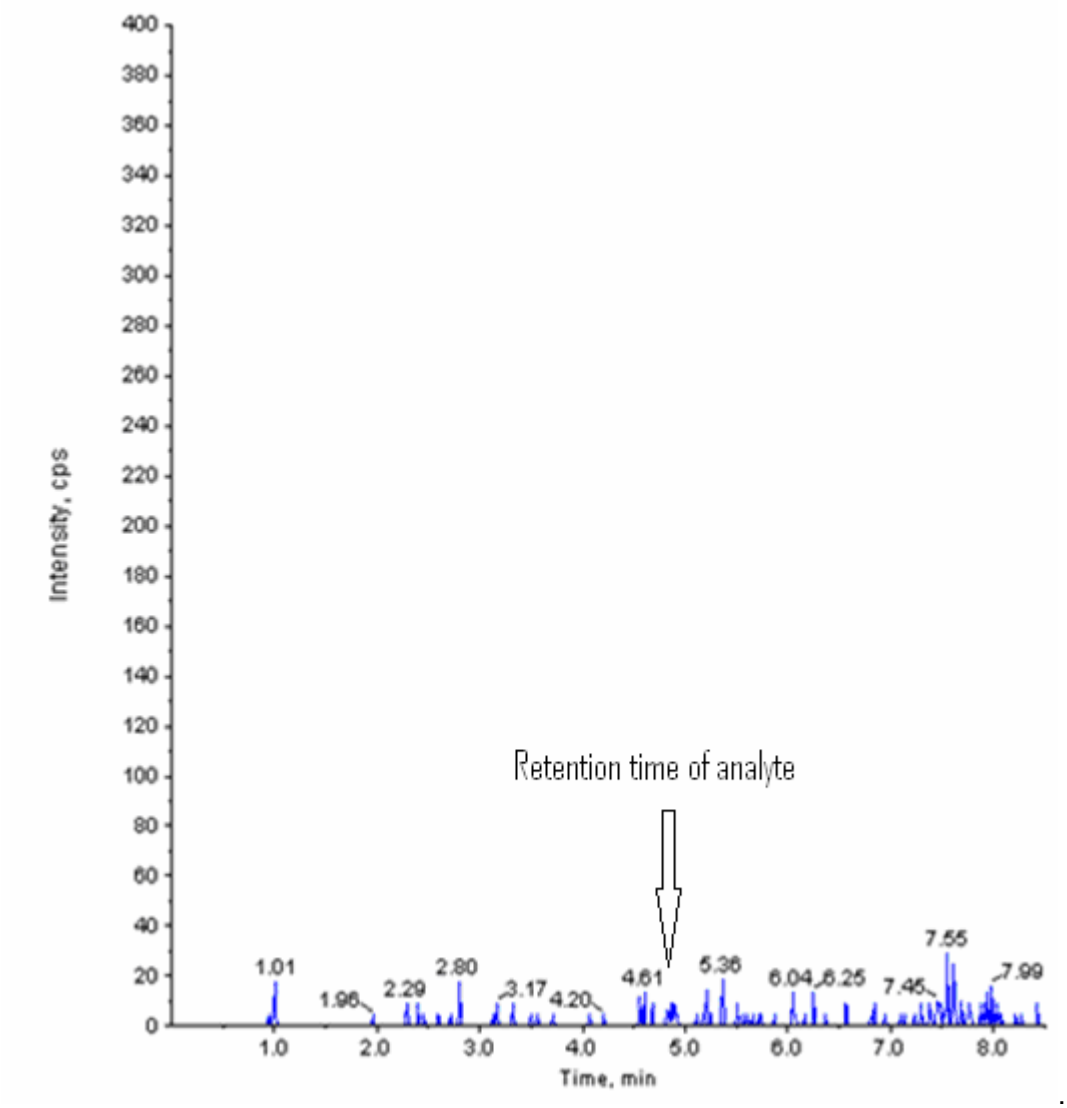
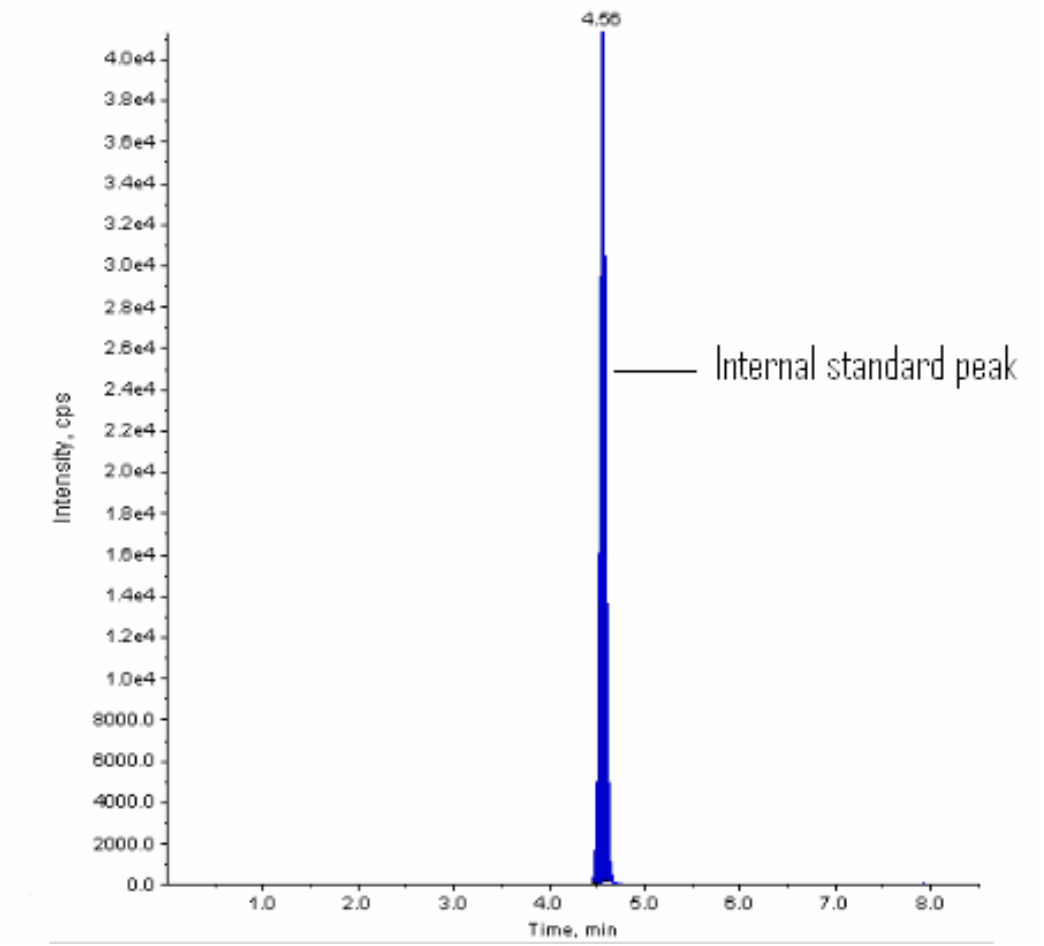


