The Investigation and Application of Alternative Strategies for LC/MS based Bioanalytical Research

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Dedication

For my family and mentors

Acknowledgements

Behind any project, endeavor or task that may be at hand, there are always people involved whose: advice, influence, and guidance may not be readily recognized by those who view the final result most often than not presented by one, single person.

The origin of the work contained within this document starts with the instruction of values and principals my parents, Robert D. and Sharon L. Rainville instilled in me at a very early age. For without this essential establishment of values and structure, I would have never learned the basics of applying: determination, use of knowledge, and pragmatism. Essential components needed to successfully fulfill the requirements of a doctoral thesis.

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Norman S. Smith, Ph.D. and Professor David Cowan, Ph.D., my supervisors during these studies have been a major source of influence and inspiration.

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Lastly but without the least importance of note or mention, the acknowledgement of the sacrifice my beloved wife, Christine has endured through this journey. Her understanding and awareness of both the process and time required in this endeavor can never be overlooked, or underappreciated.

Abstract

The work presented here demonstrates two unique approaches in the application of LC/MS for the analysis of drugs and endogenous compounds in biofluids. First, the use of basic aqueous mobile phases in conjunction with both methanol and acetonitrile by LC/MS operating in electrospray positive ionization mode was investigated. Second, the design and development of a prototype ceramic micro-fluidic device (CMFD) and optimized MS source was carried out. The CMFD was packed with sub 2 µm bridged ethyl hybrid (BEH) chromatographic particles that could withstand operating pressures of up to 12,000 psi. The MS source was built to operate in both positive and negative electrospray ionization mode with the operating flow rates corresponding to the 300 um i.d. of the CMFD.

The results generated from studies utilizing the addition of a base, such as ammonium hydroxide to modify the aqueous mobile phase, showed significant benefits for LC/MS/MS based bioanalytical assays when analyzed with electrospray positive ionization mode. Increases in the signal-to-noise values were observed for twenty-two out of twenty-four of the probe pharmaceuticals tested. Increase in the chromatographic retention of poorly retained compounds was also observed however, this increase in analyte retention did not occur for many highly polar compounds that eluted in the column void when chromatographed with the basic mobile phase conditions. The effect of the pH of the mobile phase further showed that the phospholipid fraction, present in protein precipitated plasma, was only slightly affected by the change in mobile phase pH. It provided complementary data to that obtained with traditional acidic based

mobile phases and results in an increased number of ions detected overall for metabonomic studies. Further it was observed that by changing the pH to a basic system it was possible to resolve compounds that had previously co-eluted on traditional acidic reversed-phase LC/MS.

A 0.3 x 100 mm i.d. CMFD and compatible MS source was successfully designed for the analysis of biological samples. The device showed average chromatographic efficiencies of 9038 plates compared to 10219 plates for standard silica capillary columns. Gradient performance utilizing a diverse mix of compounds yielded a peak capacity of 55 as the average peak widths for all analytes was 0.11 minutes for a 6 minute separation. Resolution of the probe pharmaceutical alprazolam and associated hydroxylated metabolite was maintained between 1.2 and 1.5 for four different devices. Testing of the device with plasma samples prepared by protein precipitation resulted in over 1000 injections being carried out over approximately a one week period. The system was however unable to withstand the high pH (10.5) utilized in the previous study but could operate with a mobile phase pH of 10. The resulting MS source built to operate under these conditions and with the flow rates required to operate the 0.3 x 100 mm CMFD yielded signal to noise increase in the range of 8.3 to 38.6 for the molecules tested.

The CMFD/MS system was successful in the analysis of biofluid samples in both ESI + and ESI – ionization modes and was shown to allow for the analysis of small sample injection volumes from plasma prepared by protein precipitation, and dried blood spots. This device was successfully utilized for the profiling of metabolites from the beta blocker drug, propranolol. Further separation of complex biofluid samples derived from axenic rats and bile from dogs again illustrated the separation and sensitivity capabilities offered by the CMFD/MS system.

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A	Contribution of Eddy diffusion to band broadening or aqueous mobile phase
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, excretion
ADMET	Absorption, distribution, metabolism, excretion, toxicity
API	Atmospheric pressure ionization
APCI	Atmospheric pressure chemical ionization
AUC	Area under the curve
AZ	AstraZenaca
aq	Aqueous
API	Atmospheric ionisation
B	Contribution of longitudinal diffusion to band broadening or organic mobile phase
BEH	Bridged ethyl-hybrid
CI	Chemical ionisation
Cmax	Maximum plasma drug concentration
CMFD	Ceramic microfluidic device
DMPK	Drug metabolism pharmacokinetics
dp	Chromatographic particle size
EC	Electrochemical
EI	Electron impact
ELS	Evaporative light scattering

ESI	Electrospray ionization mode
ESI +	Positive electrospray ionization mode
ESI -	Negative electrospray ionization mode
F	Bioavailability
FAB	Fast atom bombardment
GC	Gas chromatography
GC/MS	Gas chromatography coupled with mass spectrometry
GSK	GlaxoSmithKline
H	Plate height
HETP	Height equivalent to a theoretical plate
h	Hour
HPLC	High pressure liquid chromatography
HTCC	High temperature co-fired ceramic
i.d.	Internal diameter
IND	Investigation new drug application
IPA	Isopropanol
K	Elimination rate constant
k	Capacity factor, or retention factor
k_g	Gradient retention factor
kpsi	Kilo pounds per square inch
kV	Kilovolt
L	Length
LC	Liquid chromatography
L/Hr	Liters per hour

LC/MS	Liquid chromatography coupled to mass spectrometry
LOD	Limit of detection
LLOQ	Lower limit of quantification
LC/UV	Liquid chromatography coupled with ultra violet detection
M	Mass
MALDI	Matrix assisted laser desorption ionization
MALLS	Multiple angle laser light scattering
MeOH	Methanol
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
MRM	Multiple reaction monitoring
MS	Mass spectrometry
m/z	Mass/charge ratio
n	Number of replicates
N	Efficiency
NaOH	Sodium hydroxide
NDA	New drug application
nl	Nanoliter
nm	Nanometer

NMR	Nuclear magnetic resonance
P_c	Peak capacity
PCA	Principal compnent analysis
PCB	Printed cercuit board
PD	Phamacokinetics
pg	Picogram
PK	Pharmacokinetics
psi	Pressure per square inch
Pw	Peak width
Q	Single quadrupole
QQQ	Triple quadrupole
Q-Tof	Quadrupole time of flight mass spectometer
R	Resolution (mass spectometer)
RCF	Relative centrifugal force
RI	Refractive index
RP	Reversed-phase
R_s	Resolution
R^2	Coefficient of determination
s	Second
SIR	Single ion recording
SPE	Solid phase extraction
SRM	Selected reaction monitoring
t_g	Gradient time
Tmax	Time where Cmax is observed
TOF	Time of flight

t_0	Void time
t_R	Retention time
$t_{½}$	Drug half life
u	Linear velocity
µL	Microliter
µm	Micrometer
UPLC	Ultra performance liquid chromatography
US FDA	United States Federal Drug Administration
UV	Ultraviolet
V	Applied voltage
V_o	Volume of mobile phase pumped for an unretained analyte to reach the detector
V_R	Volume of mobile phase pumped for a retained analyte to reach the detector
w	Chromatographic peak width
α	Selectivity
˚C	Degree Celsius
ΔM	Delta M is the difference of two neighboring m/z values
%	Percent
% v/v	Percent volume by volume

Chapter 1

Introduction

Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information upon it- Samuel Johnson

1.1 Bioanalytical aspects of pharmaceutical drug development

The primary function of any bioanalytical department working within the pharmaceutical industry is to carry out identification, and quantification of biomarkers, candidate drug compounds and their related metabolites in biological fluids. This activity is critical to the successful development of new medicines since the majority of new chemical compounds that are synthesized by medicinal chemists never become suitable drug compounds [1]. Reasons for failure vary and can be contributed to safety, efficacy, cost of goods, formulation, bioavailability (F), pharmacokinetics (PK), and toxicology [2, 3]. Of the previously listed reasons for failure, efficacy, toxicity, and unacceptable pharmacokinetics are the three main causes for clinical failure of drug compounds [4, 5]. Even when a new medicine is approved by regulatory agencies, issues can arise regarding drug product safety due to idiosyncratic toxicity or unexpected drug-drug interactions [6-9]. Furthermore, the formation of toxic metabolites by the liver can inhibit enzymes that can change the rate or metabolism of other compounds [10, 11]. Because of these facts and the enormous cost of producing a drug to treat a particular disease from concept through to commercialization, it is critical to have the correct clinical study design and analytical techniques to obtain precise and accurate data that can be used by the clinicians and drug development teams to successfully navigate a drug candidate through the drug discovery/development process. **Figure 1.1.1** illustrates the time it can take to deliver a drug to market and the number of compounds that may be screened or tested for potential use as therapy for a designated disease cure. Here one can observe that it can take up to 16 years to produce a drug for the market as well as the fact it may take the evaluation of 10,000 candidate

drug compounds in order to correctly identify the best compound that has the desired pharmacodynamics (PD), PK, and low toxicity required. **Figure 1.1.2** Illustrates the cost that can be associated with the drug discovery process.

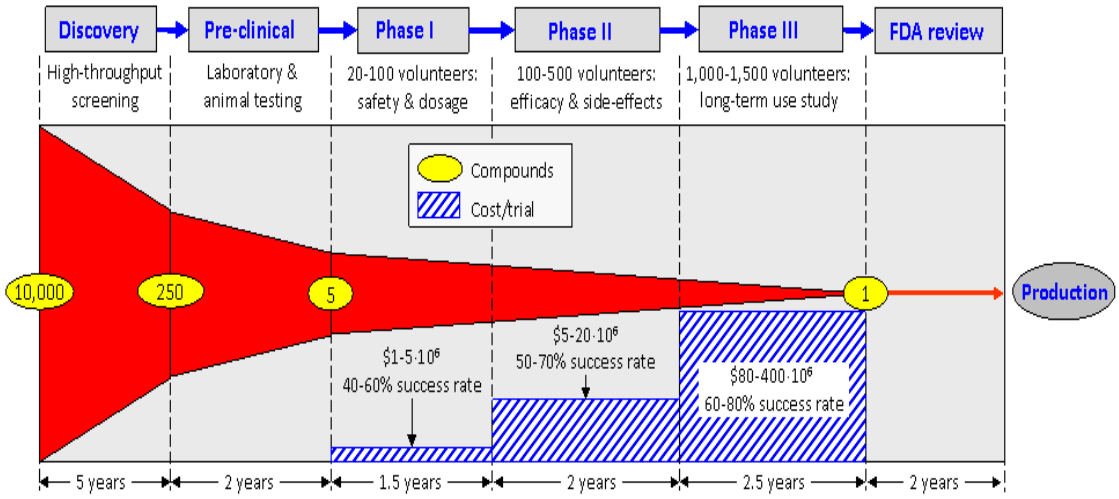

Figure 1.1.1 Attrition rate of drug compounds from discovery to approval [12]**.**

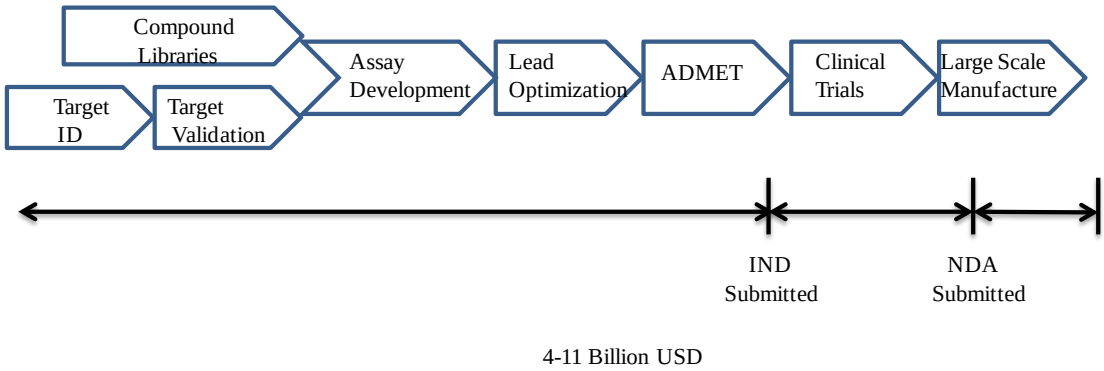

Figure 1.1.2 Cost associated with the development of a new drug compound from discovery to commercialization phase.

As can be seen the costs that are associated with the drug development process are as of 2012 estimated to be between 4-11 billion US dollars [13]. Therefore due to the data shown in **Figures 1.1.1** and **1.1.2** it is imperative that drug characteristics such as PK, toxicity, and PD of a candidate drug molecule are well understood so that resources and investment costs are not wasted on drug candidates that may prove to have poor characteristics**.** Therefore due to the important nature of correctly defining or acquiring high quality, precise and accurate data on critical drug characteristics they will be further defined in the following sections.

1.1.1 Pharmacokinetics

Pharmacokinetics (PK) is a branch of pharmacology focused on the determination of the fate of substances administered to a living organism. These substances of interest can include pharmaceutical drugs, hormones, nutrients, and toxins. PK is often studied in conjunction with pharmacodynamics (PD), which is defined as the effect of a substance to a living organism. PK also includes the study of the mechanisms of absorption, distribution, metabolism, and excretion (ADME) of an administered pharmaceutical drug and determines the rate at which any drug action begins and duration of the effect. The study of PK is an important step in the drug discovery process as it determines the effective dose at which a drug is to be administered to a patient population. Inhaled therapeutics and low dose new chemical entities (NCE) present one of the most significant analytical challenges due to their low systemic concentration. Standard curves and internal standards are used for quantification of usually a single pharmaceutical extracted from a patient sample. The samples represent different time points as a pharmaceutical is administered and then metabolized or cleared from the body. Data collected from the different time points is plotted and a PK curve is produced. **Figure 1.1.3** illustrates a generic PK curve. Typical useful measurements that are derived from this plot are: the maximum drug concentration present in the sample (C_{Max}), the time it takes to reach the maximum drug concentration (T_{Max}), area under the curve (AUC),

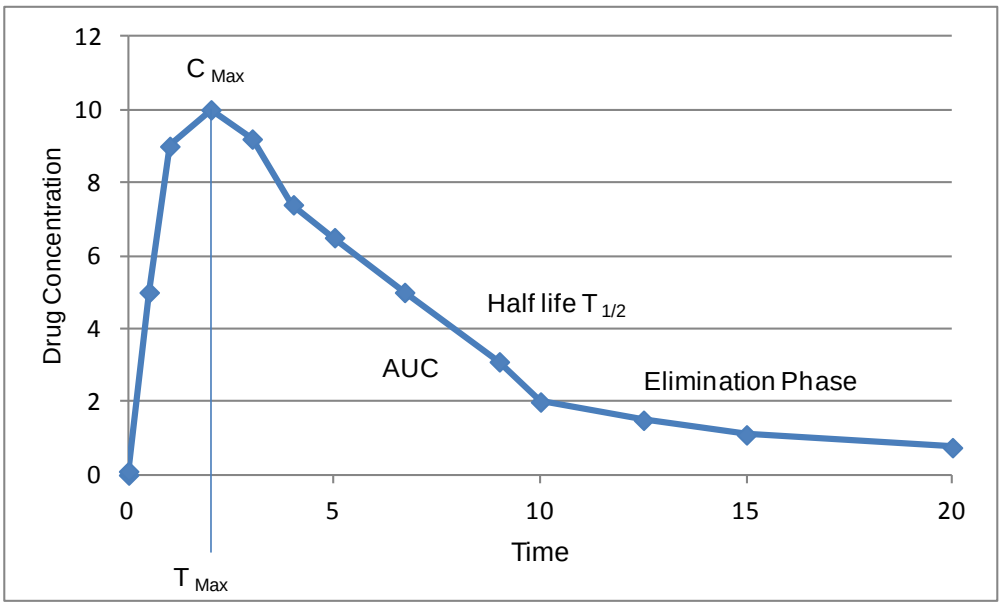

Figure 1.1.3 Generic PK curve for oral administered drug.

1.1.2 Drug metabolism

As previously described the metabolism or biotransformation of a drug can influence the PD or toxicity, and therefore safety exhibited by the drug once it is administered into an organism The overall response of the organism to metabolize a drug is based on the fact that any drug molecule is usually xenobiotic in nature and an organism's response to the drug, once detected, is to eliminate from the body as soon as possible. Drug metabolism is generally divided into two areas: Phase I (functionalisation reactions) and Phase II (conjugation reactions). A list of these reactions is shown in **Table 1.1.1**

Phase I metabolism includes a number of chemical reactions that modify the drug molecule in order that it may be eliminated by the body directly, or conjugated with another molecule, such as a sugar, and then eliminated from the body. Phase I reactions can include: oxidation, hydrolysis, reduction, and hydration and uncover functional

groups. The process whereby another molecule is conjugated with these functional groups produced by one of the Phase I reactions is termed Phase II. **Table 1.1.1** lists the phase I and phase II pathways of drug metabolism. [10].

Phase I	Phase II
Oxidation	Glucuronidation
Hydrolysis	Sulfation
Reduction	Acetylation
Hydration	Methylation
Dethioacetylation	Amino acid conjugation
Isomerisation	Fatty acid conjugation
	Glutathione conjugation

Table 1.1.1 Common phase I and phase II metabolites found in mammals.

Because drug metabolism and drug metabolites can cause a myriad of problems from lack of efficacy, and toxicity, to cases of inhibition and induction of p450 enzymes it is an important area of investigation for any new drug compound. Due to the potential issues drug metabolites can cause, the United States Food and Drug Agency (FDA) incorporated the Metabolites in Safety Testing or MIST guidelines in February of 2008. This document gives recommendations to the pharmaceutical industry on when the characterization of drug metabolites should be carried out. This document indicates that those metabolites that are detected in humans but not detected with the animal model

and those metabolites having a systematic exposure in humans of greater than 10 % of parent drug at steady state must be considered for additional safety testing [14].

1.1.3 Biomarkers

The determinations of biomarkers can aid in the indication of predisposed risk or the state of a disease. Biomarkers can further be utilized in determining toxicity or the PD of a drug and are therefore a useful tool in drug development [15]. Recently the US FDA has encouraged the incorporation of biomarkers in drug development and use in clinical diagnostics and has issued a guidance regarding the use of biomarkers [16]. One reason for this approach is the belief that this tactic will aid in the alleviation of stagnation of drug development and lead to innovation in the development of new drug compounds and personalized medicine [17]. Biomarkers can include molecules such as: mRNAs, proteins, metabolites, and electrolytes [18].

One method utilized in the discovery of biomarkers are shotgun or so called –omics approaches such as metabonomics. Metabonomics has been defined as “The Quantitative measurement of time-related multiparametric metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification” [19, 20]. Metabonomic studies are usually carried out by the analysis of mammalian biofluids such as urine, plasma, or bile. The analytical techniques often employed in metabonomic studies include NMR, GC/MS, and LC/MS [21-23]. The complex data produced from these analytical techniques is then interrogated by mathematical techniques or algorithms such as principal component analysis (PCA). It is here during this process that the algorithms during PCA analysis identify analytes that may be potential biomarkers.

1.2 High-Performance Liquid Chromatography

High-performance liquid chromatography is a form of column chromatography used in biochemistry and analytical chemistry in order to separate, identify, purify, and quantify compounds. It is utilized in many application areas including: clinical, pharmaceutical, environmental and agricultural [24-26]. It has been noted that approximately one million liquid chromatographic analyses are carried out daily [25]. It is one of the most powerful techniques in analytical chemistry due to the compatibility of the technique with a wide range of samples with varying chemical properties, polarities, and structures.

1.2.1 Origins of Chromatography

The general acceptance is that liquid chromatography was defined in the early 1900s by Mikhail S. Tswett and his work is considered the start of chromatographic analysis. His work focused on separating compounds extracted from plants using a solvent, in a column packed with particles. Tswett filled an open glass column with particles, poured his sample into the column and allowed it to pass into the particle bed. This was followed by a different solvent. As the sample passed through the column by gravity, different colour bands could be seen separating due to the fact that some components were moving faster than others. He related these different coloured bands to different compounds contained in the sample. The compounds that were more strongly attracted to the particles slowed down, while other compounds more strongly attracted to the solvent moved faster. Tswett coined the name chromatography from the Greek words

chroma, meaning colour, and *graph,* meaning writing [24, 27, 28]. Chromatography changed little after Tswett's work until the mid-20th century where Martin and Synge developed partition chromatography to separate chemicals with only slight differences in partition coefficients between two liquid solvents. After unsuccessful experiments with complex countercurrent extraction machines, Martin had the idea of using silica gel in columns to hold water stationary while an organic solvent flows through the column. The preparation of the silica gel was tedious so they instead utilized filter paper as the inert support. Martin and Synge demonstrated the potential of the methods by separating amino acids. In a series of publications beginning in 1941, they described increasingly powerful methods of separating amino acids and other organic chemicals. Further they described the concepts of the height equivalent to a theoretical plate (HETP) and band spreading in these early publications [29] .