Figure 35. Representative UHPLC-MS/MS selective reaction monitoring chromatogram of a human CMS sample spiked with internal standard at 500 ng/mL.



3.3.3.2 Carryover

Auto-sampler carryover was assessed by injecting a matrix blank (plasma matrix containing no analyte) after the highest calibration standard. No significant peak was observed in this or any other matrix blank (an insignificant peak is less than 20% of the lowest calibration standard response).

3.3.3.3 Matrix effects

Matrix effects are variation in the amount of analyte detected in the presence of plasma produced from different donors. The different components found in plasma can interfere with ionisation in the mass spectrometer source resulting in analyte suppression or enhancement. Therefore the FDA requires that matrix effects are assessed. Matrix effects were assessed by extracting six different individual lots of matrix and three water samples. Once fully extracted a pure standard solution (at the same concentration as an extracted LoQC level and internal standard, assuming 100% recovery) was added to all samples. These samples were then injected on a LC-MS/MS system.

A ratio of analyte response detected in the presence of matrix to the analyte response in the absence of matrix provides a matrix factor. A matrix factor of 1 indicates that no matrix effects are effecting the analyte response. Matrix factor less than 1 suggests suppression (matrix components competing for ionisation within the mass spectrometer source). A matrix factor greater than 1 suggest enhancement (ionisation efficiency increased due to matrix components). A normalised matrix factor for analyte takes into account any matrix effects observed for the internal standard.

The matrix effects data for the analyte are presented in table 15 and internal standard data in table 16. The matrix factors for both the analyte and internal standard range from 0.91 to 1.04 (normalised matrix factor for the analyte range from 0.88 to 1.03). These results suggest little or no matrix effect were observed for both oligonucleotides.

Another experiment to assess matrix effects is to spike six different lots of plasma with analyte at the LoQC level. Variations in response between LoQC could be a result of matrix effects. Table 14 shows the data obtained from six different lots of matrix spiked with analyte at the LoQC. All six individual lots of matrix at the LoQC level are within 15% of nominal concentration.

Table 14LoQC sample in six different lots of matrix data

LoQC level (ng/mL)	Matrix sample number	Observed concentration (ng/mL)	Accuracy (%)
15	Individual 1	14.7	97.9
	Individual 2	15.0	99.8
	Individual 3	14.2	94.8
	Individual 4	14.4	96.0
	Individual 5	12.9	85.9
	Individual 6	14.5	96.6

Table 15 Quantification of matrix effects for analyte

LoQC level (ng/mL)	Peak area of water blank spiked post extraction	Mean peak area of pure standard (RSD%)	Matrix sample number	Peak area of individual matrix sample spiked post extraction	Matrix Factor	Matrix Factor Normalised*	Mean Matrix Factor Normalised (RSD%)
15	10280.5	10992.7	Individual 1	10742.7	0.98	1.02	0.98
	11519.2	(5.8)	Individual 2	11014.0	1.00	1.03	(5.1)
	11178.3		Individual 3	10517.0	0.96	0.98	
			Individual 4	11261.5	1.02	0.99	
			Individual 5	10038.5	0.91	0.88	
			Individual 6	10566.0	0.96	1.00	

* Calculated using the Internal Standard Matrix Factor from table 16

Table16 Quantification of matrix effects for internal standard

Quality control level (ng/mL)	IS peak area of pure standard	Mean peak area of pure standard (RSD%)	Matrix sample number	IS Peak area of sample spiked post extraction	Matrix Factor	Mean Matrix Factor (RSD%)
15	53498.6	51822.0	Individual 1	49861.9	0.96	0.99
	51417.2	(2.9)	Individual 2	50067.4	0.97	(4.0)
	50550.1		Individual 3	50536.8	0.98	
			Individual 4	53365.3	1.03	
			Individual 5	53660.1	1.04	
			Individual 6	49702.5	0.96	

3.3.3.4 Selectivity

An insignificant peak is anything below 20 % of the lowest limit of quantification (the lowest calibration standard) or below 5% of the mean internal standard response. There were no significant interfering peaks detected in reagent, matrix blanks and ULOQ calibration standard (without internal standard) in the retention window of analyte and internal standard respectively. All six different lots of matrix showed no interference at the retention time of either the analyte or internal standard (figure 6-8).

3.3.3.5 Short-term stability in matrix

Room temperature stability was assessed by storing MeQC samples on an open bench at room temperature for 24 hours. These were then analysed (six replicates) using a freshly prepared series of calibration standards and QC samples that had been stored at -20 °C. The accuracy and precision of the results of the 24 hour room temperature samples are presented in table 17. The accuracy and precision of these measurements conform to internationally recognised acceptance criteria (accuracy of +/- 15% and precision (RSD) less than 15%). However, the accuracy of the data for the 24 hour stability samples was noticeably higher than those stored at -20 °C.

Freeze-thaw stability (three cycles) data are presented in table 18. These samples failed acceptance criteria (acceptance criteria was +/- 15 of the actual concentration of the analyte added to plasma (Section 1.6), with the average accuracy of 116%. Further investigation is required to fully understand why the freeze-thaw samples showed an increase in analyte response. Possible reasons for the observed results could be a chemical change in the matrix caused by repeated freezing and thawing or breaking of the analyte-protein bonds.

Table 17 Room temperature 24 hour stability at medium quality control concentration level.

Replicate	QC 175 ng/mL (MeQC) Observed concentration (ng/mL)
1	211
2	195
3	190
4	198
5	198
6	189
Mean (ng/mL)	197
Standard deviation (n-1)	7.94
RSD (%)	4.0
Accuracy (%)	112.6

Table 18 Three freeze-thaw cycle stability data at medium quality control concentration level.