Martin, in collaboration with Anthony T. James, went on to develop gas chromatography beginning in 1949 and published on the subject in 1952. During his lecture for the Nobel Prize in Chemistry (shared with Synge, for their earlier chromatography work) Martin announced the successful separation of a wide variety of natural compounds by gas chromatography [30, 31].

The major components of a modern high-performance liquid chromatography system are shown below in **Figure 1.2.1**. The system consists of (A) containers for mobile phase, (B) a pump to move solvent through the system, (C) an autosampler to inject sample onto the system, (D) column and temperature control device, (E) detector to measure analytes in the separation, (F) waste container to collect the used mobile phase and (G) a data collection system [25, 26].

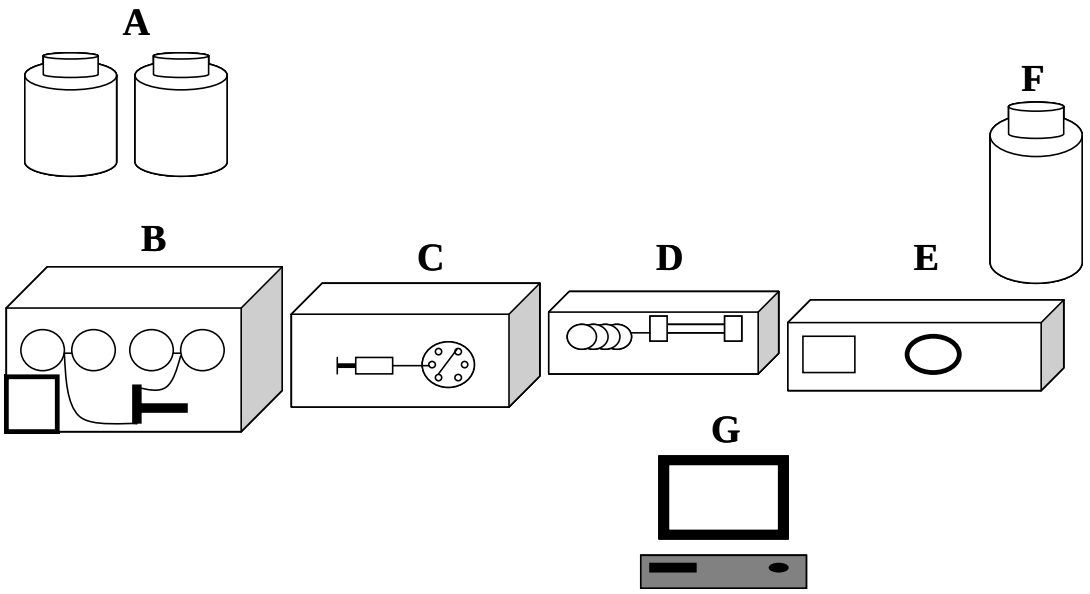

Figure 1.2.1 Components of a modern chromatography system.

The pump is the major part of the chromatography system. Its purpose is to deliver mobile phase through the chromatographic system in a precise manner. The pump is comprised of check valves, flow controllers, and transducers in order to ensure the delivery of mobile phase in a reproducible manner with minimal baseline noise. Modern pump assemblies also contain degassing features and a means to mix mobile phases for gradient type separations.

The autosampler provides a means to inject sample from the container in which the sample is stored, usually a glass vial or microtiter plate, onto the chromatographic system. The sample is injected onto the system utilizing a 6 port valve. The valve works in two positions: load, where the sample is introduced into the sample loop, and inject, where sample is placed into the flow path of the system and then onto the head of the

column. The autosampler is usually equipped with a means of temperature control so that thermally labile samples will not decompose during the course of an analytical run. It is imperative that the autosampler injects sample volumes reproducibly as inconsistencies will influence the accurate and precise measurement of analytes during quantification.

The column is the heart of the chromatographic system. The column provides a means for separation of analytes from one another. The selectivity, efficiency, capacity, and resolution of a separation can all be affected by the type and performance of the packing or stationary phase material contained within the column hardware. The packing material consists of particles in µm diameter dimension. The particles can be porous, non-porous or a combination or both, so called pellicular particles. Silica-based packings are the most common type, mainly because silica is easy to obtain and to derivatize. Chromatographic columns are usually kept in a column heater/cooler device that maintains the temperature of the column while separations are being carried out. Ensuring constant column temperature is important due to the fact that selectivity can be influenced by temperature [28]. If one is running a routine or validated analysis, the retention times of analytes must be reproducible if they are to meet established criteria.

Detectors are a key component, as the analytes that are separated by chromatography must be qualitatively and/or quantitatively measured in order to realize any meaningful results [32]. The detector produces an electrical signal that is proportional to the mass or concentration of a sample and is interpreted by the data handling system. Detectors are often categorized by the physical or chemical means by which they operate. Some detectors are very selective in their response while others tend to be more universal. There are many different types of detectors available that are readily coupled with high-performance liquid chromatography. They include: ultraviolet/visible absorbance

(UV/Vis), refractive index (RI), electrochemical (EC), evaporative light scattering (ELS), fluorescence, and mass spectrometry (MS). (7) The performance of a detector is based on sensitivity, noise and baseline stability. The most common type of detector used is UV-based whether tunable UV or photodiode array type [33].

1.2.3 Separation in a Chromatographic System

Separation of analytes or components in a chromatographic system is carried out on the column. The basic mechanism for separation is the difference in affinities an analyte has for the stationary phase or packing material and the mobile phase. The higher the degree of affinity an analyte has for the stationary phase the longer it will stay on the column. The higher the affinity an analyte has for mobile phase the faster it will elute from the column, too high an affinity for the mobile phase will result in no retention or separation.

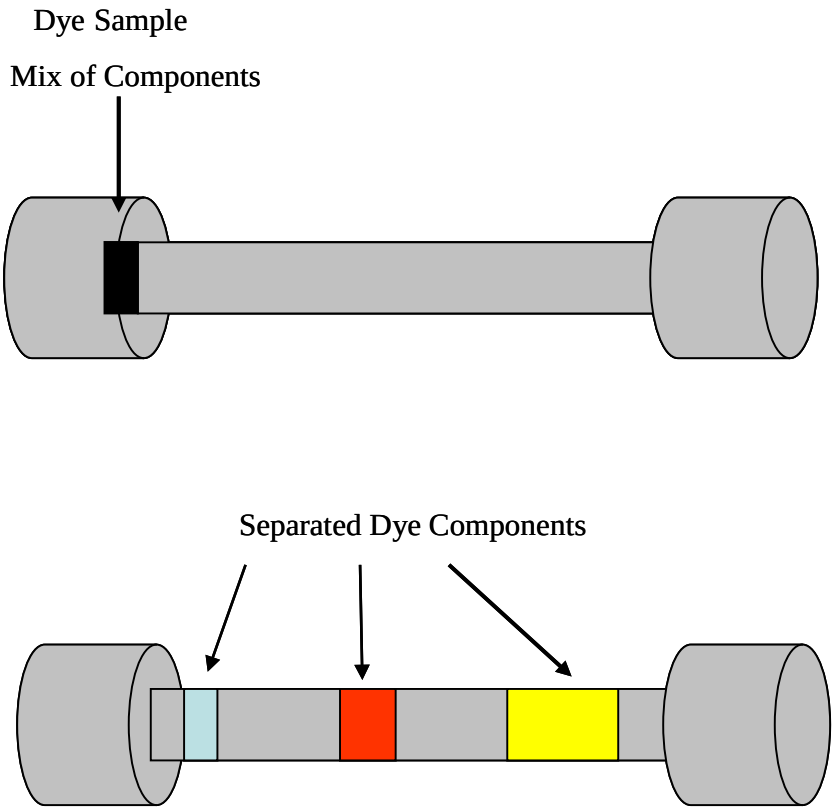

Figure 1.2.2 Separation of components on a chromatographic column.

To illustrate this, a dye is injected onto a chromatographic column, **Figure 1.2.2,** the dye being a mixture of individual components. Each of the individual components in the dye is different and therefore has varying chemical and structural properties that influence their affinity for the stationary and mobile phases. We see that the dye is separated into three discreet bands: blue, red, and yellow. The yellow band has the highest affinity of the dye components for the mobile phase, and therefore elutes from the chromatographic column first. This is in contrast to the blue band which has the highest affinity for the stationary phase and is the last of the dye components to elute

from the column. The red component exhibits properties that are in between the yellow and blue components.

What an analyst operating a modern chromatographic system observes during this process is shown in **Figure 1.2.3.** Here we see that as the individual dye components are eluted from the column there are measured by a detector. This data is collected by a computer and observed on a computer screen. Below we see that the yellow component eluted first from the column is seen on the left of the computer screen as a triangular or "peak" shape. Later eluting peaks will be seen to the right of the yellow peak as illustrated below. This process continues until all components have been eluted from the column.

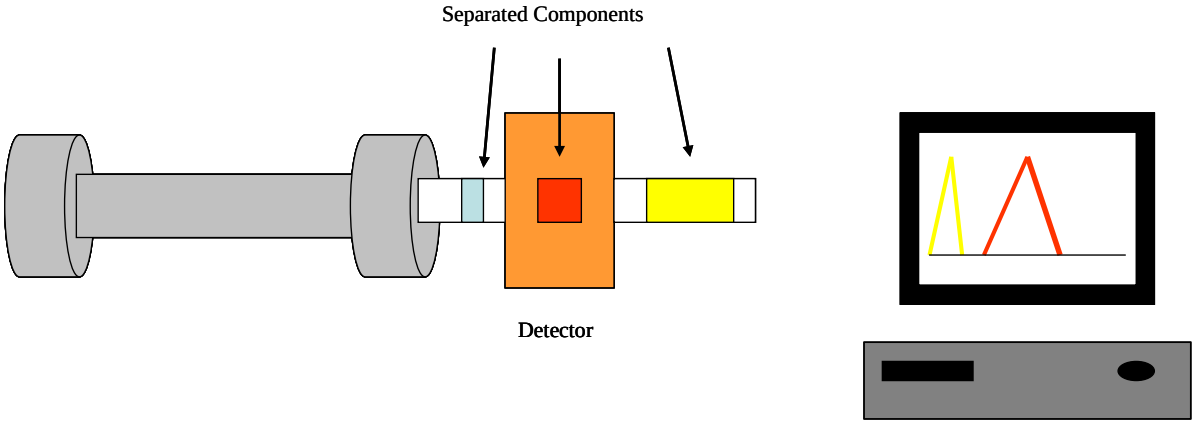

Figure 1.2.3 Detection and viewing of analytes separated by a chromatographic column.

1.2.4 Modes of Chromatography

A variety of chromatographic techniques exist. The key modes of chromatography are reversed-phase, normal-phase, ion-exchange, size-exclusion, and affinity with each mode of chromatography being categorized by a specific mechanism of retention and operation [25]. There are advantages and disadvantages to each of the different modes. Further, each mode is applied depending on the type of sample or analyte and the desired result one is seeking [26].

1.2.5 Reversed-phase Chromatography

Reversed-phase chromatography is the most popular chromatographic technique; in fact approximately 70 to 80% of all LC separations are carried out by reversed-phase [34]. This is due to the relative ease of use, compatibility with analytes, reproducible retention times, and fast equilibration [34]. The retention mechanism of reversed-phase is as follows: The surface of the stationary phase is non-polar. This hydrophobic surface interacts with the hydrophobic part of the analyte. The larger or stronger the hydrophobic interactions are of an analyte, the longer it will be retained by reversed-phase. Converse to this are analytes with polar functional groups, and these analytes will be less retained.

Stationary phases are typically long chain hydrocarbons, usually C_8 or C_{18} in length chemically attached to a base particle. The most popular reversed-phase columns are made from silica-based particles with different functional groups bonded to them. There are many different functional groups that can be bonded to the silica-based particle

providing different selectivities. The mobile phase is polar and usually a mixture of water or buffer with a miscible organic solvent such as methanol or acetonitrile.

The applications of reversed-phase LC are vast. As stated earlier a majority of small molecule separations are carried out by reversed-phase. Peptides and oligonucleotides are also readily separated by reversed-phase, as well as large biopolymers such as proteins. The advantage of reversed-phase chromatography is it is so widely applicable, as many compounds can be separated with few technical problems [34].

1.3 Mathematical Description of the Chromatographic Process

Over the last one hundred years many scientists have investigated the chromatographic process. Martin and Synge, van Deemter, Knox and Guiochon, [34, 35] and others developed numerous mathematical equations to describe and compare the chromatographic process and parameters. Due to the previously stated fact that 70-80 percent of all chromatographic separations are accomplished by reversed-phase, the following section will describe the mathematical measurements and theory behind reversed-phase separations. The values for capacity, retention, selectivity, resolution, linear velocity, efficiency and peak capacity are explained in the following section. The plate and rate theory will also be discussed. These measurements can become useful in developing a set of optimal conditions for the successful separation of a mixture of components.

1.3.1 Capacity factor

In order to achieve effective chromatographic separations, chromatographic columns must have the ability to retain and separate sample analytes. The capacity factor, k is a measure of the strength of interaction of the analyte with the column packing material. The capacity factor for a given separation is defined by the following equation: [25, 32, 35, 36].

$$k = \frac{t_R - t_0}{t_0} = \frac{V_R - V_0}{V_0}$$

Equation 1.3.1 Definition of capacity factor.

Whereby t_R is the time taken for the analyte to reach the detector and t_o is the time for unretained analytes to reach the detector. The same value for k can be determined by volume instead of time. V_R represent the volume of solution pumped for an analyte to reach the detector and V_0 is the volume of solvent pumped whereby unretained analytes reach the detector.

The capacity factor is mostly dependant on the type of packing material in the column [25]. However, the capacity factor can be influenced by the strength of the mobile phase. As a rule, a calculated capacity factor of between 2 and 5 represents an acceptable balance between the length in time of the analysis and the resolution of the analysis. A capacity factor that lies outside the 2 to 5 region may lead to insufficient resolution from unretained analytes in the void volume (V_0), if < 2, or an analysis time that is too long, > 5.

1.3.2 Selectivity

The selectivity of a chromatographic separation is the measure of the difference in retention time or volume for two given analytes in a separation. Selectivity is defined by the symbol α and is described in the following equation: [25, 32, 34-36]

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0} = \frac{V_2 - V_0}{V_1 - V_0} = \frac{k_2}{k_1}$$

Equation 1.3.2 Definition of selectivity.

The selectivity of a separation can be a function of the column packing material. It can also be influenced by the composition and pH of the mobile phase as well as the separation temperature. Selectivity values can range from values of as little as 1 to very large values. Values of 1 represent no difference in the selectivity between analytes in a separation and therefore no separation between analytes will occur. One of the easiest ways to affect the selectivity of a separation is to modify the mobile phase. This can carried out by changing the organic modifier, the pH or the aqueous modifier.

1.3.3 Resolution

The resolution in a chromatographic separation is utilized in order to define the degree of separation between analytes in the chromatogram. The three previously discussed concepts of capacity, selectivity, and efficiency all have a role in the resolution of a separation. This can be seen in the following formula: [25, 34-36]

$$Rs = \frac{1}{4} \quad \frac{\alpha - 1}{\alpha} \quad (N^{1/2}) \quad \frac{k}{1 + k}$$

Equation 1.3.3 Definition of resolution.

N (efficiency, described later in Chapter 1) is controlled by physical and mechanical factors while selectivity and capacity are influenced by chemical factors.

The resolution between two peaks in a separation can be measured by utilizing the following formula: (9)

$$Rs = \frac{\Delta t_R}{½ (w_{t,1} + w_{t,2})}$$

Equation1.3.4 Calculation of chromatographic resolution of two adjacent peaks.

Where Δt_R is the difference in the retention time between two adjacent peaks and w_{t1} and w_{t2} are the widths of the peaks. The width of the peak can be measured at different

points. Common peak widths utilized to calculate chromatographic resolution are at 4.4, 13.4 and 50 percent peak height.

1.3.4 Linear Velocity

The linear velocity of the mobile phase passing through the column is an important parameter to consider and is represented by the symbol μ. The linear velocity determines how long an analysis will take and also the backpressure on the chromatographic system. The linear velocity can also affect efficiency or plate count [34].

1.3.5 Efficiency, plate count

Efficiency or plate count is a measure of the peak broadening as a function of retention time. The origin of this measure of chromatographic separation is based on the distillation theory in gas chromatography. In this theory, a column of fixed length is occupied by a theoretical plate. This fixed length represented the height equivalent to a theoretical plate. The efficiency or plate count of a separation can therefore be determined by the formula: [25, 32, 34-36]

$$N = \frac{L}{H}$$

Equation 1.3.5 Definition of efficiency.

In this equation N represents the efficiency or plate count. L represents the length of a column and H is the height equivalent of a theoretical plate. This equation can further be manipulated so that it can be readily measured from a separation to: [34]

$$N = f \frac{t^2_R}{W^2_P}$$

Equation 1.3.6 Calculation of efficiency.

In this derivation of the efficiency formula t_R represents the retention time of the analyte and W_P represents the width of the peak at a certain height. The position where W_P is measured dictates the value for the factor f. For example a common W_P taken for the measurement of efficiency is at 13.4 % of the peak height. Here the value of the factor f is 16.00. It should be noted that the efficiency or plate count of a separation can only be applied to isocratic separations and not to gradient separations. Two major theories are often utilized to describe column efficiency. The simplest of these is the plate theory. The second is the rate theory developed by van Deemter and others which takes into consideration different causes of peak broadening that affect column efficiency [25, 35].

1.3.6 Peak Capacity

In isocratic mode peak capacity (P_c) describes the number of analytes that can theoretically be separated as peaks with a given resolution in a given analysis time.

$$P_C = 1 + \frac{\sqrt{N}}{4Rs} \ln (1 + k_{max})$$

Equation 1.3.7 Definition of peak capacity.

Peak capacity increases with column efficiency, but the major factor influencing peak capacity is the capacity factor of the last eluting peak. Therefore, any aspect of the

chromatographic system that might limit the value of k_{max} for the last peak will also limit the peak capacity [34].

When using a gradient elution, peak capacity can be calculated by the following equation where t_g = time for the gradient and w is the width of the chromatographic peak (12).

$$P_C = 1 + \frac{t_g}{w}$$

Equation 1.3.8 Calculation of peak capacity in gradient mode.

1.3.7 Plate Theory

The plate theory and expressions derived from the theory are generally applied to different types of column chromatography. In the plate theory it is assumed that the analyte passing through the column is in equilibrium with both the mobile and stationary phases [25]. However equilibrium of the analyte between the two phases never actually happens and furthermore the plate theory does not address the effects of band broadening on the chromatographic separation. The plate theory also does not consider particle size, loading, mobile phase viscosity, and flow rate. In the plate theory the chromatographic column is divided into a number of sections termed “plates”. The greater the number of plates or theoretical plates (N) the higher the column efficiency. The smaller or thinner each plate is, the more plates can be fit into a column. This is expressed mathematically as: [25, 34, 35]

$$H = \frac{L}{N}$$

Equation 1.3.9 Definition of HETP.

Where H is the height equivalent to a theoretical plate or HETP, L is the length of the column, and N is the column efficiency. Therefore from the equation we can see that a column with a very high N will have a small H. The plate theory was developed from distillation theory and applied to chromatography by Martin and Synge in 1941. The work they did established the plate concept as an approach for the mathematical explanation of the chromatographic process [35].

1.3.8 The Rate Theory

Because the plate theory does not take into consideration factors that can cause band broadening in a column, other theories have been developed to explain the chromatographic process. Some of the theories that have addressed the contribution of band broadening during the chromatographic process are: the Gidding equation, the Knox equation, and the van Deemter equation [35].

The most popular of the rate theories is the van Deemter. The most general form of the equation is written as: [34, 35, 37]

$$H = A + B/\mu + C\mu$$

Equation 1.3.10 Definition of van Deemter equation.

Where H represents the efficiency of the column and μ represent the linear velocity of the mobile phase. The A term is independent of the linear velocity and is related to the size and the distribution of the inter-particle channels within the packed bed of the column. The B term is inversely proportional to the linear velocity and describes diffusion in the axial direction. Last the C term, which is directly proportional to the linear velocity and describes mass transfer from the flowing mobile phase to the particle and back again. A typical van Deemter plot can be seen in **Figure 1.3.2**.

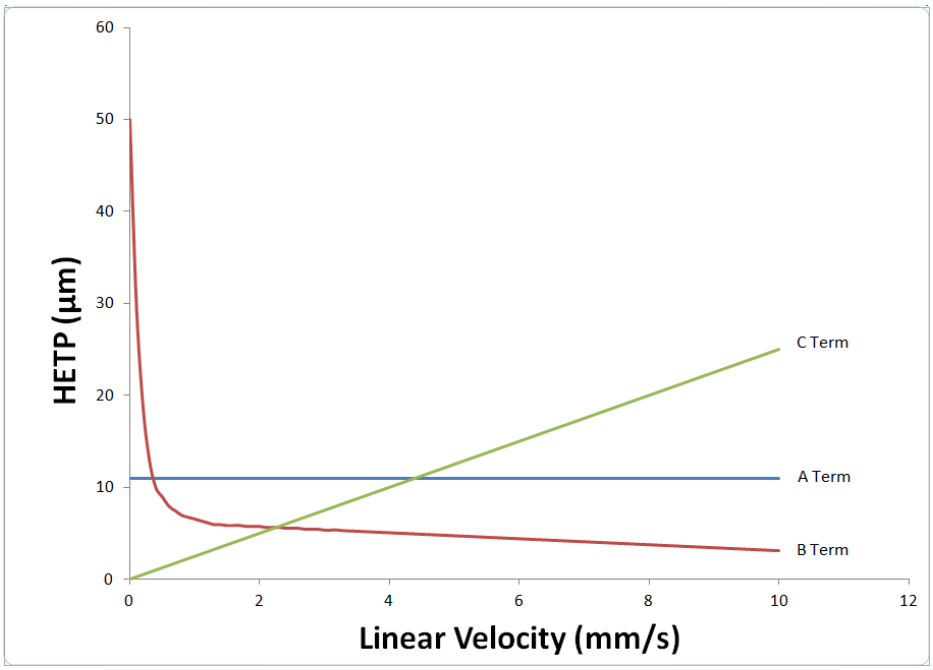

Figure 1.3.1 van Deemter Plot showing contribution of A, B, and C terms on HETP. Figure courtesy of Waters Corporation

The three terms or dispersion mechanisms of the van Deemter curve are affected in different ways by the flow rate of the mobile phase. To reduce dispersion due to longitudinal diffusion a high flow rate can be used, whereas a low flow rate is required to reduce dispersion from mass transfer. The van Deemter equation represented in a graphical format demonstrates the contribution to overall HETP by the three dispersion mechanisms. The point at which the total dispersion from the three effects produces minimum dispersion gives the optimum linear velocity and flow rate of the mobile phase. This represents the flow rates required if one is to achieve maximum benefit [34, 37].

1.3.9 Band Spreading

Two sources of band spreading exist; they are the contributions from the column and external contributions or extra column effects. Both of these will affect the N of a separation [38]. Contributions from the column include: end fittings, column volume, packed bed uniformity and particle size. Extra-column dispersion may arise from the volumes of the injection valve, the detector cell, the tubing between the injector and column, the tubing between column and detector, as well as the connecting fittings. These extra-column volumes should be kept to a minimum as they affect separation by causing band broadening and tailing. In addition, the diameter and total length of tubing between the injector and detector has been shown to be crucial to resolution with narrow diameter columns such as columns with less than 2.1mm i.d. and 3μm diameter particles [38].

1.3.10 Effect in the reduction of particle size on van Deemter equation

Reduction in particle size (d_p) is a means of increasing chromatographic performance. This can mathematically be illustrated in the van Deemter equation. The van Deemter equation can be rewritten in the following way to illustrate the effect of particle size on H.

$$H = A(d_p) + \frac{B}{\mu} + C(d_p)^2\mu$$

Equation 1.3.11 Modified van Deemter to illustrate effect of particle size.

In the above equation we can see that only the A and C term are affected by the particle size of the stationary phase in the chromatographic column. The A term is affected by particle size in the following way: large particles or irregular particles packed into a column will lead to a larger interstitial volume between the particles. The effect of this is a broader band or peak resulting in a less efficient column due to the large value of the A term. Smaller particles or spherical particles will pack within the column in a tighter configuration. This tighter packing of the particles reduces the interstitial volume between the particles and results in a narrow band or peak and a column with greater efficiency due to the smaller A term.

The C term, or mass transfer, is affected by particle size in the following way: Larger particles have a greater distance, through the pores, which analytes in the mobile

phase must travel in order to interact with the stationary phase. Smaller diameter particles have a shorter distance through the pores which analytes must travel in order to interact with the stationary phase. This shorter distance associated with smaller particles reduces the C term, which reduces band broadening resulting in sharper chromatographic peaks.

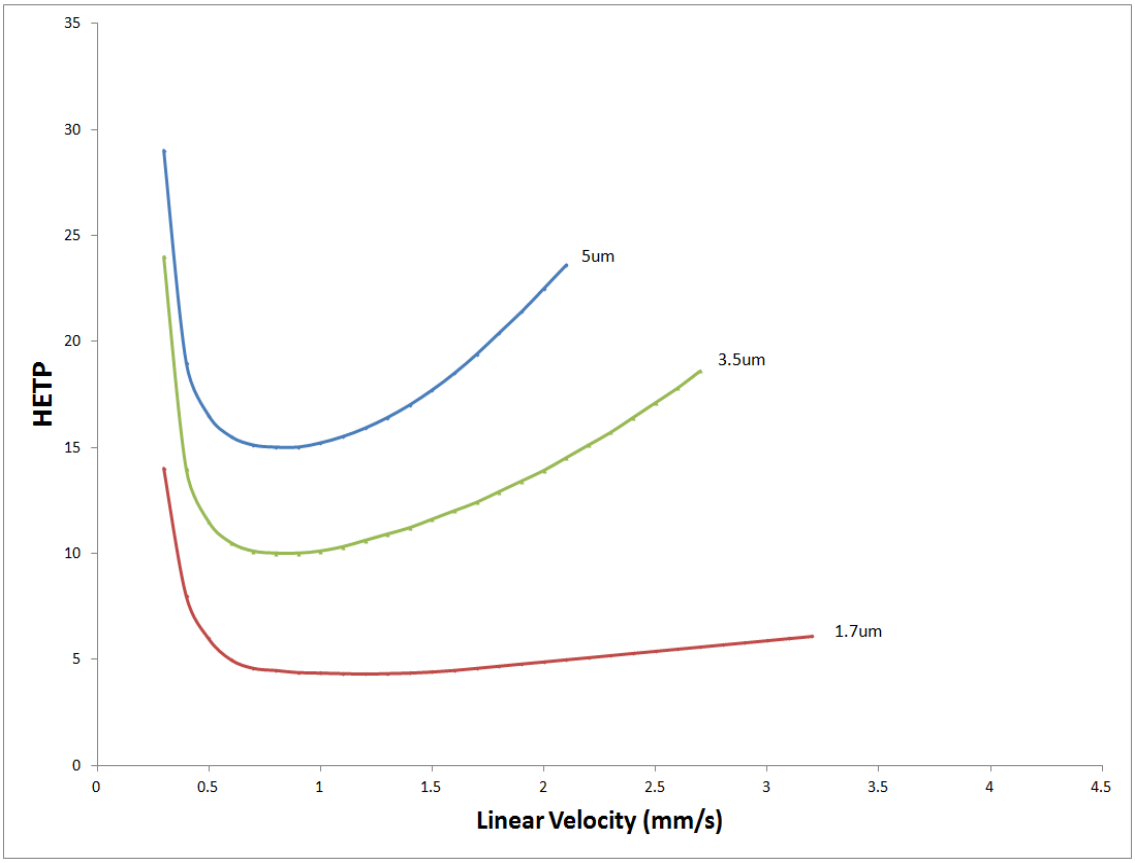

Figure 1.3.2 Effect of particle size on van Deemter curve**.** Figure courtesy of Waters Corporation.

Figure 1.3.2 illustrates the effect of reducing the chromatographic particle size on the van Deemter curve. The plot illustrates three main points. First, as you decrease the particle size H is reduced, increasing chromatographic efficiency. Second, as the particle size is decreased, the optimal linear velocity whereby H is at its lowest value increases. Third, the optimal flow rate where H is at its lowest value has a much wider operating range for small particles compared to larger particles. In practical terms the effect of smaller particles is: First, a higher flow rate must be utilized in order to gain the maximum benefit for the use of smaller particles. Second, we can shorten the analysis time by moving to smaller particles due to the wide operating range of optimal linear velocity. However, although reduction in particle size does have the effect of lowering H and therefore increasing chromatographic performance, it increases the pressure at which the chromatographic system must operate.

1.3.11 Effect of the Reduction in Particle Size on Back Pressure

System pressure increases inversely with the square of the chromatographic particle size, however, the relationship between particle size and back pressure is inversely proportional to the third power of the particle size at the optimal flow rate. [39]. The increase is system pressure as a function of particle size is shown in **Figure 1.3.3** Standard HPLC instrumentation has an upper back pressure limit of 6000 psi. Due to the greater pressure generated by the 1.7 µm columns, instrumentation must be capable of operating at these higher pressures. This must include the fittings, pumps, injector valve and seals. The instrumentation must also have minimal volume if the narrow chromatographic bands generated by the 1.7 µm column are to be maintained.

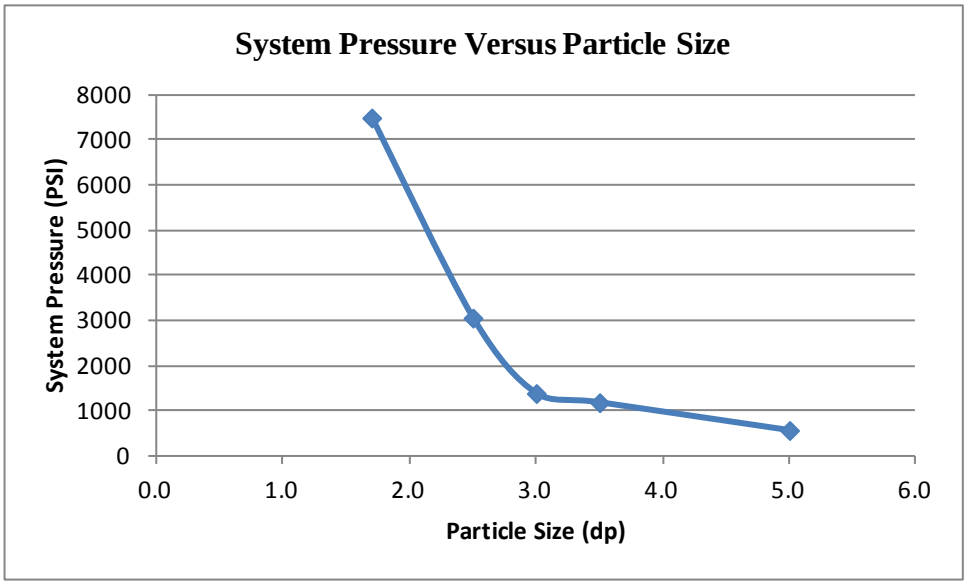

Figure 1.3.3 System pressure versus chromatographic particle size utilizing 2.1 x 50 mm columns and optimal linear velocities for each particle size. Conditions: column temperature = 40 °C, mobile phase = 50/50 H_2O/CH_3CN, LC system = Waters ACQUITY UPLC.

1.4 Mass Spectrometry

A Mass Spectrometer (MS) is an analytical instrument that measures the masses of individual molecules which have been converted into gas-phase ions. Ions are separated, detected and measured by their mass-to-charge ratio (m/z). The m/z ratio is by definition the mass of the ion (m) divided by the number of charges (z) that the ion carries. MS is an important analytical technique that can provide very selective and sensitive detection. The technique can provide information on the molecular weight, composition, and quantification of an analyte. Scientists from various disciplines within chemistry and biochemistry depend on mass spectrometric analysis. Pharmaceutical

industry scientists involved in the process of drug discovery and development rely on the specificity, dynamic range, and sensitivity of MS.

1.4.1 Origins of Mass Spectrometry

Eugen Goldstein in 1886 observed rays under low pressure travelled away from the anode and through channels in a perforated cathode, opposite to the direction of negatively charged cathode rays. Goldstein called these positively charged anode rays "Kanalstrahlen"; the standard translation of this term into English is "canal rays". Later Wilhelm Wien found that the influence of electric or magnetic fields deflected the canal rays and, in 1899, constructed a device whereby parallel electric and magnetic fields separated the positive rays according to their charge-to-mass ratio. Wien found that the charge-to-mass ratio depended on the nature of the gas in the discharge tube [40, 41]. The English scientist J.J. Thomson later improved on the work of Wien by reducing the pressure to create the first mass spectrograph, at the time referred to as a parabola spectrograph. The word spectrograph relates to the terms spectr-um and phot-ograph-ic plate [42]. The early devices that measured the m/z ratio of ions were referred to as mass spectrographs and recorded the mass spectra on photographic plates [43, 44]. Thomson is considered the founder of MS and he obtained, for the first time, mass spectra of many elements and compounds such as neon, oxygen, nitrogen, carbon dioxide and others. Later Thomson’s student Francis Aston made many accurate mass measurements with his velocity focusing instrument. He found the atomic weight of many elements and found that chlorine consisted of two isotopes, 35 and 37, which was

in contrast to the atomic weight of 35.4 which chlorine has previously been known by [45].

1.4.2 Principles of Mass Spectrometry

There are three basic steps in the analysis of molecules by MS. The first step is the production of gas phase ions from the sample molecules. This step in the process may involve the removal of electrons or protons, and is referred to as the ionization process. The second step is the separation of the ions by a mass analyzer based on the ions mass-to-charge ratio (m/z). Here at this step the trajectory or motion of charged ions through the mass analyzer are manipulated via the application of electric or magnetic fields in order to successfully separate, accumulate, or fragment dependant on their m/z ratio and direct or not direct the ions introduced into the mass analyzer to the detector. The third step is the detection step whereby the ions that reach the detector component are measured, the signal amplified, and a mass spectrum is displayed.

1.4.3 Components of a Mass Spectrometer

The mass spectrometer is comprised of six basic elements: First, a means to introduce sample into the MS. The second, is a source, whereby ionization of sample occurs. Third, the mass analyzer, which separates ions based on their mass-to-charge ratio. Fourth, is a detector, to count ions exiting the mass analyzer and to convert this data into

a useful output. Fifth, a computer system to process data. **Figure 1.4.1** below illustrates the basic components of a modern MS system. Last a vacuum system.

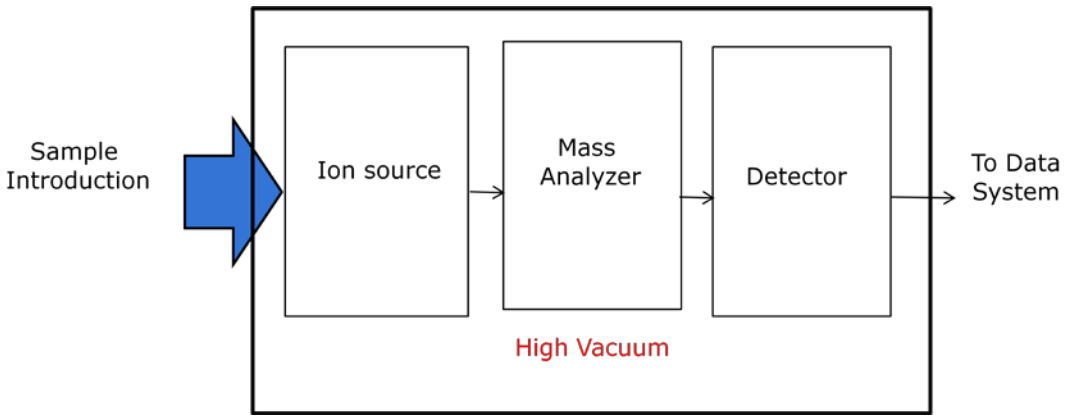

Figure 1.4.1 Components of a mass spectrometer.

There are many different methods in which a sample can be introduced into the MS. These methods can involve direct introduction by a probe, plate, a syringe, a liquid or gas chromatographic system. The sample then must undergo a process whereby the sample is converted into a gaseous state and ionized. There are numerous methods that can produce ions that are then separated in the mass analyzer. Ionization techniques include: electron impact, (EI) chemical ionization, (CI) fast ion bombardment, (FAB) matrix-assisted laser desorption ionization (MALDI), atmospheric pressure chemical ionization, (APCI), and electrospray ionization (ESI). Of the ionization techniques listed, ESI is most utilized for bioanalytical studies due to the ability of the technique to ionize analytes over a wide polarity range and the fact that the parent ion is often left intact during the ionization process. The mass analyzer is the heart of the MS system much in the same sense that the chromatographic column is the heart of the LC system.

Mass analyzers can include: quadrupoles, ion traps, time-of flight, ion cyclotron resonance, and magnetic sector types. Mass analyzers can also exist as a combination of mass analyzers such as a quadrupole time-of-flight or Q-ToF. The three main attributes of a mass analyzer are the ion transmission, the resolution, and the mass limit. The mass analyzer is under vacuum in order to ensure proper transmission of ions through the mass analyzer to the detector without interference from the atmosphere. The detector component of the MS provides a means to convert the signal from the MS into useful data that can then be utilized by the analyst. Detectors utilized by mass spectrometers can be micro-channel plates, electron multiplier, or photomultiplier. A data system is required in order to record, process, store and display the data in a form that can be interpreted by the user. A vacuum system is the last component of the MS system. It is utilized in order to remove atoms or molecules from specific components of the MS system. Pumping systems can require the ability to maintain a pressure of 10^{-4} to 10^{-8} Torr and are usually oil-based rotary and turbo-molecular pumping systems that are electrically driven.

1.4.4 Electrospray Ionization Mode

Electrospray ionization (ESI) is an ionization technique that is carried out at atmospheric pressure and therefore is referred to as an atmospheric pressure ionization (API) technique. It is the most successful interface for LC/MS applications [40]. The concept of electrospray was founded by Malcom Dole in 1968 [40]. However, the successful development of the technique for LC/MS is credited to John Fenn. The mechanism of ESI is a debated topic however it is generally believed to follow a process of droplet formation, droplet shrinkage, and gas phase ion formation [40, 41]. The gas

phase ions are then introduced into the mass analyzer component of the MS system for separation, fragmentation, or accumulation. **Figure 1.4.2** below illustrates this process.

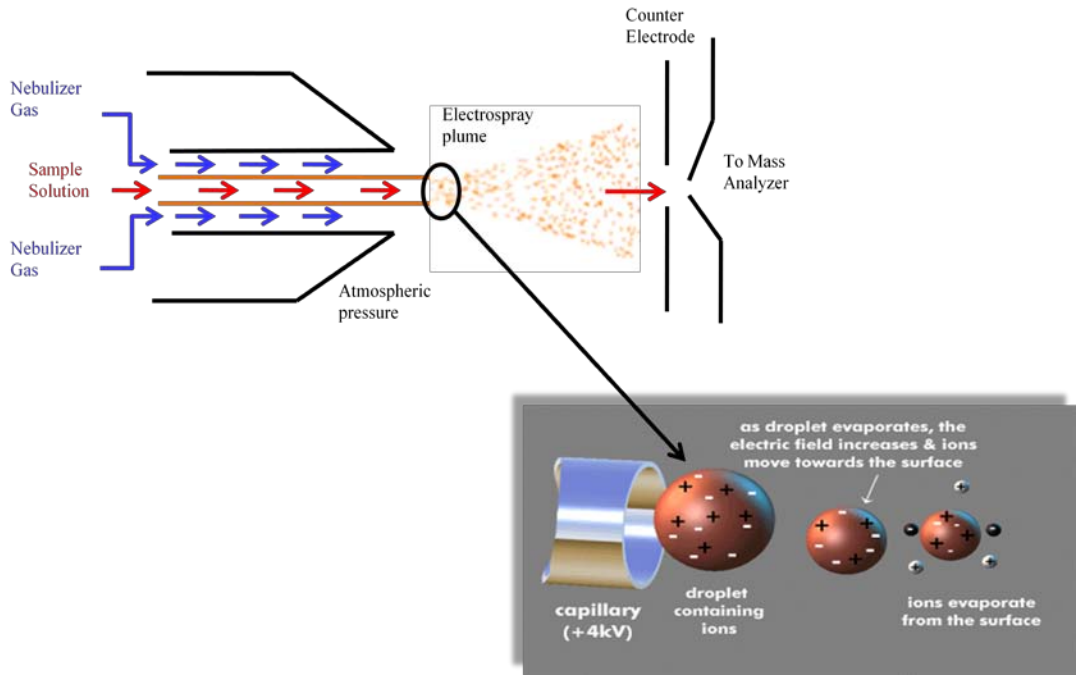

Figure 1.4.2 Electrospray ionization process. Figure courtesy of Waters Corporation

As we see in **Figure 1.4.2,** ions present in solution are introduced into the MS source via a steel capillary to which a voltage has been applied. The application of voltage and nebulizer gas produces a fine spray or plume. A counter electrode attracts the ions toward the sample cone and into the mass analyzer. Over the distance whereby the ions travel into the mass analyzer the droplets containing the ions are evaporated and undergo a process of fission. The end result of this process is a gas phase ion that can be introduced into the mass analyzer [40, 41]. ESI can be performed in either positive or negative modes and the chemical composition of the analyte will predict whether positive or negative mode is utilized in analysis. In general, compounds with basic

moieties in their composition will prefer electrospray positive ionization mode (ESI+) and compounds with acidic moieties in their composition will prefer electrospray negative ionization mode (ESI-).