Replicate	QC 175 ng/mL (MeQC) bserved concentration (ng/mL)
1	209
2	200
3	211
4	176
5	210
6	209
Mean (ng/mL)	203
Standard deviation (n-1)	13.6
RSD (%)	6.7
Accuracy (%)	116.0

3.3.3.6 Recovery

The recovery of the analytical method for the analyte was assessed by comparing the detector response of an extracted MeQC against post spiked (analyte added to an extracted matrix blank sample) matrix blank samples at a concentration to simulate MeQC with 100% recovery. The recovery of the assay was 83 %, indicating that the complex extraction procedure used was working well.

3.4 Conclusions

A bioanalytical method for the quantification of a phosphorothioted oligonucleotide in human plasma capillary micro-sample using LC-MS/MS has been successfully developed and validated. The compatibility of CMS in oligonucleotide analysis has been successfully demonstrated. The accuracy and precision, dilution process, room temperature stability, selectivity and matrix effects are within international recognised acceptance criteria and therefore the developed method is a robust analytical method.

The main difficulty faced during the method development stage was creating a sensitive, selective and reproducible extraction and quantification method. It was relatively simple to modify the developed plasma method for CMS analysis. Therefore using CMS for oligonucleotide analysis does not dramatically increase the amount of method development time required. One main drawback is that the extraction time for capillary samples is increased by 20 minutes due to the equilibration of the washout solution and the CMS (a shorter mixing time was not tested). Another issue with using CMS is that currently it is difficult to physically handle small glass tubes. The amount of interest surrounding CMS means it may only be a matter of time before new handling equipment is developed (equipment such as capillary holders, 96-well plates large enough to place CMS samples into and automation robots).

Chapter 4

Conclusion and further work

4.0 Conclusion

The overall aim of this research was to increase the use and popularity of micro-sampling. The first stage of this research focused on developing techniques which limit the requirements for re-analysis of samples, which fall outside the calibration range for any given method. The two techniques developed and partially validated (MSSD and ISRM) have the potential to drastically reduce the amount of re-analysis required for all matrices. The limit sample volume provided by micro-sampling means that reanalysis is not always possible. However, MSSD and ISRM would alleviate these issues. Also, the reduction in repeat analysis would result in a saving in cost and time.

Concerns surrounding DBS analysis were brought to light midway through the research (discussed in section 1.1). As a result, the use and popularity of DBS has decreased. Industry attention has switched in the direction of CMS because the same reduction of animals used for research can be achieved.

Therefore, my research also switched to CMS. The aim of the second part of my research was to develop a bioanalytical method for the quantification of a phosphorothioted oligonucleotide in human plasma capillary micro-samples using LC-MS/MS and in doing so demonstrate the compatibility of CMS with a challenging class of compounds. A sensitive, robust and precise method was successfully developed to quantify 18 mer oligonucleotide using CMS. This research was presented at the European Bioanalysis Forum (November 2013, in Barcelona), attended by 500 influential individuals in bioanalysis. CMS featured heavily in the discussion and presentation signalling the industries intention to implement CMS in the majority of pre-clinical studies. The oligonucleotide method developed in this research has been modified and successfully used on actual pre-clinical and clinical discovery studies (analysing a total of 30 study samples).

Further work

Covance laboratories have recently set up a global team to research and implement CMS. As a member of this team I can build on the research conducted in this thesis. The next step is to dose actual animals and collect CMS, to compare the data generated with that of conventional sampling methods (currently this has been scheduled for June and I will be the responsible scientist for the bioanalysis). I will also be visiting GlaxoSmithKline (Ware, UK) for a two week secondment to build on my experience in analysing micro-samples. This will enable me to share this research and develop it further.

References

Abu-Rabie, P., Denniff , P., Spooner, N., Brynjolffssen, J., Galluzzo, P., Sanders, G.**(2011).**Method of applying internal standard to dried matrix spot samples for use in quantitative bioanalysis. *Anal Chem*. Nov 15, 83(22), 8779-86

Aburuz, S., Millership, J., McElnay, J. (2006) Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin. *J Chromatogr B AnalytTechnol Biomed Life Sci.* Mar 7;832(2):202-7 . Epub Jan 30 (2006).