ESI is referred to as a soft ionization technique. This is due to the fact that a molecule introduced into the MS source operating in ESI mode is often left intact as the protonated molecular ion in positive ionization or as the deprotonated anion in negative ionization mode. This is in contrast to other ionization techniques such as electron ionization where the molecule is often fragmented. The value of this intrinsic feature on this mode of ionization is that information of the molecular weight can be readily obtained and can be useful in properly identifying components and their structure. An example of this would be attempting to identify all of the associated metabolites of a new pharmaceutical compound. The ability to detect intact mass of the pharmaceutical or the conjugated pharmaceutical can lead to insight on how a pharmaceutical compound is metabolized, an important aspect in drug development.

1.4.5 Quadrupole and Time-of-flight Mass Analyzer Principles

Quadrupole and time-of-flight based mass analyzer MS instruments represent two very common types of MS instrument that are currently used by bioanalytical scientists in order to perform both quantification and qualitative analyses. This is based on the fact that the attributes of these two types of mass analyzer *i.e.* mass range, resolution, sensitivity, duty cycle, dynamic range, and speed are sufficient to perform LC/MS/MS analysis for bioanalysis.

The quadrupole mass analyser was developed in parallel with the quadrupole ion trap by Wolfgang Paul [46]. A quadrupole mass analyser consists of four parallel rods, with opposite rods connected electrically, (**Figure 1.4.3**) that have fixed DC and alternating RF potentials applied to them. Ions produced in the source of the instrument are then focused and passed along the middle of the quadrupoles. Their motion will depend on the electric fields so that only ions of a particular m/z will have a stable trajectory and thus pass to the detector. Specific combinations of the potentials and frequency will result in specific ions having a stable trajectory through the quadrupole to the detector. All other m/z values will have unstable trajectories and will hit the quadrupoles and not be detected (**Figure 1.4.3**).

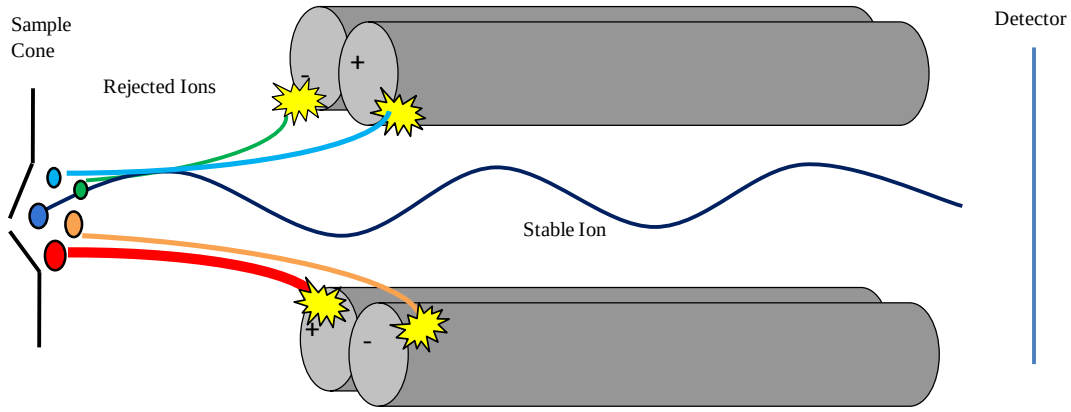

Figure 1.4.3 Separation of ions in a quadrupole mass analyzer. Figure courtesy of Waters Corporation.

Quadrupole mass spectrometers generally have two configurations in the modern laboratory, either single (Q) or tandem configurations (QQQ). The tandem configuration enables basic fragmentation studies to be carried out [47]. Both configurations are

commonly used in conjunction with LC for use as a simple high-throughput screening system.

Time-of-flight (TOF) MS was first described by Stephens in 1946 [48]. Later Wiley and McLaren produced the design of a linear TOF mass analyzer that became the first commercially available instrument [41] . TOF MS is a method in which an ion's m/z ratio is determined by measurement of time. Here ions are accelerated through the mass analyzer by an electric field of known strength. This acceleration results in an ion having the same kinetic energy as any other ions being separated at the same point in time [49]. The time taken for an ion to reach the detector at a known distance is measured and will depend on the m/z ratio of the ion. Therefore ions that have a larger m/z ratio will reach the detector after ions that have a smaller m/z value. This process is illustrated in **Figure 1.4.4** below.

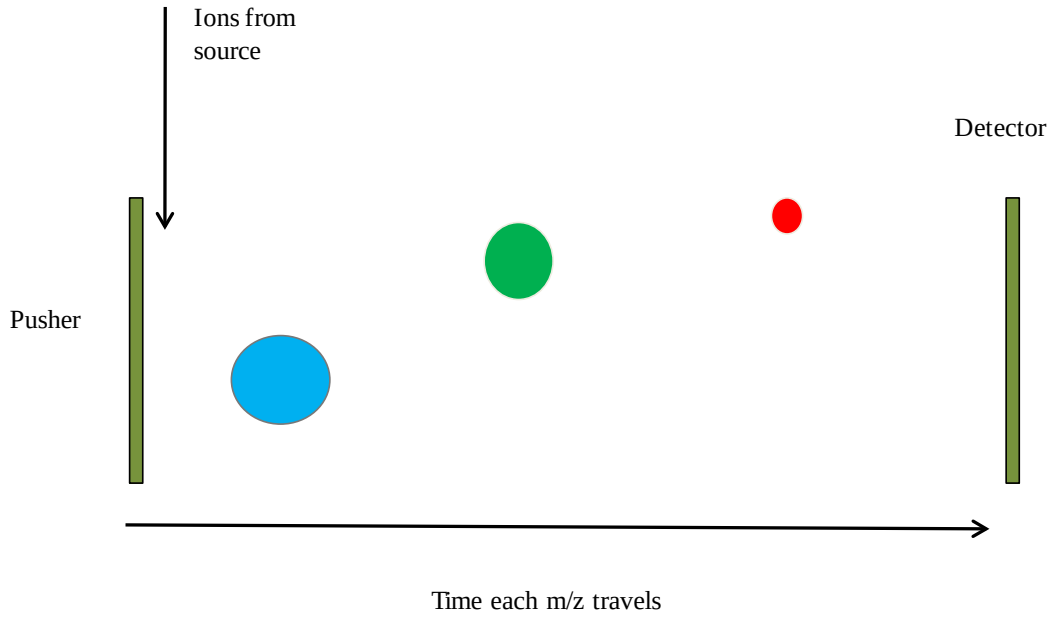

Figure 1.4.4 Separation in a time of flight mass analyzer.

Important attributes of this type of MS are the increased resolution of the instrument compared to the quadrupole MS previously described. **Figure 1.4.5** below shows a comparison of the resolution of a TOF MS compared with that of a quadrupole MS.

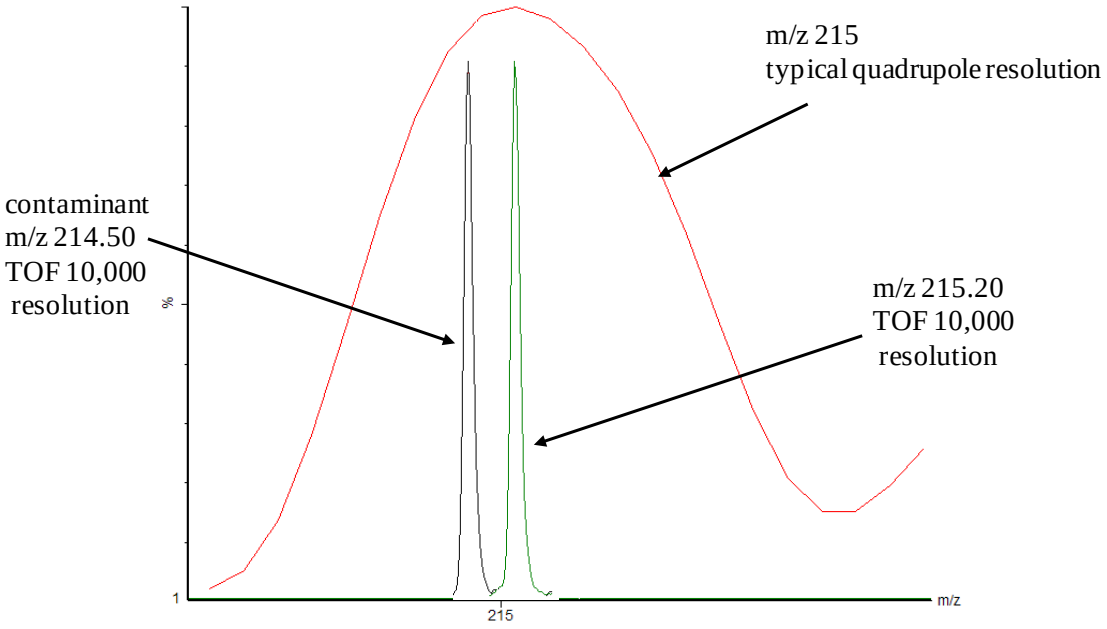

Figure 1.4.5 Resolution (FWHM) using a time of flight versus quadrupole mass analyzer. Figure courtesy of Waters Corporation.

The advantage of this increase in resolution is that it enables for the identification of analytes with much greater mass accuracy. This can be of great importance in DMPK work where on may be carrying out experiments in order to correctly identify and elucidate the structure of a drug metabolite or potential biomarker [50]. The resolution of a MS is calculated from Equation **1.4.1** [49]

$$R = \frac{M}{\Delta M}$$

Equation 1.4.1 Calculation of resolving power of a mass analyzer.

Where R is the resolution of a MS, M is the average m/z value for a detected ion, and delta M is the difference of two neighbouring m/z values measured at half height (FWHM). The larger the resolution of the MS indicates better separation of the spectral peaks and is similar to the convention used with chromatographic separations. An example of this is shown in Figure **1.4.5**. Here we observe the typical resolution of a quadrupole MS compared with that of a TOF MS of resolution 10,000. Typical resolution for a quadrupole MS is 1000, as is termed a unit mass resolution instrument due to the fact that it can resolve m/z of unit mass *i.e.* m/z 215 versus 216.

Along with resolution the mass accuracy of a mass spectrometer is a key parameter in the performance of the mass spectrometer and is of particular importance when performing structural elucidation of compounds. An example of this would be in the identification of drug metabolites. The mass accuracy is measured by dividing the mass error by the exact or calculated mass and multiplying by 10^6 and is expressed in parts per million or ppm. as shown in **Equation 1.4.2**.

mass error = exact mass-accurate mass

ppm = (mass error/exact mass) x 10^6

Equation 1.4.2 Calculation of the mass accuracy of a mass spectrometer.

1.5 Bioanalytical LC/MS and the drive for improvement

As previously stated, bioanalytical methods must effectively determine a drug's PK profile and any associated metabolites that would be produced once administered to a patient. Further the quantitative measurement of biomarkers can indicate the PD or toxicity related to a new drug compound is essential. The analytical technique of LC/MS is often utilized for this purpose. The primary reason for the use of LC/MS is due to the fact that all determinations of analytes in bioanalysis are carried out in the presence of biological matrix. All biological matrices including: blood, plasma, urine, bile, or cerebrospinal fluids (CSF) are complex mixtures of multiple analytes that all contain multiple chromophores. Because of this it is quite difficult to develop a very sensitive and selective methods whereby one can perform quantification of a target analyte by utilizing LC with UV detection.

Figure 1.5.1 illustrates this point. Here we observe the separation of a mix of pharmaceutical probe compounds spiked into rat plasma that has been precipitated with acetonitrile and analyzed by LC/UV and by LC/MS. In this example the UV detector was set at 254 nm and the MS was operating in selected reaction monitoring (SRM) mode. From this comparison of the two analytical techniques it is observed that the LC/MS separation provides a separation of greater selectivity and sensitivity. Such is

the sensitivity of current LC/MS/MS instrumentation that analyte levels of pg/mL can be determined regularly [51-53].

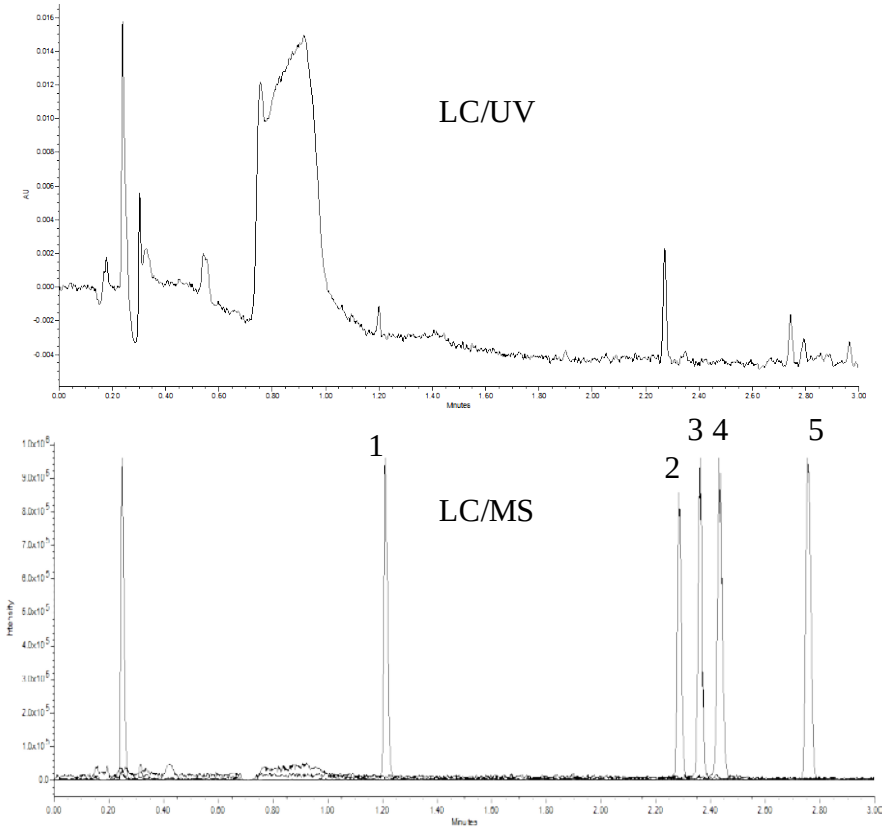

Figure 1.5.1 Separation of pharmaceutical compounds spiked into rat plasma by LC/UV and by LC/MS. Analytes separated are: lidocaine, alprazolam, warfarin, tolbutamide, clopidogrel (in order peaks 1-5).

The use of a tandem quadrupole mass spectrometer in combination with liquid chromatography is often the most utilized configuration for quantification methods [54-56]. However, the identification of drug metabolites and relevant biomarkers is frequently carried out by time-of-flight or trap type mass analyzers. The reason for this is the inherent higher mass resolution and accurate mass measure characteristics of these mass analyzers which enables for easier structural elucidation and determination of the chemical composition of compounds such as metabolites or potential biomarkers.

Despite the current sensitivity and ability of LC/MS/MS to extract relevant data from complex biological samples, there still exists the need for improvement of the technique. The reasons for this increased need in LC/MS sensitivity can be categorized into two main areas: i) low circulating drug levels resulting from low exposure drug compounds, such as inhaled medications, and ii) low volume of sample available, such as from serially bled mice or new sample formats such as dried blood spot samples (DBS) provides new sensitivity challenges that must be addressed [57-59].

The sensitivity of bioanalytical LC/MS/MS can be increased by utilizing a variety of different techniques. These techniques are listed in **Table 1.5.1**. There exist three areas whereby the sensitivity of a bioanalytical LC/MS method can be influenced: i) sample pre-treatment, ii) chromatographic parameters, and iii) mass spectrometric parameters.

Sample pre-treatment	Chromatography	Mass Spectrometry
Concentration	Increase injection volume	Acquisition mode
Protein-precipitation	Longer column length	Mass analyzer
Liquid-liquid extraction	Increase selectivity	Ionization
Lipid depletion devices	Sub 2 µm particles	Selected ion for detection
Solid liquid extraction	Pellicular particles	
Solid-phase extraction	Reduction of column i.d.	
	2 dimensional LC	

Table 1.5.1 Parameters of a Bioanalytical LC/MS method that can influence sensitivity.

The first part of a bioanalytical LC/MS method that can be addressed in order to increase the sensitivity of a method is sample pre-treatment. Sample pre-treatment methods fall into two main areas: sample concentration or sample separation. The first of these, sample concentration, is carried out by reduction of the sample volume by reduction or removal of the liquid component and can be accomplished by dry down or microelution SPE methods. This process results in an increase in analyte concentration. In the case whereby the whole of the liquid component of the sample is removed, reconstitution of the sample into a smaller than original volume, must be carried out prior to injection onto the LC/MS system.

Sample pre-treatment methods can also include methods where removal or purification of the target analyte from the endogenous biological sample components is a desirable option. Methods that utilize precipitation, liquid-liquid and solid phase extraction are often implemented for this purpose [60, 61]. There are two main reasons for implementation of a pre-treatment strategy. The first is to put the sample into a format that is ready to inject onto a LC system, The second is that removal of the target analyte from other biological endogenous materials present in the sample reduces the probability of a process referred to as ion suppression.[62, 63]. Ion suppression is part of a phenomenon referred to as matrix effects and is particular to MS detection. This

process occurs when analytes of interest co-elute with endogenous components from the biological sample or matrix and are introduced into the source of the MS simultaneously and can lead to erroneous results [62, 64, 65]. The importance of ion suppression in bioanalytical assays carried out by LC/MS/MS is well recognized by the US FDA who have included requirements for the calculation of matrix-derived ion suppression in assay development and validation.

In addition to sample pretreatment, different chromatographic method parameters can influence the sensitivity of LC/MS based analysis either by influencing chromatographic peak shape or resolution of target analytes from matrix components in order to reduce matrix effects. The first of these is the injection volume, one of the simplest methods to increase sensitivity of a LC/MS based assay is to inject more sample onto the LC system. This is of course predicated on the fact that there exists enough sample to accomplish this. Column length can also be considered, as an increase in the column length can provide for increased separation efficiency thereby resolving components and minimizing any chance of matrix effects. Selectivity changes produced by either mobile phase composition or column stationary phase can also be manipulated in order to increase the resolution of a separation. Furthermore, a change in chromatographic particle properties, such as utilizing a smaller particle, can produce sharper chromatographic peaks thus increasing resolution from matrix component present in biofluids [66, 67].

Reduction of column i.d. has been explored in the past as a means to increase sensitivity in bioanalytical LC/MS [68-70]. With this technique analytes can be eluted from a column in a smaller elution volume thus increasing the concentration introduced into the detector resulting in a greater signal being produced. A further advantage of this technique is the overall reduction in sample that has to be consumed for analysis. Along

with this is the reduction in overall solvent consumption compared to standard 2.1 mm i.d. columns utilized by most LC/MS methods.

Recently the use of 2-dimensional chromatography has been explored for use in increasing sensitivity in bioanalytical LC/MS [71]. This technique relies on the increase in resolution not offered by single column LC/MS therefore resulting in a reduction in the possible event of co-elution of the analyte of interest with endogenous matrix components resulting in matrix effects. Two main configurations that are utilized for this approach are the trap and elute and heart-cut configurations. In both of these cases increased resolution of the target analyte from the matrix components is accomplished prior to MS detection. The use of this technique also offers a means whereby the MS source is kept cleaner through the use of the required valves and diversion of flow from the MS to waste during the high organic column wash step usually incorporated into any bioanalytical method in order to wash the column clean of any highly hydrophobic compounds present in the sample that may be bound to the column during analysis.

Mass spectrometric detection parameters that can be manipulated for increased assay sensitivity are based on four areas: The type of acquisition mode, the mass analyzer, ionization of the analyte under investigation, and the selected m/z used for detection. The first of these, the acquisition mode, can be changed dependent on the type of mass analyzer being used during the analysis. For a tandem quadrupole MS, the acquisition modes that are typically available are full scan, product scan, precursor scan, neutral loss, selected ion monitoring (SIM), and selected reaction monitoring (SRM) sometimes, by several instrument manufacturers, referred to as multiple reaction monitoring (MRM). The most sensitive of these is SRM mode which is most commonly used for quantification studies due to the fact that it is the most sensitive. This mode is the most sensitive because it is the most selective of all the modes of acquisition

available. The type of mass analyzer can also influence sensitivity, and as stated earlier tandem quadrupole mass analyzers operating in SRM mode are very sensitive due to the selectivity of the instrument operating in this mode. TOF instruments unlike tandem quadrupole instruments cannot operate in SRM mode of acquisition simply due to the components that this type of mass analyzer is fabricated from. This instrument therefore is operated mainly in a full scan type of acquisition thus detecting all of the ions present in an analysis producing a very non-selective analysis. Therefore they are not as sensitive as the tandem quadrupole instruments when operating in SRM mode.

The ionization of the analyte under investigation can be controlled by the ionization technique used. There are many types of ionization techniques however for the purposes of bioanalysis, most are carried out using electrospray ionization, however, atmospheric pressure chemical ionization (APCI) is sometimes used [72, 73]. With electrospray ionization parameters such as capillary and cone voltages as well as desolvation temperatures must all be optimized in order to realize the best sensitivity that can be offered by the technique. In addition to these software controllable parameters, manipulation of ionization can be accomplished through modification of the mobile phase. Most often formic acid is added to the mobile phase and either acetonitrile or methanol used as the strong eluent during analysis with ESI in the positive mode LC/MS/MS [74-76].

The m/z that is being monitored during an analysis can also influence sensitivity. Often a different m/z can be monitored that may lead to increase in sensitivity. Typically when developing an SRM method multiple fragment ions of choice can be evaluated to assess which is the best to use. This approach will almost always be limited to SRM mode of acquisition and the fragment ions as most small molecule drugs only offer a single charge state of the parent.

In the work presented here, the use of conventional and non-conventional mobile phase additives and compositions was explored in conjunction with a prototype ceramic microfluidic device for use in bioanalytical studies. The prototype ceramic microfluidic device was packed with sub 2 µm bridged ethyl hybrid chromatographic particles and operated with pressures of up to 10,000 psi. This liquid chromatography approach was coupled to MS detection with either a tandem quadrupole operating in SRM mode or Q-TOF operating in full scan mode. Both instruments were operated in the electrospray positive ionization mode. The aims of this work was to investigate the effect of these techniques on chromatographic performance, assay sensitivity and ability to extract increased information gathered during the analysis of small molecules derived from or spiked into samples containing biological matrix.

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Chapter 2

Comprehensive Investigation of the Influence of Acidic, Basic, and Organic Mobile Phase Compositions on Bioanalytical Assay Sensitivity in Positive ESI Mode LC/MS/MS

This chapter is based on the following publication:

Comprehensive Investigation of the Influence of Acidic, Basic, and Organic Mobile Phase Compositions on Bioanalytical Assay Sensitivity in Positive ESI Mode LC/MS/MS

Paul D. Rainville, Norman W. Smith, David Cowan, and Robert S. Plumb

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Success comes to the man who so works that his efforts will bring the most and the best results-not to the man who simply works hard. - Agriculture for Beginners Burkette, Stevens, and Hill

2.1 Introduction

As described in Chapter 1 several LC/MS parameters can be adjusted in order to increase the MS response and signal-to-noise of a compound being analyzed. One approach that can be taken is to improve the ionization efficiency of the compound. Ionization efficiency can be increased by proper adjustment of desolvation temperature, desolvation gas flows, capillary and cone voltage settings on the MS. All of these parameters influence the ability of an analyte to exist in a charged gaseous state, required for introduction into the MS. Another approach to increasing the ionization of a compound is to remove or separate it from compounds that co-elute and interfere with the ionization process as previously mentioned in section 1.6.

The addition of formic or acetic acid to the LC mobile phase is commonly utilized to increase ionization with positive-ion ESI MS [1-4]. The fundamental rational for the acidification of the mobile phase is that most pharmaceuticals or analytes that are basic are analyzed in ESI positive mode because when exposed to an acidic environment they exist in a protonated state readily promoting the gas phase ionized state [5] see **Figure 2.1.1**. Here lidocaine, a basic pharmaceutical compound with a pKa value of approximately 7.9 is shown in a neutral state and its charged state when in an acid environment.

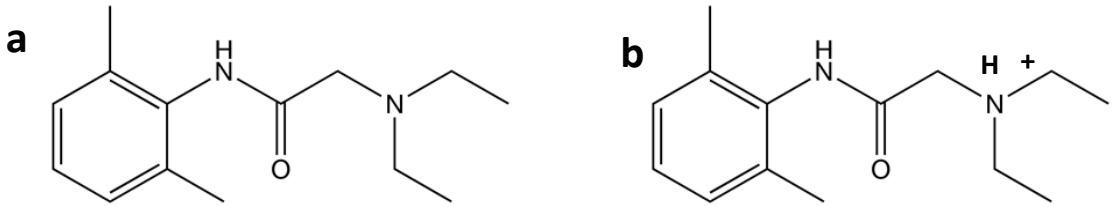


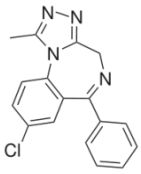
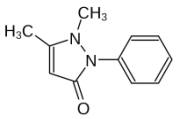
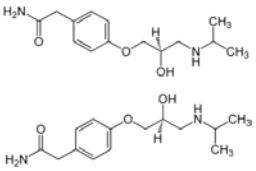
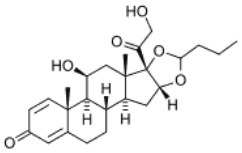
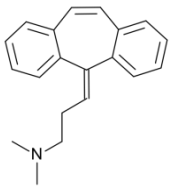
Figure 2.1.1 Lidocaine in neutral (a) and charged state (b).

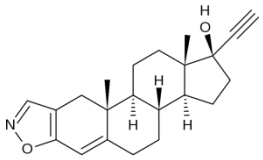
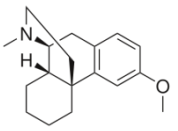
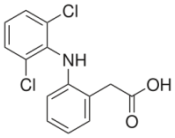
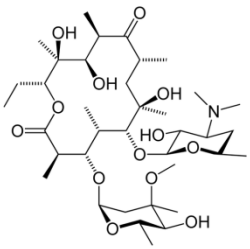
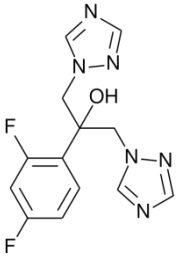
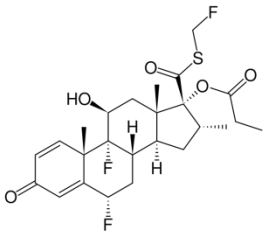
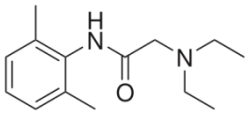
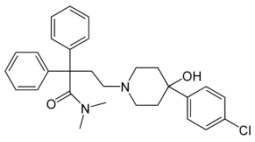
However the vast majority of pharmaceutical LC/MS analysis being performed with acidic based mobile phases, early work by Leclercq *et al.* in 2005 and that by Farkas *et al.* in 2008 had illustrated some benefit of using high pH mobile phases for the analysis of basic pharmaceutical compounds in simple solvent matrices under positive ionization ESI mode[6, 7]. These two investigations showed that it was possible to use basic instead of the widely utilized acidic mobile phases for ESI positive mode without detriment to the MS signal. Secondly, in many cases the MS signal for basic compounds was greater when analyzed under basic mobile phase versus acidic mobile phase compositions. Thirdly, these basic mobile phases produced some advantage to the improvement in serial UV detection and chromatographic peak shape.

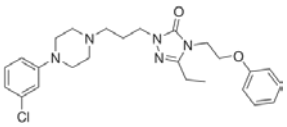
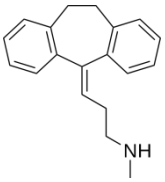
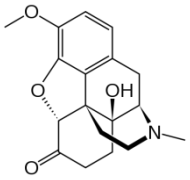
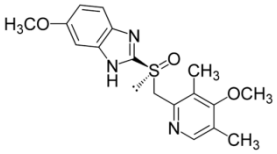
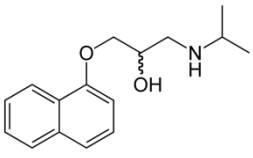
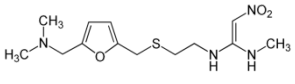
One of the major reasons for not considering the use of high pH mobile phases was the widespread usage of silica based LC columns which have an upper operating range of pH 7.5-8. At greater pH these types of LC columns begin to dissolve and collapse. The use of high pH mobile phases for LC/MS was enabled by developments in stationary phases such as hybrid organo-silica stationary phases which have an increased chemical stability to allow for operating ranges of up to pH 12 [8, 9]. One of

the main advantages of using high pH mobile phases is the decrease in chromatographic peak tailing associated with basic compounds that can occur when analyzed with acidic mobile phases. This peak tailing is normally associated with chemical interaction between the basic analyte and active groups on the surface of the silica stationary phase but can also be as a result of mass overload [10]. This decrease in chromatographic peak tailing therefore can lead to narrower, more symmetrical peak shape that can result in an increase in the signal-to-noise during analysis.

As a result of these pH related observations and need for improved performance and sensitivity of bioanalytical assays, the use of basic mobile phases for use in LC/MS based bioanalytical assays was investigated. The data presented in this chapter compares the MS response of twenty-four probe pharmaceutical compounds spiked into protein precipitated plasma and into solvent when analysed using pH 2.5 or pH 10.5 aqueous mobile phases. The probe pharmaceuticals chosen varied in pKa, molecular weight, hydrophobicity, and functional groups (**Table 2.1.1**). The signal-to-noise, peak area, peak width, and retention time were calculated for each probe compound. The influence of the organic eluents acetonitrile and methanol was evaluated with the acidic and basic aqueous mobile phase components, since these two organic solvents are the most commonly used. The effect of the acidic and basic mobile phase on the choline-containing lipid fraction, a major matrix component present in the protein-precipitated plasma samples, was also evaluated as it is a major source of interference in bioanalytical assays.

Number	Compound	Structure	Class
1	Alprazolam		• Benzodiazepines
2	Antipyrine		• Pyrazolones • Benzene and Derivatives • Anilines
3	Atenolol		• Phenols and Derivatives • Ethers • Phenethylamines • Anisoles
4	Budesonide		• Corticosteroids • Glucocorticoids
5	Cyclobenzaprine		• Dibenzocycloheptene

Number	Compound	Structure	Class
6	Danazol		• Steroids • Steroid Derivatives
7	Dextromethorphan		• Morphinans • Benzylisoquinolines
8	Diclofenac		• Aminobenzoates • Phenylacetates
9	Erythromycin		• Macrolides
10	Fluconazole		• Benzyl Alcohols and Derivatives • Cumenes and Derivatives • Phenethylamines
11	Fluticasone		• Steroids and Steroid Derivatives
12	Lidocaine		• Acetanilides
13	Loperamide		• Phenylpiperidines • Diphenylmethanes

Number	Compound	Structure	Class
14	Nefazodone		• Phenols and Derivatives • Ethers • Halobenzenes • Anisoles • Phenyl Esters • Anilines
15	Nortriptyline		• Dibenzocycloheptene
16	Oxycodone		• Morphinans • Benzylisoquinolines
17	Omeprazole		• Phenols and Derivatives • Benzimidazoles • Ethers • Anisoles
18	Propanolol		• Naphthalenes
19	Ranitidine		• Furans

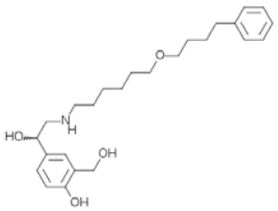
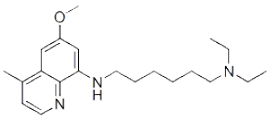
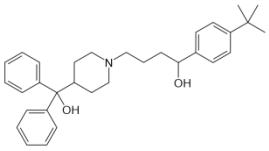
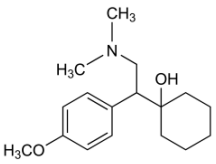
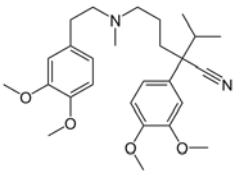
Number	Compound	Structure	Class
20	Salmeterol		• Benzyl Alcohols and Derivatives • Phenols and Derivatives • Phenethylamines
21	Sitamaquine		• Aminoquinolines
22	Terfenadine		• Diphenylmethanes
23	Veneflaxine		• Phenols and Derivatives • Ethers • Cumenes and Derivatives • Phenethylamines • Anisoles
24	Verapamil		• Catecholamines and Derivatives

Table 2.1.1 Probe pharmaceuticals studied.

2.2 Methods and Materials

2.2.1 Chemicals and Materials

Methanol and acetonitrile were obtained from Fisher Scientific (Waltham, MA USA). Formic acid, dimethyl sulfoxide and ammonium hydroxide were obtained from Sigma – Aldrich Chemicals (St Louis, MO, USA). Rat plasma was purchased from Equitech-Bio (Kerrville,TX, USA) and stored frozen at -20 ˚C prior to use. Purified water was generated in house using a Millipore water system. Authentic standards for acetaminophen, antipyrine, atenolol, budesonide, cyclobenzaprine, danazol, dextromethorphan, diclofenac, erythromycin, fluconazole, lidocaine, loperamide, nefazodone, nortriptyline, oxycodone, omeprazole, propranolol, ranitidine, sitamaquine terfenadine, venlafaxine, verapamil were purchased from (Sigma-Aldrich St. Louis, MO. USA). Sample standard for alprazolam was purchased from Cerilliant (Round Rock, TX, USA). Fluticasone propionate and salmeterol was obtained from Toronto Research Chemicals (North York ON, Canada).

2.2.2 Sample Preparation

The standard compounds were dissolved in methanol, with the exception of fluticasone, which was dissolved in dimethyl sulfoxide, to produce 1 mg/mL solutions. The resulting solutions were then diluted with aqueous methanol: water (5:95 v/v) to provide a series of spiking solutions. The standard solutions were then spiked into rat plasma or into aqueous methanol: water (5:95 v/v). The solvent composition was kept at less than five percent in the plasma samples. Samples were then protein-precipitated by the addition of 100 µL of acetonitrile to 50 µL of sample in a 1.5 mL Eppendorf tube.

Sample mixtures were then vortex mixed for one minute and centrifuged at 13,000 RCF for 5 minutes. The supernatant was then removed and diluted with water using 1:5 protein-precipitated sample: water in order to match the initial generic gradient conditions utilized in the study. Samples prepared in order to investigate the phospholipid component present in the rat plasma, were prepared utilizing the same previously described protein-precipitation method.

2.2.3 Liquid Chromatography

A 5 µL injection of each sample was injected onto an ACQUITY® Ultra Performance LC® separations module (Waters Corporation, MA, USA). Chromatography was performed on a 2.1 x 50 mm 1.7 µm ACQUITY BEH C_{18} column. Mobile phase A consisted of water with formic acid (0.1%v/v) or water with ammonium hydroxide (0.1 % v/v). Mobile phase B consisted of methanol or acetonitrile. The column was maintained at 45 ˚C and eluted under linear gradient conditions from 5 to 95 percent B over 2.5 minutes at a flow rate of 0.6 mL/min.

2.2.4 Mass Spectrometry

Mass spectrometry was performed on a Waters Xevo™ TQ mass spectrometer (Waters, Manchester, UK) equipped with an electrospray interface. Settings were as follows: source temperature = 150 ˚C, desolvation temperature = 600 ˚C, desolvation gas flow rate = 1000 L/h, and cone gas flow = 40 L/h. Cone and collision energies were optimized for each compound. Argon was utilized as the collision gas. The mass spectrometer was operated in SRM, SRM with precursor ion scan or SRM with full scan MS mode. **Table 2.2.1** lists the transitions and conditions utilized for each of the compounds analyzed in this assay

Compound Name	Cone (V)	Collision Energy (eV)	SRM (m/z)
Alprazolam	40	25	309.2 > 281.0
Antipyrine	36	26	189.3 > 106.3
Atenolol	30	24	267.2 > 144.9
Budesonide	16	12	431.3 > 323.1
Cyclobenzaprine	28	38	276.2 > 215.2
Danazol	30	24	338.3 > 148.0
Dextromethorphan	40	38	272.3 > 171.0
Diclofenac	16	32	296.1 > 214.1
Erythromycin	28	32	716.8 > 158.1
Fluconazole	28	16	307.2 > 220.0
Fluticasone	18	20	501.2 > 293.3
Lidocaine	22	26	235.2 > 86.1
Loperamide	28	28	477.3 > 266.1
Nefazodone	48	34	470.3 > 246.1
Nortriptyline	24	14	262.2 > 233.0
Oxycodone	28	30	316.2 > 241.1
Omeprazole	16	10	345.9 > 198.1
Propranolol	25	20	260.1 > 183.1
Ranitidine	22	24	315.2 > 130.1
Salmeterol	28	24	416.5 > 232.2
Sitamaquine	30	21	344.3 > 271.2
Terfenadine	22	26	472.4 > 436.3
Venlafaxine	8	28	278.3 > 121.1
Verapamil	38	30	455.3 > 165.0

Table 2.2.1 SRM transitions and MS conditions utilized for each compound.

2.3 Results and Discussion

2.3.1 Comparison of acidic and basic mobile phase pH on probe pharmaceuticals

The benefits of high pH mobile phases in reversed-phase chromatography are known for compounds containing basic moieties. Analyzing these compounds at a pH level above their pKa can allow the compounds to be chromatographed as neutrals. This results in longer retention times as well as more symmetrical peak shapes. As previously stated, Farkas and Peng were the first to report a thorough investigation of the use of high pH for the analysis of over 45 basic drug substances in 2008.

The data presented here highlights the benefits of high pH mobile phase for the LC/MS/MS analysis of pharmaceutical compounds that ionize under electrospray positive ion mode. These compounds tested were selected due to their variation in chemical structure, polarity and lipophilicity. Six consecutive, replicate injections were carried out for each compound under the acidic and basic mobile phase conditions. The effect of the mobile phase pH in combination with organic modifier on endogenous molecules, such as phospholipids, present in biological matrix was also investigated as well as the contribution of each of the mobile phase component to the overall background noise detected by the mass spectrometer.

The effect of utilizing a high pH mobile phase is shown in **Figure 2.3.1.** The data displayed here shows the effect of mobile phase pH on the LC/MS/MS analysis of omeprazole spiked into 95/5 (H_2O/CH_3OH). The data from **Figure 2.3.1** shows roughly a two-fold increase in response when omeprazole was analyzed with the basic mobile phase compared to the traditional acidic mobile phase. This experiment was then repeated for the same analyte, omeprazole, spiked into rat plasma that was prepared by

protein precipitation. From **Figure 2.3.2** we observe again the same approximate two-fold increase in response that was observed when omeprazole was spiked into solvent. These results indicate that the endogenous matrix components present in the protein-precipitated rat plasma are not contributing to the increased response observed under the basic mobile phase conditions *i.e.* matrix effects are not the cause for the increase in MS response.