Agarwal, K. **(1981).***J. Biol. Chem*. 256:1023-1028

Apffel, A., chakel, JA., Fishcher, S., Lichtenwalter, K., Hancock, WS.**(1997).** Analysis of oligonucleotides by HPLC-electrospray ionization mass spectrometry. *Anal. Chem*. 69, 1320-1325

Agrawal,K., Frank, J.**(2000)**.A ratchet-like inter-subunit reorganization of the ribosome during translocation.*Nature* 406, 318-322

Amsterdam, P., Waldrop, C.**(2010)**Theapplication of dried blood spot sampling in global clinical trials. *Bioanalysis*.Nov, 2(11), 1783-6.

Barfield, M., Spooner, N., Lad, R., Parry, S., Fowles, S.**(2008)** Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies**.***J Chromatogr B* 870(1):32-7. Epub May 22.

Brown, P.R., Hartwick, R.A.**(1989)** High performance liquid chromatography in chemical analysis. John wiley and son, Vol 98 P277-295

Burnett, J.E. **(2011)**Dried blood spot sampling: practical considerations and recommendation for use with preclinical studies. *Bioanalysis*.May, 3(10), 1099-107

Chang, M., Zellner, M., Winkler, W., Hayden, H., Dlestinger, M., Roth, E., Miller, I. **(2005)** Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets.*Electrophoresis*. 26(12):2481-9.

Chen, B., Bartlett, M.**(2012)** A one step solid phase extraction method for bioanalysis of a phosphorothioate oligonucleotides and its 3' n-1 metabolite from rat plasma by uHPLC-MS/MS. *AAPSJournal* 14, 772-780.

Clark, G.T., Haynes, J.J., Bayliss, M.A.**(2010)**Burrows L. Utilization of DBS within drug discovery: development of a serial microsampling pharmacokinetic study in mice. *Bioanalysis*.Aug, 2(8), 1477-88.

Crooke, ST. **(2008)**. Antisense drug technology.Principles, strategies and application.*taylor and francis group, new york,usa.*

Dainty, T.C., Richmond, E.S., Davies, I.,Blackwell, M.P. **(2012)** Dried blood spot bioanalysis: an evaluation of techniques and opportunities for reduction and refinement in mouse and juvenile rat toxicokinetic studies. int J Toxicol.; 31(1):4-13. Epub 2012 Jan 6.

Dole, M*.* **(1968).***J Chem. Phys*. 49, 2240-2249.

Fan, L., Lee, J., Hall,J., Tolentino, E.J., Wu, H., Shourbagy, T.**(2011)** Implementing DBS methodology for the determination of Compound A in monkey blood: GLP method

validation and investigation of the impact of blood spreading on performance.*Bioanalysis*. 3(11), 1241-52.

Fenn, J.B., Yamashita, M. **(1984)** Electrospray ion source. *J phys. Chem*, 88, 4451-4459.

Fenn, J.B.,Yamashita, M.**(1985)** Electrospray interface for liquid chromatographs and mass spectrometers *Anal Chem*.57(3):675-9.

Fenn, J.B., Mann,M., Meng, C.K., Wong,S.F., Whithouse, C.M.**(1989)** Electrospray ionization for mass spectrometry of large molecules *science* 246 64-71

Ganz, N., Singrasa, M., Nicolas, L., Gutierrez, M., Dingemanse, J., Döbelin, W., Glinski M.**(2012)** Development and validation of a fully automated online human dried blood spot analysis of bosentan and its metabolites using the Sample Card and Prep DBS System.*JChromatogr B AnalytTechnol Biomed Life Sci*. Feb 15, 885-886, 50-60.

Guidance for Industry, Bioanalytical Method Validation, FDA, CDER and CVM, May 2001.

Guthrie, R., Susi, A., **(1963).**A simple phenylalanine method for detecting phenylketonuria in large populations of newborninfants.*Paediatrics*.32, 338-43.

Hagerman, A. E. **(1978)**. Protein precipitation method for the quantitative determination of tannins *J. Agric. Food Chem.*, *26* (4), pp 809–812

Hennion, MC. **(1999)**Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. Volume 856, Issues 1–2, 24, Pages 3–54

Hemsley, M., Ewles. M., Goodwin, L. **(2012)** Development of a bioanalytical method for quantification of a 15-mer oligonucleotide at sub-ng/ ml concentrations using LC- MS/MS. *Bioanalysis*4, 1457-1469.

Hendrike, G. **(2009)** Theoretical Model in LC based bioanalytical method development. Journal of pharmaceutical and bioanalytical analysis (49) 1-10.