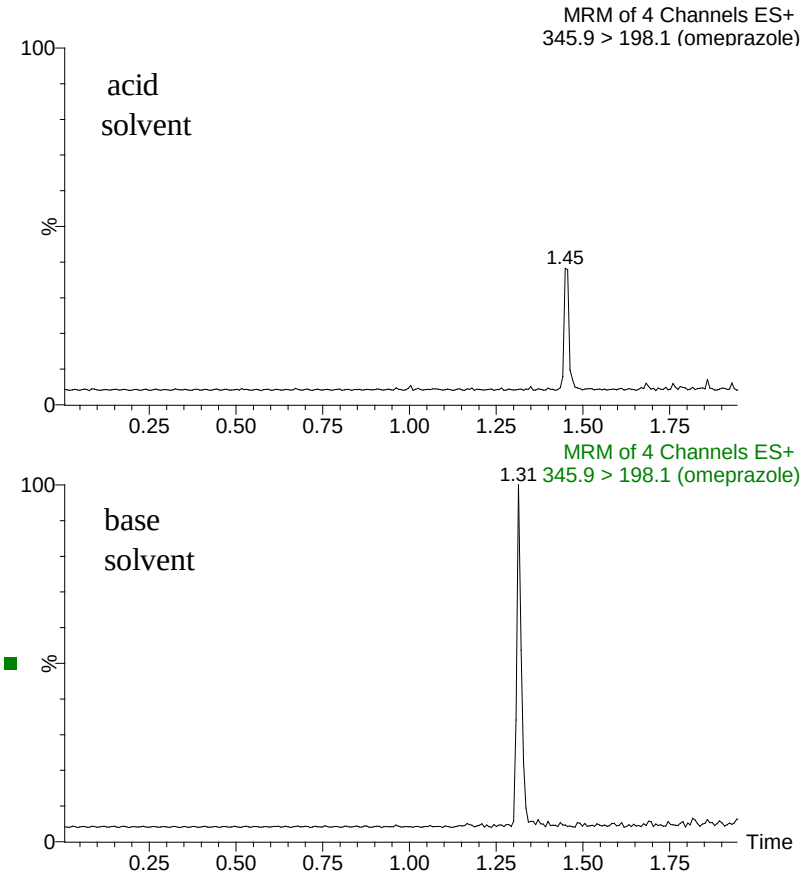


Figure 2.3.1 Analysis of omeprazole spiked into 95/5 (H_2O/CH_3OH) by LC/MS under ESI+ ionization mode with either acidic or basic modified mobile phase. The chromatograms are scaled to the largest response. Chromatographic conditions: flow rate = 0.6 mL/min, gradient elution = 5-95 B/2.5 minutes, column temperature = 40 °C, injection volume = 5 µL, column = Waters ACQUITY BEH 1.7 µm C_{18}.

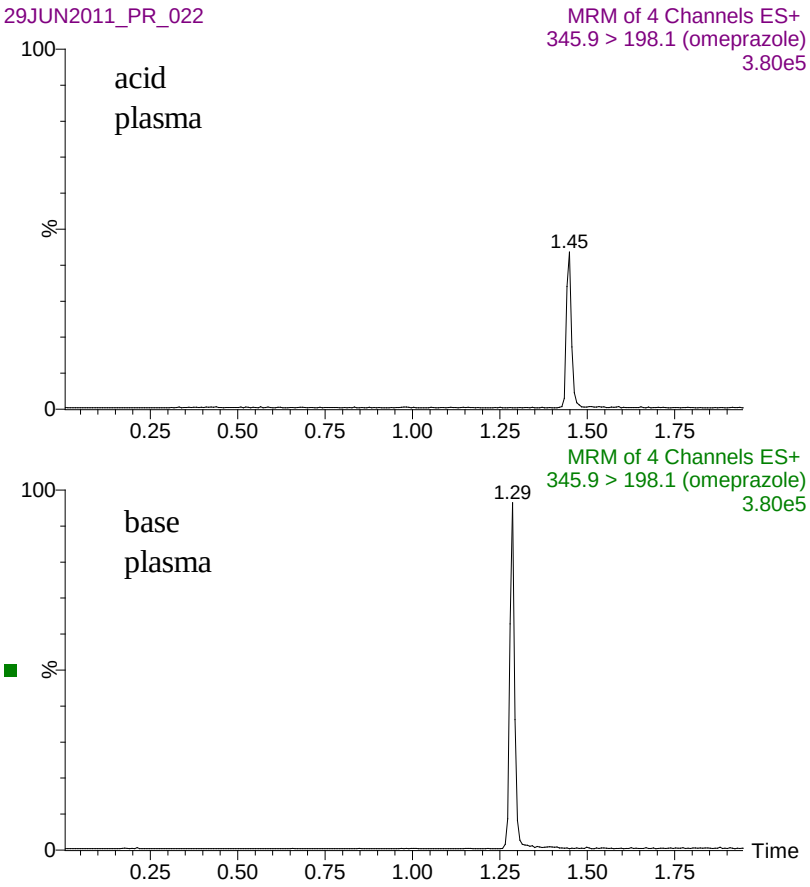


Figure 2.3.2 Analysis of omeprazole spiked into rat plasma by LC/MS under ESI+ ionization mode with either acidic or basic modified mobile phase. The chromatograms are scaled to the largest response.

Previously reported studies have suggested that the increase in response of compounds analyzed under basic mobile phase conditions may be due to an increase in retention time and therefore elution in a higher concentration of organic modifier [7]. However in this example, the retention time for omeprazole was decreased by 0.16 minutes when analyzed with the basic mobile phase and the response was greater than when analyzed with traditional acidic conditions. This result suggests that the increase in analyte response may not be due to a later elution time and higher organic solvent composition of the peak. A similar result was also observed for diclofenac analyzed under the acidic and basic conditions as again the higher pH mobile phase produced a reduced retention

time and increased MS response compared to the acidic mobile phase analysis where diclofenac was retained on the column for a longer time and yet produced a much smaller MS response compared with the basic analysis.

An increase in MS response was also observed even when the retention time did not change with the change in mobile phase pH. This is illustrated in **Figure 2.3.3**. Here fluticasone propionate was analyzed in the same manner as the previous omeprazole example.

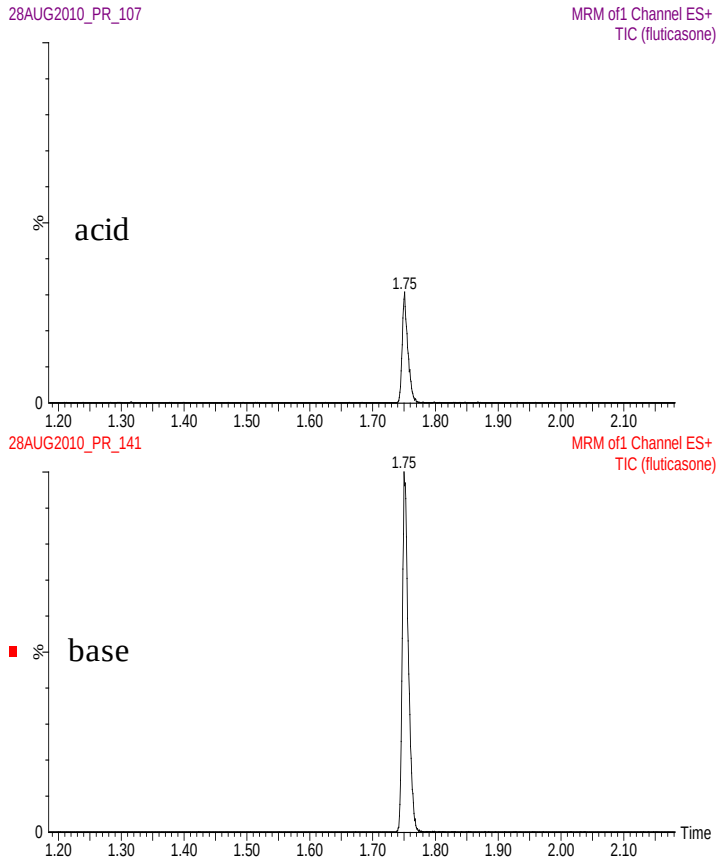


Figure 2.3.3 Analysis of fluticasone propionate under acidic and basic mobile phase conditions. The chromatograms are scaled to the largest response.

However, in this case, the retention time for fluticasone propionate was identical under both acidic and basic modified mobile phase conditions. Thus the organic solvent composition of the analyte peak entering the mass spectrometer is identical. This result was observed for five of the twenty-four compounds, (approximately twenty percent) tested during this study. These compounds were: alprazolam, antipyrine, danazol, fluconazole, and fluticasone propionate. This data again indicates that the increase in MS response with the basic mobile phase may not be due to chromatographic peak elution in a higher organic solvent composition.

A significant increase in analyte retention times was observed for the more polar compounds when analyzed with the basic versus acidic mobile phase. An example of this retention time shift is illustrated with the analysis of the H2-receptor antagonist, ranitidine, as shown in Figure **2.3.4**.

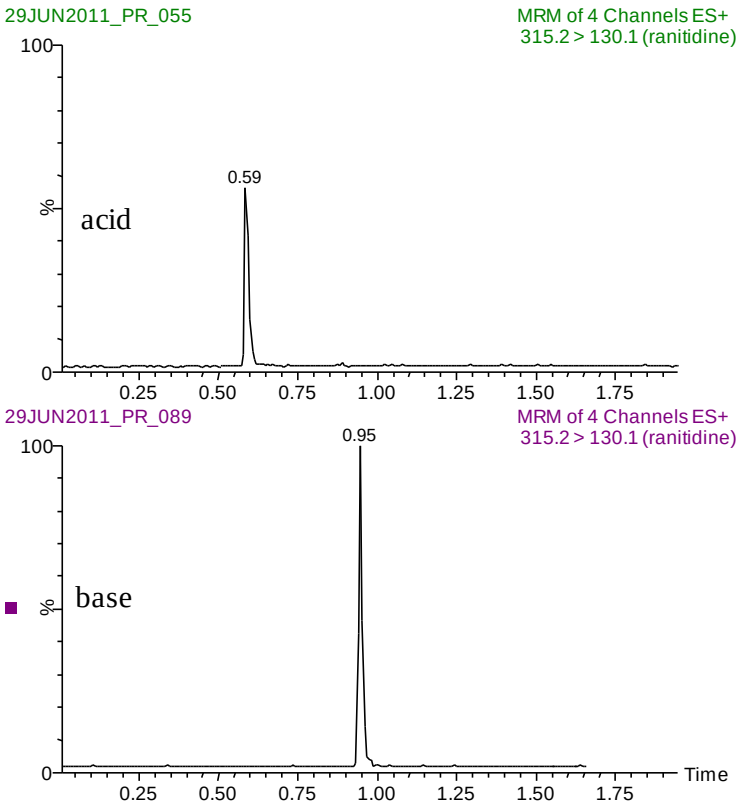


Figure 2.3.4 Analysis of ranitidine under acidic and basic mobile phase conditions.

In this example, a retention time increase of 0.31 minutes was observed, producing an increase in the gradient retention factor from 1.0 to 2.2. This observed increase in retention time under basic conditions for polar compounds has significant benefit in bioanalytical assays. By increasing analyte retention there is greater opportunity to separate the analyte from matrix interferences eluting near the void volume of the column and improve assay robustness. These matrix interferences could potentially lead to the incorrect measurement of the drug in a biofluid sample, leading to erroneous results due to ion suppression as discussed in Chapter 1. Table 2.3.1 list the gradient retention factor (k_g) for all of the twenty four probe pharmaceutical tested in this study. k_g was calculated in the same manner as capacity factor.

Compound	n = 6 (minutes)	ACID/CH_3CN	ACID/CH_3OH	BASE/CH_3CN	BASE/CH_3OH
Alprazolam	AVG	3.92	5.50	3.96	5.54
	ST DEV	0.00	0.00	0.00	0.00
	%RSD	0.00	0.00	0.00	0.00
Antipyrine	AVG	2.13	3.31	2.06	3.35
	ST DEV	0.02	0.02	0.03	0.04
	%RSD	0.93	0.47	1.56	1.26
Atenolol	AVG	1.08	1.72	2.15	4.01
	ST DEV	0.02	0.02	0.00	0.06
	%RSD	1.80	1.16	0.00	1.44
Budesonide	AVG	4.76	6.98	4.74	6.99
	ST DEV	0.02	0.02	0.02	0.02
	%RSD	0.42	0.30	0.42	0.28
Cyclobenzaprine	AVG	4.20	7.05		
	ST DEV	0.04	0.05		
	%RSD	0.90	0.66		
Danazol	AVG	5.77	7.04	5.77	7.05
	ST DEV	0.00	0.00	0.02	0.00
	%RSD	0.00	0.00	0.28	0.00

Compound	n = 6 (minutes)	ACID/CH_3CN	ACID/CH_3OH	BASE/CH_3CN	BASE/CH_3OH
Dextromethorphan	AVG	4.08	5.53	6.39	7.49
	ST DEV	0.04	0.02	0.02	0.02
	%RSD	1.10	0.36	0.25	0.21
Diclofenac	AVG	5.22	8.94	2.88	6.17
	ST DEV	0.03	0.08	0.02	0.02
	%RSD	0.55	0.89	0.55	0.32
Erythromycin	AVG	4.87	6.54	5.69	7.06
	ST DEV	0.03	0.02	0.00	0.02
	%RSD	0.66	0.37	0.00	0.28
Fluconazole	AVG	2.19	3.46	2.20	3.46
	ST DEV	0.00	0.02	0.02	0.00
	%RSD	0.00	0.45	0.71	0.00
Fluticasone	AVG	5.74	6.63	5.74	6.63
	ST DEV	0.02	0.02	0.02	0.02
	%RSD	0.35	0.30	0.35	0.30
Lidocaine	AVG	2.50	3.35	5.17	6.31
	ST DEV	0.00	0.02	0.02	0.00
	%RSD	0.00	0.47	0.38	0.00
Loperamide	AVG	5.06	5.77	5.94	7.08
	ST DEV	0.02	0.00	0.02	0.00
	%RSD	0.39	0.00	0.33	0.00
Nefazodone	AVG	5.53	6.85	6.08	7.62
	ST DEV	0.03	0.02	0.00	0.00
	%RSD	0.52	0.23	0.00	0.00
Nortriptyline	AVG	4.09	5.67	6.58	7.29
	ST DEV	0.02	0.02	0.00	0.02
	%RSD	0.51	0.35	0.00	0.29
Oxycodone	AVG	2.04	2.39	4.27	5.33
	ST DEV	0.00	0.02	0.00	0.05
	%RSD	0.00	0.66	0.00	0.88
Omeprazole	AVG	2.92	4.62	2.15	4.04
	ST DEV	0.00	0.02	0.00	0.00
	%RSD	0.00	0.34	0.00	0.00

Compound	n = 6 (minutes)	ACID/CH_3CN	ACID/CH_3OH	BASE/CH_3CN	BASE/CH_3OH
Propranolol	AVG	3.60	5.44	5.08	6.83
	ST DEV	0.02	0.02	0.00	0.02
	%RSD	0.55	0.39	0.00	0.29
Ranitadine	AVG	1.09	1.50	2.26	3.42
	ST DEV	0.02	0.00	0.02	0.00
	%RSD	1.82	0.00	0.69	0.00
Salmeterol	AVG	5.23	7.01	5.35	7.19
	ST DEV	0.03	0.03	0.00	0.00
	%RSD	0.66	0.41	0.00	0.00
Sitamaquine	AVG	5.22	6.42	6.02	8.35
	ST DEV	0.06	0.03	0.11	0.00
	%RSD	1.08	0.45	1.75	0.00
Terfenadine	AVG	5.52	8.38	7.71	8.12
	ST DEV	0.06	0.10	0.02	0.00
	%RSD	1.06	1.20	0.27	0.00
Venlafaxine	AVG	3.60	4.85	5.65	6.73
	ST DEV	0.02	0.02	0.00	0.00
	%RSD	0.55	0.32	0.00	0.00
Verapamil	AVG	3.89	4.90	6.05	6.81
	ST DEV	0.02	0.02	0.02	0.00
	%RSD	0.40	0.41	0.33	0.00

Table 2.3.1 Probe pharmaceutical gradient retention factors analyzed under acidic and basic mobile phase pH with either CH_3CN or CH_3OH as the organic modifier.

2.3.2 Effect on Peak Area

The chromatographic peak area is a measure of the sensitivity or response of the LC/MS system. The data displayed in **Figure 2.3.5** shows the average peak area values

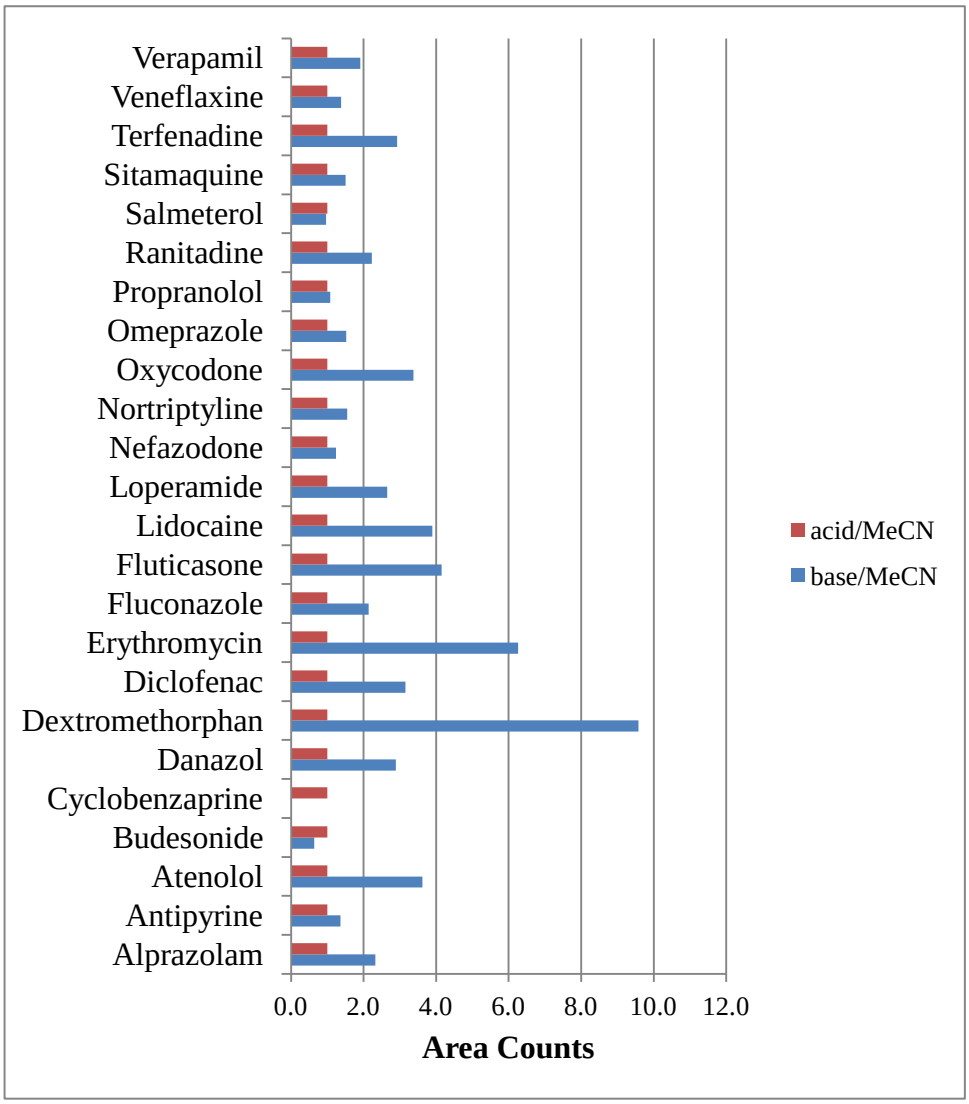


Figure 2.3.5 Bar graph illustrating the effect of mobile phase pH with CH_3CN as the organic modifier on the area count for the probe pharmaceuticals tested.

for the twenty-four probe pharmaceutical compounds that were spiked into plasma and analyzed under basic and acidic conditions with acetonitrile as the strong eluent. The results obtained for the basic mobile phase analysis were normalized against the peak

areas obtained with standard acidic conditions. One can see from **Figure 2.3.5** that of the twenty four compounds tested, twenty-one compounds had greater average peak area counts when run with the basic mobile phase, the exceptions being budesonide, cyclobenzaprine and salmeterol, which showed no increase in their average peak area The increases in peak area ranged from 1.2 to 9.6 fold with propranolol and dextromethorphan having respectively the lowest and highest increases in peak areas.

The experimental procedure was then repeated utilizing methanol as the organic eluent. The data from this experiment is shown in **Figure 2.3.6**. In this figure one can observe that twenty out of twenty-four compounds showed an increase in average peak area count when analyzed with the basic mobile phase. Three of the compounds gave similar results as the previous experiment when acetonitrile was used as the strong eluent. These compounds were: budesonide, cyclobenzaprine and salmeterol. Terfenadine, which exhibited a higher area count when analyzed with base and acetonitrile, showed a decrease in peak area when analyzed with base and methanol compared with the acidic mobile phase.

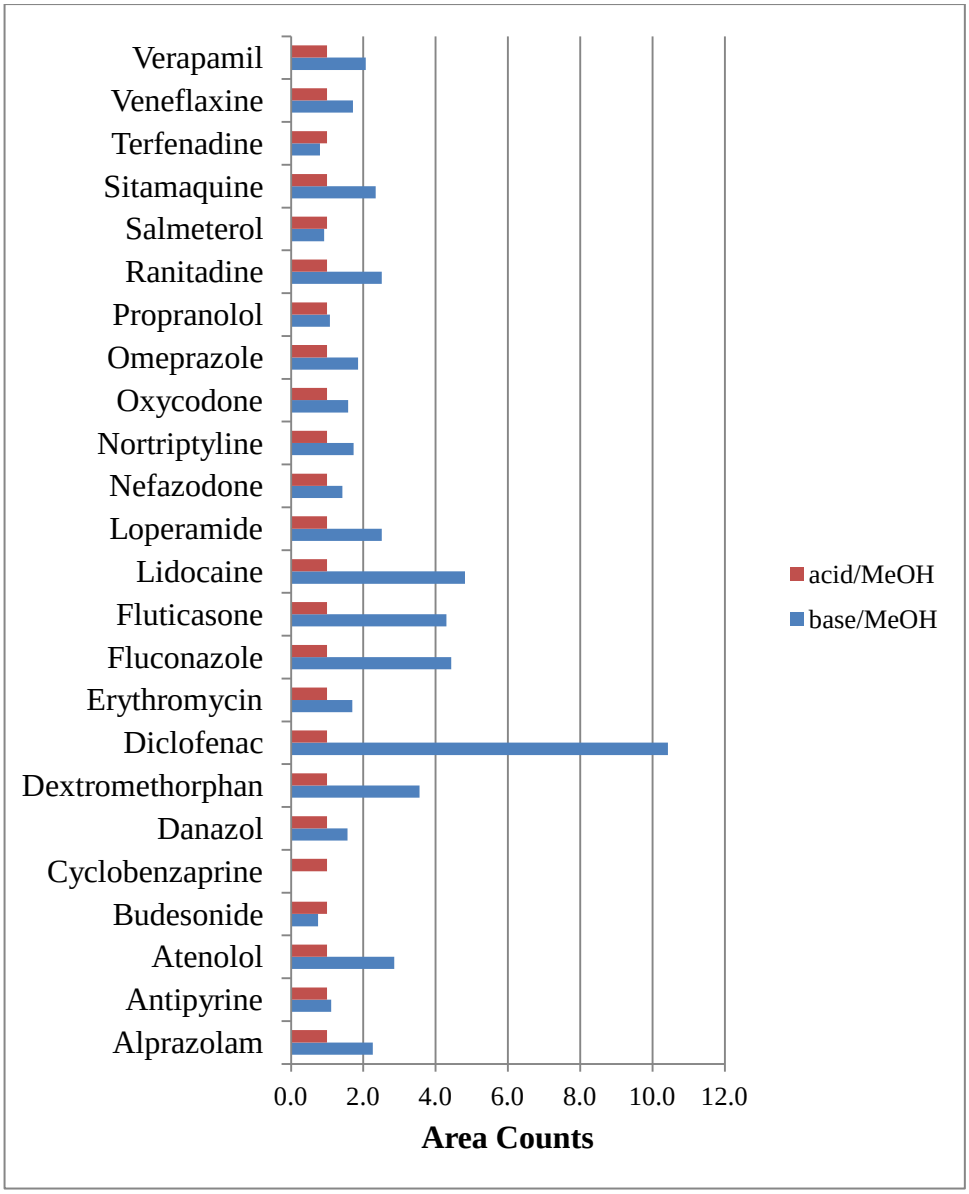


Figure 2.3.6 Bar graph illustrating the effect of mobile phase pH with CH_3OH as the organic modifier on the area count for probe pharmaceuticals tested.

2.3.3 Impact on Signal-to-Noise

Whereas an increase in area response or count may indicate an increase in the detection capability of the LC/MS system towards the compounds analyzed using a basic mobile phase, it is the signal-to-noise measurement which is the most important measurement as this is the parameter which determines the lower limit of quantification of an assay[11, 12]. **Figure 2.3.7** shows the average signal-to-noise values for the test compounds spiked into plasma, and run with acetonitrile as the organic modifier under acidic and basic conditions. The signal-to-noise was calculated using the peak-to-peak algorithm within the operating system, MassLynx software version 4.1. The data obtained under the basic mobile phase conditions is normalized against the signal-to-noise values for the acidic mobile phase analyses in the same manner as was performed with the area count measurements.

From **Figure 2.3.7**, the number of compounds showing an increase in the average signal-to-noise analyzed when using the basic/acetonitrile mobile phase was seventeen of the twenty-four compounds.

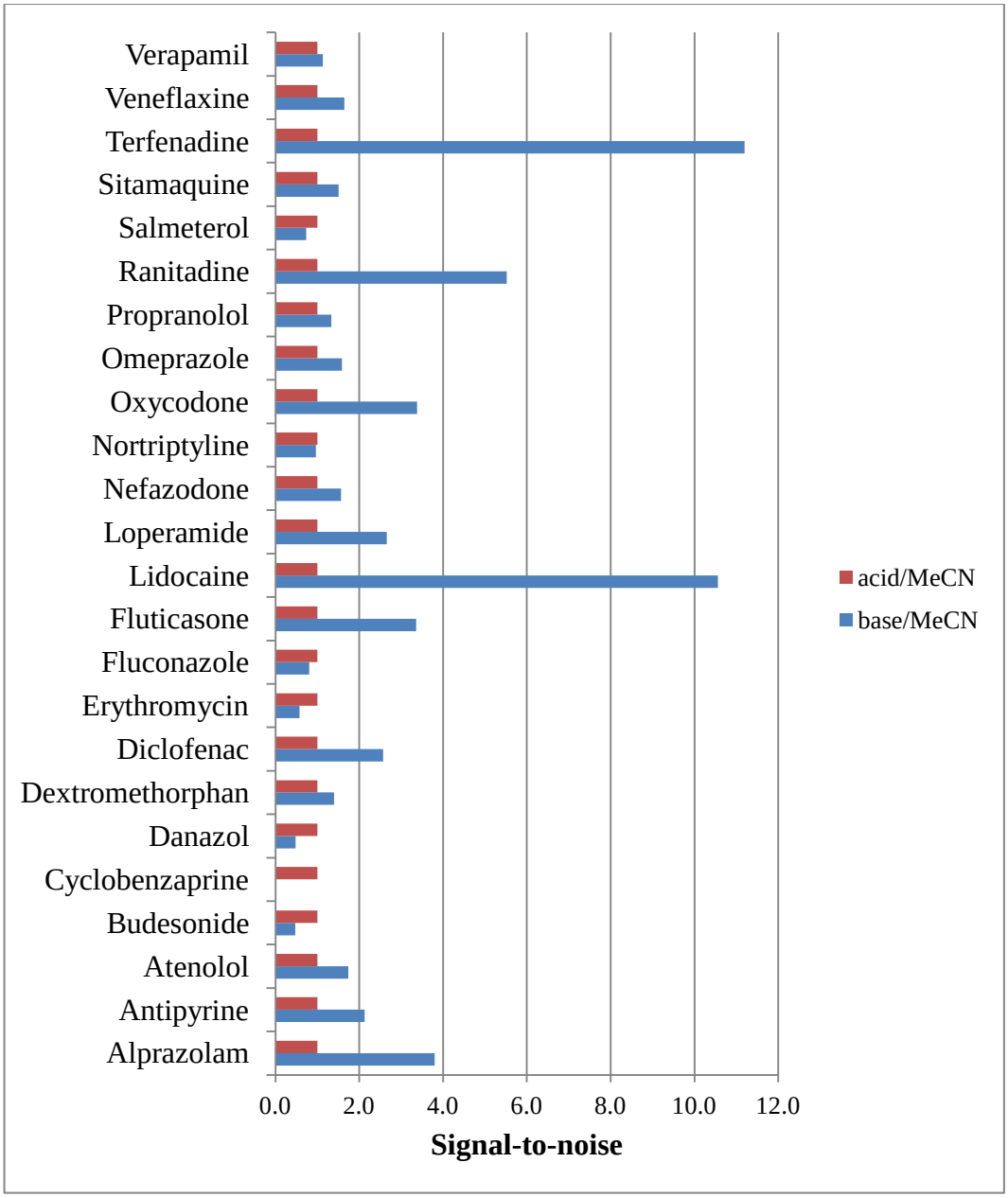


Figure 2.3.7 Bar graph illustrating the effect of mobile phase pH with CH_3CN as the organic modifier on the signal-to-noise for probe pharmaceuticals tested.

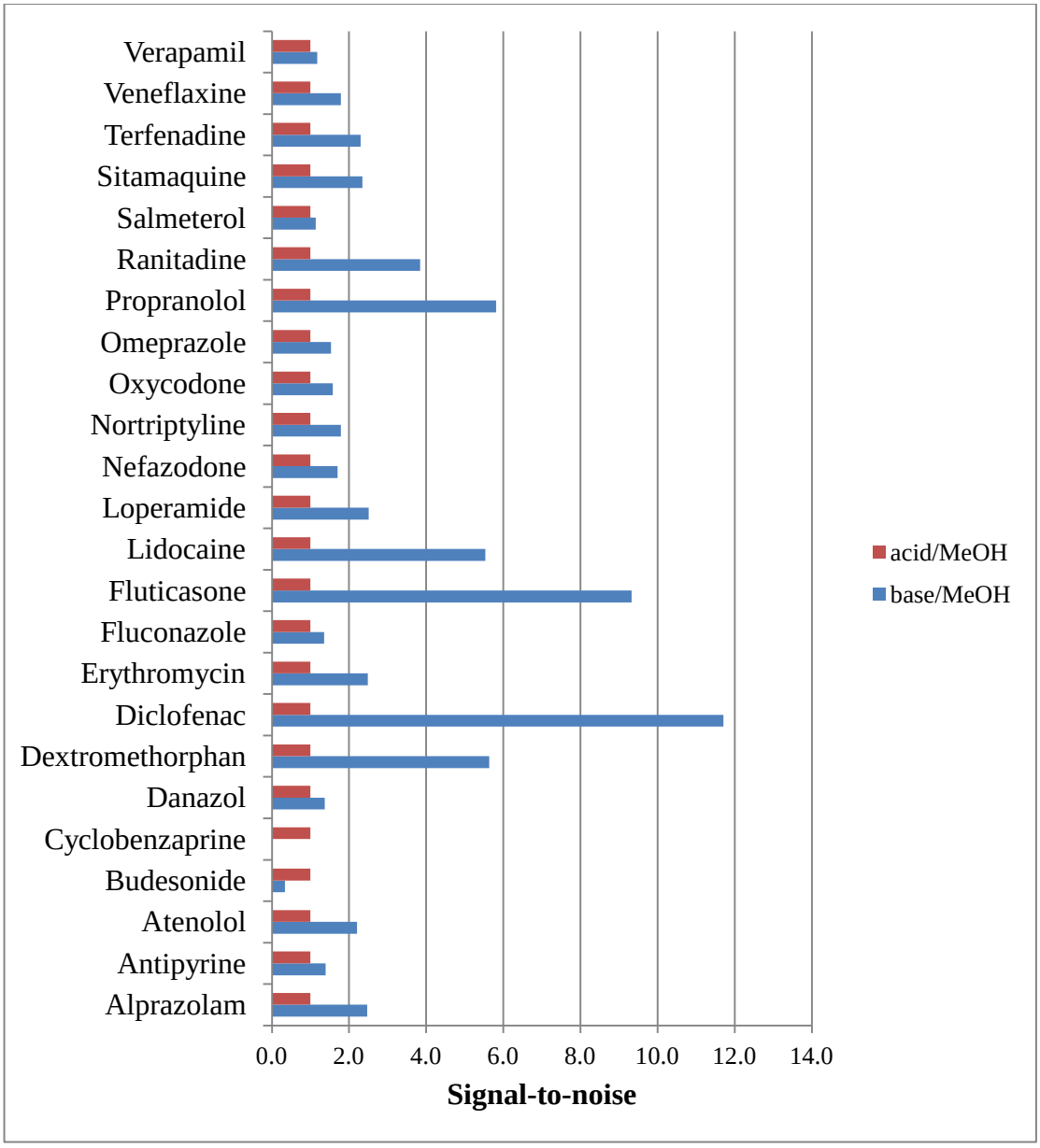


Figure 2.3.8 Bar graph illustrating the effect of mobile phase pH with CH_3OH as the organic modifier on the signal-to-noise for probe pharmaceuticals tested at a concentration of 1 ng/mL.

The signal-to-noise measurements when employing methanol showed twenty-two out of twenty-four had increased signal to noise values compared with the acidic mobile phase (**Figure 2.3.8**). The signal-to-noise values were more scattered compared to that of the peak area data. The chromatographic peak area data when analyzed with either acetonitrile or methanol as the organic mobile phase gave the same general trend for

increases in area counts for the test compounds when the pH of the mobile phase was altered. The only exception to this trend was terfenadine. This can be contrasted with the signal-to-noise data where the trend is not as obvious. For example, the highest and lowest signal-to-noise responses with base and acetonitrile as the mobile phase were terfenadine and verapamil respectively. Whereas with the basic methanol mobile phase combination the compounds that exhibited the highest and lowest signal-to-noise values were diclofenac and salmeterol. It was also noted that the basic mobile phases produced greater signal-to-noise values for twenty-two out of twenty-four compounds tested when compared to traditional acidic mobile phases irrespective of the organic modifier. This result indicates that the organic modifier as well as the mobile phase pH needs to be considered when optimizing for assay sensitivity. One possible explanation for the variance in the signal to noise data may be due to interferences present in the mobile phase, sample, or sample diluents. These interferences may lead to higher background or matrix suppression [12, 13] .

2.3.4 Effect on Peak Width

Chromatographic peak widths have a direct effect on the signal-to-noise, as the narrower or sharper a chromatographic peak, the greater is the peak height resulting in improved signal-to-noise values. The chromatographic peak widths for the test compounds were measured at ten percent peak height. **Figure 2.3.9** and **Figure 2.3.10** show the resulting average peak width measurements using the four mobile phase combinations of the study. From the graph one can observe that eight of the twenty-four compounds tested had narrower peak widths with the basic/acetonitrile mobile phase combination. While fourteen of the twenty-four compounds tested had narrower peak

widths with the basic/methanol mobile phase combination compared to the standard acidic conditions. The entire test compounds that had decreased peak widths with the ammonium hydroxide modified mobile phase also had a corresponding increase in signal-to-noise values. Therefore the narrower peak shape observed with the basic mobile phase did contribute to the improved signal-to-noise values obtained.

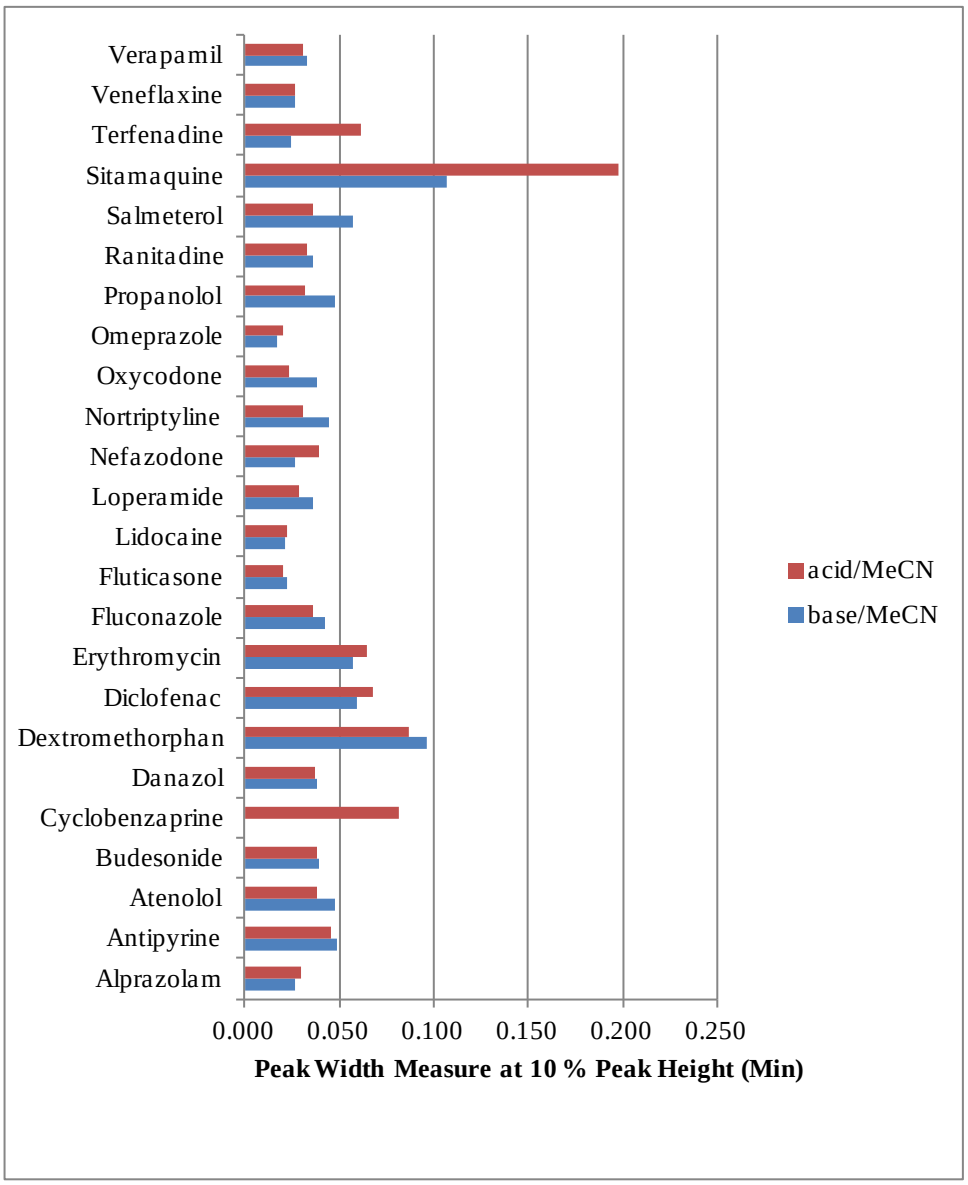


Figure 2.3.9 Bar graph illustrating the effect of mobile phase pH with CH_3CN as the organic modifier on the chromatographic peak width for probe pharmaceuticals tested.