Horning, E.C., Carroll, D.I., Stillwell, R.N., Dzidic, I.**(1975).**Atmospheric pressure ionization mass spectrometry.Corona discharge ion source for use in a liquid chromatograph-mass spectrometer-computer analytical system.*Anal. Chem.*, *47* (14), pp 2369–2373.

Horvath, J.F.K., Presiss, B. **(1967)** Fast liquid chromatography. Investigation of the operating parameters and the separation of nucleotides on pellicular ion exchangers.Anal chem. 39 (12) 1422-8.

Jonsson, European Bioanalysis forum (EBF) presentation 2012, provided by Astra Zeneca

Kebarle, P., Verkerk, U.H., **(2009).** "Electrospray: from ions in solution to ions in the gas phase, what we know now". *Mass Spectrom Rev*28 (6): 898–917.

Kirby, KS.,**(1956)** A new method for the isolation of ribonucleic acids from mammalian tissues *Biochem J* 64,405.

Lagerwerf, FM. **(2000)**Exploring the boundaries of bioanalytical quantitative LC–MS–MS. TrAC Trends *Analytical Chemistry*. Volume 19, Issue 7, Pages 418–427.

Lawson, G., Cocks, E., Tanna, S., Quantitative determination of atenolol in dried blood spot samples by LC-HRMS: a potential method for assessing medication adherence. *J Chromatogr B AnalytTechnol Biomed Life Sci.* May 15, 897, 72-9 (2012).Epub Apr 16 (2012).

Lin, Z.J., Li, W., Dai, G., **(2007)** Applications of LC-MS for quantitative analysis and metabolite identification of therapeutic oligonucleotides. *J. Pharm. Biomed. Anal.*44, 330-341.

Liu.G., Snapp, H.M., Ji, Q.C. **(2011)** .Internal standard tracked dilution to overcome challenges in dried blood spots and robotic sample preparation for liquid chromatography/tandem mass spectrometry assays. Rapid Communications in Mass Spectrometry.Volume 25, Issue 9, pages 1250–1256.

Martin, P., **(1998)**Molecular imprints as sorbents for solid phase extraction: potential and applications ***Anal. Commun.*35**, 13H-14H

McCarthy, S.M., Martin, G. M., Gebler, J.**(2009)**Reveresed-phase ion-pair liquid chromatography analysis and purification of small interfering RNA. *Anal.Biochem*. 390, 181-188.

Mei, H., Hsieh, Y., Nardo, X.X., Wang, S., Korfmacher, A. **(2003)** Investigation of matrix effects in bioanalytical high performance liquid chromatography tandem mass spectrometric assay. Rapid commun. Mass spectrum (17) 97-103

Methieu, E. J. **(1868)***Math pures appl.,* (J. Liouville), 13, 137.

Mess, J.N., Taillon, M.P., Côté, C., Garofolo, F. **(2012)**Dried blood spot on-card derivatization: an alternative form of sample handling to overcome the instability of thiorphan in biological matrix.*BiomedChromatogr*.Dec, 26(12), 1617-24.

Ooms, J.A., Knegt, L., Koster, E.H., **(2011)** Exploration of a new concept for automated dried blood spot analysis using flow-through desorption and online SPE-MS/MS. *Bioanalysis*. Oct, 3(20), 2311-20.

Prakash, T.P., Bhat, B.**(2007)** 2'- Modified oligonucleotides for antisense therapeutics. Curr.Top. Med. Chem. 7, 641-649.

Prakash, T.P.,**(2011)**An overview of sugar- modified oligonucleotides for antisense therapeutics. Chem. Biodiv. 8, 1616-1641.

Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays, BMV Whitepaper, Crystal City Meeting, May 2006

Roehr, B. **(1998).** "Fomivirsen approved for CMV retinitis". *J IntAssoc Physicians AIDS Care.* V4 (10): 14–6

Rossi,DT., Zhang, N. **(2000).**Automating solid-phase extraction: current aspecs and future prospekts.*Journal of Chromatography A*.Volume 885, Issues 1–2, 14, Pages 97–113

Sacchi, N. **(1987)**. Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction.Analytical.*Biochemistry* 162, Issue 1,156–159

Sahu, N.K., Shilakar,I. G., Nayak, A., Kohli, D.V. **(2007)**Antisense technology: a selective tol for gene expression regulation and gene targeting. *Curr. Pharm. Biotechnol.* 8, 291-304.

Shah, V.P., Midha, K.K., Findlay, J.W.A. **(2000),**Bioanalytical method validation – a revisit with a decade of progress.*Pharm. Res*. 17(12), 1551–1557.

Shah, V.P. **(2007)** The history of bioanalytical method validation and regulation: evolution of a guidance document on bioanalytical methods validation. *AAPS J*. 9(1) E43–E47

Sleczka, B.G., D'Arienzo, C.J., Tymiak, A.A., Olah, T.V.**(2012)**Quantitation of therapeutic proteins following direct trypsin digestion of dried blood spot samples and detection by LC-MS-based bioanalytical methods in drug discovery. *Bioanalysis*. Jan, 4(1), 29-40.

Spooner, N., Lad, R., Barfield, M**.(2009)**Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method. *Anal. Chem.81* (4), pp 1557–1563

Stein, CA. **(1993)***Science* 261: 1004-1012

Taylor, G., **(1964)** Disintegration of Water Droplets in an Electric Field". *Proceedings of the Royal Society A* 1964280 (1382): 383

Tiler, P.R., Romanyshyn, A. **(2002)** Implication of matrix effects in ultra-fast gradient or fast isocratic liquid chromatography with mass spectrometry in drug discovery RCM (16) 92-98

Thomson, B.A., Iribarne, J.V. **(1979)**. On the evaporation of small ions from charged droplets". *Journal of Chemical Physics* **64** (6): 2287–2294

Tremblay, G.A., Khalafaghian G, Legault J, Nielsen P, Bartlett AJ.**(2011)** Dual ligation hybridization assay for the specific determination of oligonucleotide therapeutics.Bioanlaysis 3, 499-508.

Tu, H., Ray, A.K., Li, K.Y. **(2005)** Charge limits on droplets during evaporation. *Langmuir*21 (9): 3786–94.

Turpin, P.E., Burnett, J.E., Goodwin, L., Foster, A., Barfield, M. **(2010)** Application of the DBS methodology to a toxicokinetic study in rats and transferability of analysis between bioanalytical laboratories.*Bioanalysis*.;2(8),1489-99 .

Van Deemter., JJ.,Zuiderweg, FJ., and Klinkenberg, A. **(1956)**. Longitudinal diffusion and resistance to mass transfer as causes of non ideality in chromatography.*Chem.Eng. Sc.***5**: 271–289.

Van Dongen, W.D., Niessen, W.M.A. **(2011)** Bioanalytical LC-MS of therapeutic oligonucleotides. *Bioanalysis 3*, 541-564.

Venn, RF. Principles and practices of Bioanalysis (Second addition)

Watt, A.P., Morrison, I. I.,Evans, D.C. **(2000)** Approaches to higher-throughput pharmacokinetics (HTPK) in drug discovery.*Drug DiscoveryToday*.Volume 5, Issue 1, Pages 17–24

Watt, AP., Morrison, D., Locker, KL., Evans DC. **(2000)** Higher throughput bioanalysis by automation of a protein precipitation assay using a 96-well format with detection by LC-MS/MS. *Anal Chem*. Mar 1, 72(5), 979-84

Wang, L. **(2011)** Oligonucleotide bioanalysis: sensitivity versus specificity. *Bioanalysis3*, 1299-1303.

Wickstrom, J., et al **(1986).**Relative Nuclease Resistance of Hybrid Oligonucleotide Phosphorothioates.*J. Biochem.Biophys*.Meth. 13:97-102. 68

Yost, RA., Enke, CG. **(1978)** Selected ion fragmentation with a tandem quadrupole mass spectrometer ,*Journal of the American Chemical Society*100 (7): 2274

Zhang, G., Lin, J., Srinivasan, K., Duncan, J.N.**(2007)** Strategies for bioanalysis of an oligonucleotide class macromolecule from rat plasma using liquid chromatograpy- tandem mass spectrometry. *Anal.chem*. 79, 3416-3423

Zhou, S., Cook, K.D.**(2001)** A mechanistic study of electrospray mass spectrometry charge gradient within electrospray droplets and their influence on the ion response. *JASMS* (12) 206-214

http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-Multiquant.pdf).

http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry.pdf).

www.sisweb.com/mstools/isotope.htm.