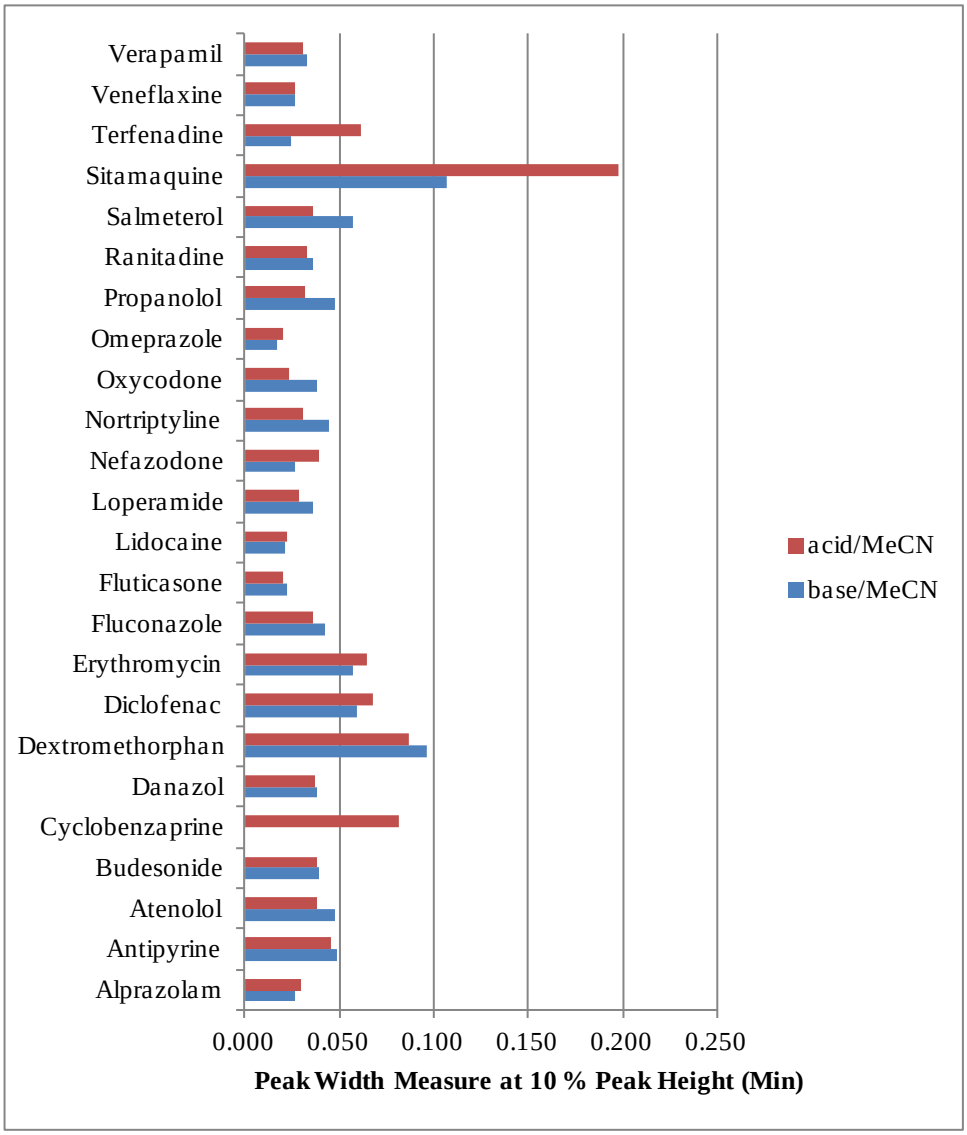


Figure 2.3.10 Bar graph illustrating the effect of mobile phase pH with CH_3OH as the organic modifier on the chromatographic peak width for probe pharmaceuticals tested.

2.3.5 Effect on choline-containing lipids

The minimization of matrix interferences can be as important as increasing the signal to noise for a bioanalytical LC/MS assay, as it adds directly to the assay robustness. Sources of matrix interferences can be due to concomitant medications, metabolites, as well as other endogenous compounds and can inhibit or enhance MS response through a process referred to as ion suppression / ion enhancement as previously stated in Chapter 1 [14-16]. One of the most common endogenous matrix components that has been indicated as a source of potential problems in blood derived matrices are the glycerophosphocholine-containing phospholipids (GPChos) [17-19]. Therefore, the effect of changing mobile phase pH and organic mobile phase on the retention time of GPChos was investigated during this study. **Figure 2.3.11** illustrates the profile for the GPChos in protein-precipitated rat plasma acquired by precursor ion scan of m/z 184, in positive ion electrospray with each of the four mobile phase conditions.

Here we can observe that the retention time of the major choline-containing phospholipids present remained only slightly affected when the pH of the mobile phase was changed from an acidic to basic conditions. This is in contrast to the effect that the organic modifier had on these GPChos components. Changing the organic modifier from acetonitrile to methanol resulted in a significant increase in the retention time as well as the overall MS response for the GPChos. Therefore if one is to consider developing a bioanalytical assay using a basic mobile phase one must consider the effect that the mobile phase pH will have on the compound under analysis. If the retention time of the analyte under basic conditions is later than that with a standard acidic modified mobile phase, special consideration to matrix effects must be investigated as co-elution with the GPChos may occur. Also multiple lots of plasma must be evaluated

as the GPCho profiles can differ not only between different species such as rat and humans but within the same species as well [20]. This point is illustrated in **Figure 2.3.12**.

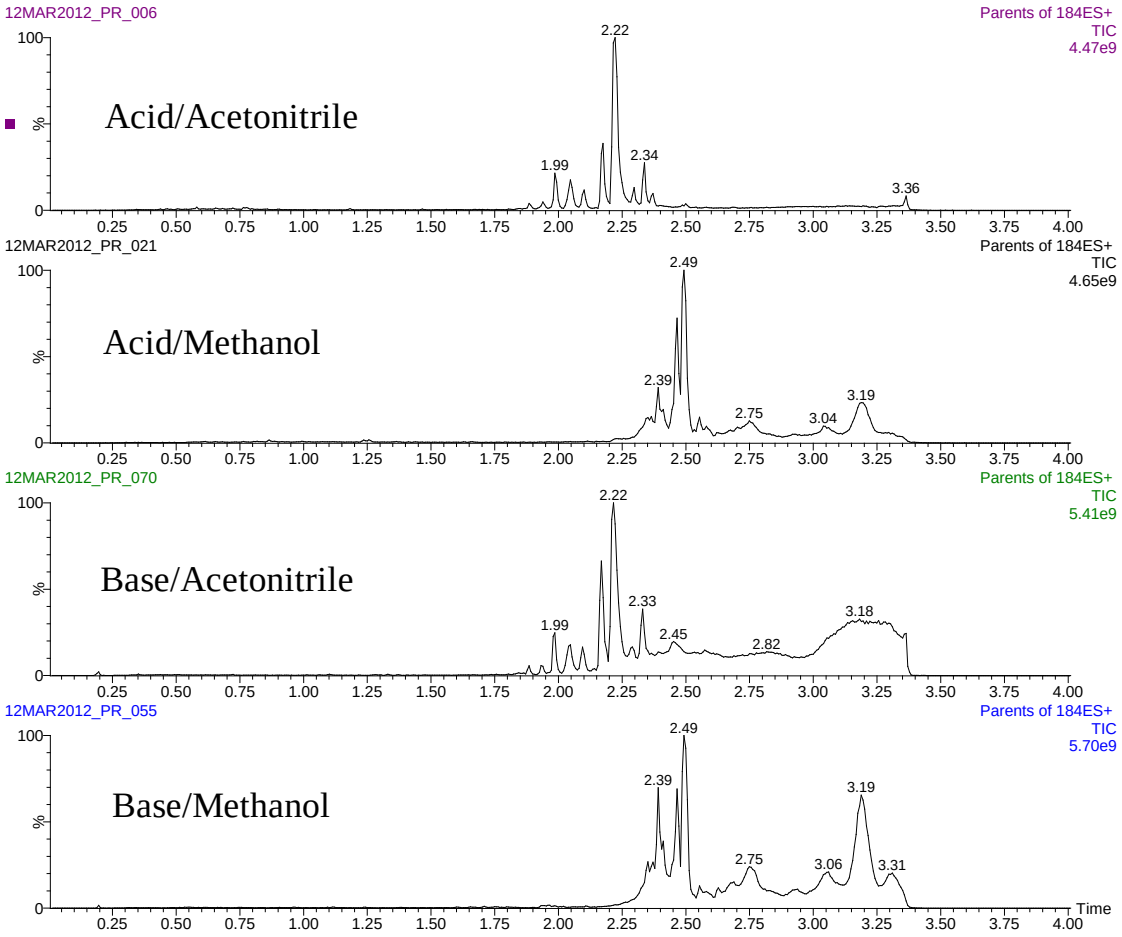


Figure 2.3.11 Profile of GPChos in protein-precipitated rat plasma acquired by precursor ion scan of m/z 184, in positive ion electrospray with each of the four mobile phase conditions.

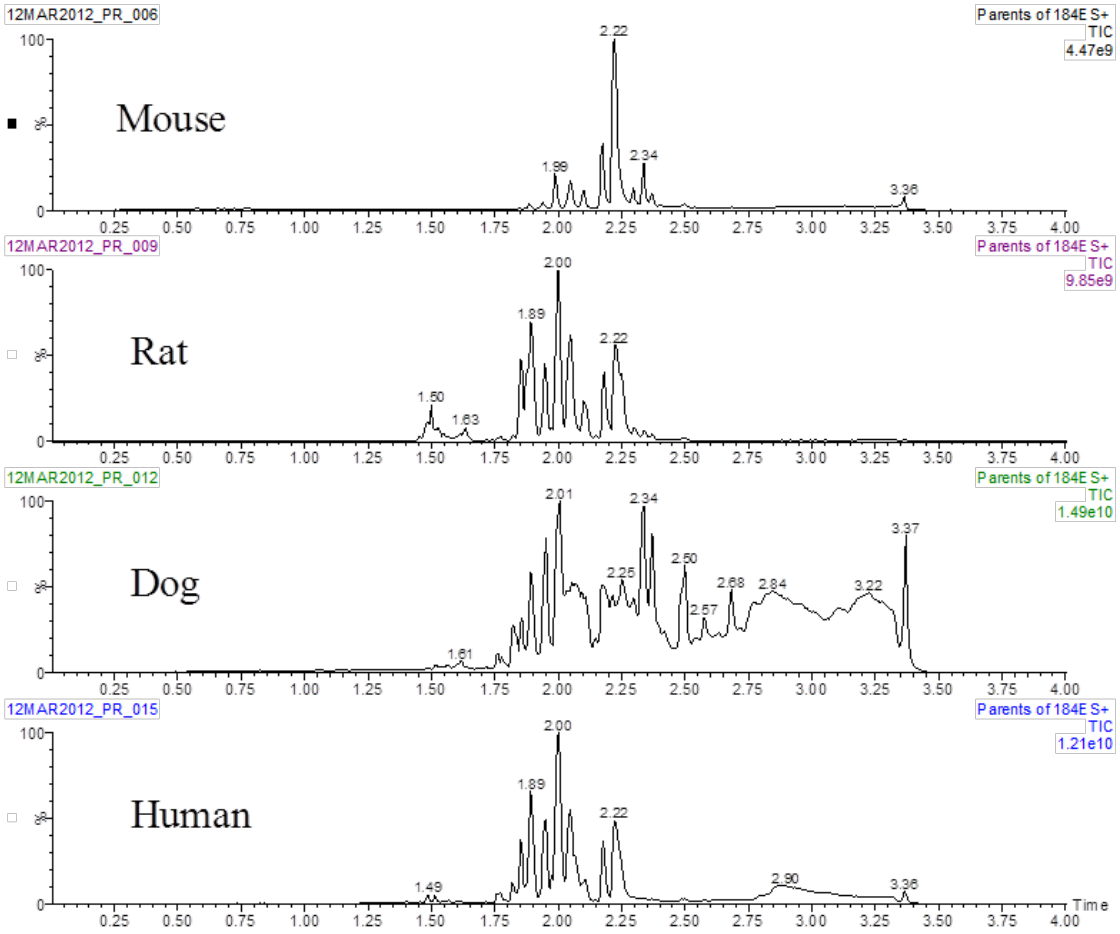


Figure 2.3.12 Profile of GPChos in protein-precipitated rat plasma acquired by precursor ion scan of m/z 184, in positive ion electrospray for different species.

While it is not clear from these experiments as to why an increase in signal-to-noise is possible with the basic mobile phases, there is a definite correlation between the use of basic mobile phase and an increase in the MS response of the probe pharmaceuticals tested in this study with the MS operating in electrospray positive ionization mode. However, as stated earlier, the increase in MS response is unlikely to be due to increased retention time with the basic mobile phase and subsequent elution of the chromatographic peak in a higher organic content. Also some of the compounds that showed increased MS response had reduced retention time. Furthermore the contribution of narrower peaks and therefore increased peak height that was observed

for a portion of the test compounds indicates that the use of a basic mobile phase in bioanalysis would be beneficial compared to traditional acidic mobile phases. The results from this study are similar to results observed by Delatour and Leclercq, who showed the potential for incorporating high pH mobile phases for use in pharmaceutical analysis by LC/MS/MS in 2005 [21]. They postulated different mechanisms to try to explain the observed MS response while using basic mobile phases. These mechanisms were based on the ionization process and included: gas-phase proton transfer from the ammonium ion to the compound undergoing analysis or collision-induced dissociation of the analyte-ammonium ion to the protonated analyte form and surface enrichment of protons in the surface layer of droplets from which ions are desorbed. An investigation across different MS instrument platforms may contribute information in order to evaluate whether the results shown here are also observed over a wide range of MS instruments that vary in source architecture. The observation that methanol gave an increased response for many of the compounds over that of acetonitrile may be explained by the fact that, depending on the nature of the probe pharmaceutical under investigation, the organic modifier can enhance or suppress the signal observed by the MS [22]. The increase in GPCho response when methanol was utilized may be due to the increased solubility of these compounds in this particular organic modifier.

2.4 Conclusion

The data presented here has indicated that the addition of a base, such as ammonium hydroxide, to modify the aqueous mobile phase, has significant benefits for LC/MS/MS based bioanalytical assays under electrospray positive ionization mode. An increase in the signal-to-noise was observed for twenty-two out of twenty-four of the probe pharmaceuticals tested indicating the importance of mobile phase pH optimization when developing an LC/MS/MS based assay. An increase in the chromatographic retention of poorly retained compounds was also observed. The increase in the MS response was not necessarily related to the later elution of the analyte in a higher organic composition under basic conditions. The results demonstrated that seven out of the twenty-four (approximately thirty percent) of the probe pharmaceuticals tested eluted earlier, or with the same retention time, compared to acidic mobile phase, produced a greater signal-to-noise when analyzed with the basic conditions. The effect of the pH of the mobile phase further showed that the phospholipid fraction present in protein precipitated rat plasma was slightly affected by the change in mobile phase pH. However, the basic modified aqueous mobile phase in combination with methanol yielded the highest response for the choline-containing lipid fraction of the prepared plasma sample. The results of these studies indicate that basic mobile phases may be an effective option in order to increase the signal-to-noise of LC/MS/MS bioanalytical assays run under ESI positive ionization mode.

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Chapter 3

The Development and Validation of a High Sensitivity UPLC/MS Bioanalytical Method for Alprazolam Utilizing Basic pH Mobile Phase

This chapter is based on the following publication:

A High Sensitivity Bioanalytical Method for Alprazolam Using Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry

Joanne Mather, Paul D. Rainville, Warren Potts III, Norman W. Smith, Robert S. Plumb

Drug Testing and Analysis Volume 2, Issue 1, January 2010, Pages: 11–18.

Learn to live and live to learn,
Ignorance like a fire doth burn,
Little tasks make large return- Bayard Taylor

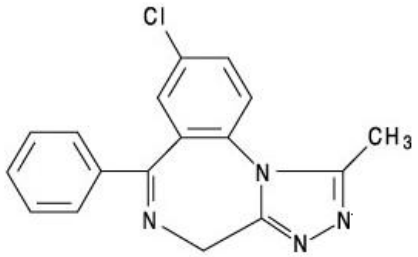
3.1 Introduction

The use of both acidic (pH 2.5) and basic (pH 10.5) pH aqueous mobile phases for positive ESI LC/MS analysis was extensively explored in Chapter 2 for a variety of pharmaceutical drug compounds varying in molecular weight, structure, pKa, and hydrophobicity. Significant gains in chromatographic peak area, retention time, and signal to noise were all observed during the study when analyzed under the basic mobile phase conditions. Also observed was a reduction in the peak width and the influence of mobile phase modification on endogenous analytes within plasma samples.

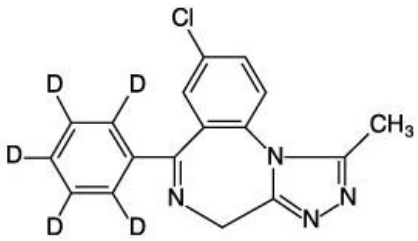
One of the compounds included in the study was the small molecule drug alprazolam. Alprazolam (Figure 3.1.1) is a short acting benzodiazepine used for the treatment of moderate to severe anxiety disorders, panic attacks, and as an adjunctive treatment for anxiety associated with clinical depression. Alprazolam is readily absorbed from the gastrointestinal tract with the peak plasma concentration achieved in 1-2 hours. Most of the drug is bound to plasma protein, mainly serum albumin. Alprazolam is hydroxylated in the liver to hydroxyl alprazolam, which is also pharmacologically active. Some of the drug is also excreted in the dosed form. The compound is typically dosed at 1-3 mg/day resulting in a C_{max} of 8-37 ng/mL with a typical half-life of 12 hours [1, 2].

In this chapter, the development and partial validation of a LC/MS/MS bioanalytical assay for alprazolam in rat plasma was carried out over a dynamic range of 0.01-10 ng/mL utilizing basic mobile phases with positive ESI LC/MS. Both accuracy and precision were estimated for five different QC samples. The sensitivity of the assay

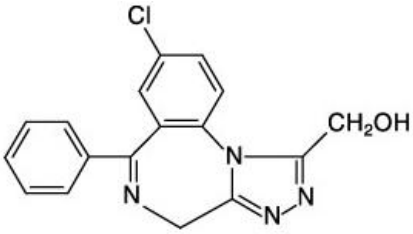
with respect to the mobile phase pH and organic modifier was further evaluated as well as the robustness of the assay over a 96 hour period.



Alprazolam



Alprazolam D5 Internal Standard



Metabolite of Alprazolam

Figure 3.1.1 Chemical structure of alprazolam, deuterated alprazolam, and the hydroxyl-alprazolam metabolite.

3.2 Materials and Methods

3.2.1 Chemicals and Materials

Methanol and acetonitrile were obtained from Fisher Scientific (Waltham, MA USA), Formic acid and ammonium hydroxide were obtained from Sigma – Aldrich Chemicals (St Louis, MO, USA). Rat plasma was purchased from Equitech-Bio (Kerrville,TX, USA) and stored frozen at -20 ˚C prior to use. Purified water was generated in house using a MilliQ water system from Millipore (MA, USA). Alprazolam (1.0 mg/mL in methanol), d5-alprazolam (internal standard) and the hydroxyl-alprazolam metabolite was purchased from Cerilliant (Round Rock, TX, USA).

3.2.2 Sample Preparation

The alprazolam solution (1 mg/mL) was volumetrically diluted in methanol : water (5:95 v/v) to produce series of spiking standards. Calibrants were prepared by spiking alprazolam from the spiking standards into control rat plasma over the range 0.01-10 ng/mL and QC samples at concentrations of 0.025, 0.25, 0.625, 2.50 and 6.25 ng/mL. The plasma samples were then prepared for analysis by protein precipitation by taking 0.375 mL of the alprazolam spiked plasma and adding 0.75 mL of acetonitrile. The samples were vortex-mixed for 1 minute and centrifuged at 13,000 RCF for 5 minutes. The supernatant was removed and an equal amount of water was added.

3.2.3 Liquid Chromatography

A 20 µL injection of each sample was injected onto an ACQUITY® Ultra Performance LC® separations module (UPLC) and onto an Alliance HPLC separations module (Waters Corporation, MA, USA). UPLC method development was performed on four columns: 2.1 x 50 mm 1.7 µm ACQUITY BEH C_{18}, 2.1 x 50 mm 1.7 µm ACQUITY BEH C_8, 2.1 x 50 mm 1.8 µm ACQUITY HSS T3 C_{18}, and 2.1 x 50 mm 1.7 µm ACQUITY BEH RP Shield C_{18}. HPLC was performed on a 2.1 x 50 mm 5 µm XBridge C18. Mobile phase A consisted of water with formic acid (0.1 % v/v) or water with ammonium hydroxide (0.1 % v/v). Mobile phase B consisted of methanol or acetonitrile. The column was maintained at 45 ˚C and eluted under linear gradient conditions from 5 to 95 percent B. UPLC was run at a flow rate of 0.6 mL/min while the HPLC was run at 0.2 mL/min.

3.2.4 Mass Spectrometry

Mass spectrometry was performed on a Waters Xevo™ TQ mass spectrometer (Waters, Manchester, UK) equipped with an electrospray interface. Settings were as follows: capillary voltage = 1 kV, source temperature = 150 ˚C, desolvation temperature = 600 ˚C, desolvation gas flow rate = 1200 L/Hr, and cone gas flow = 40 L/Hr. The mass spectrometer was operated in SRM mode. **Table 3.1.1** illustrates the transitions and conditions utilized for each of the compounds analyzed in this assay.

Compound	SRM m/z	Cone (V)	Collision Energy (eV)
Alprazolam	309.2 > 281.0	40	25
Alprazolam D5	314.2 > 286.1	40	25
Alprazolam OH	325.0 > 297.0	40	25

Table 3.1.1 SRM transitions and MS conditions utilized for each compound.

3.2.5 Validation Protocol

The assay was validated using the procedures outlined in the US FDA Guidance for Industry Bioanalytical Method Validation [3]. The calibrants (concentration range 0.01 – 10 ng/mL) and five QC levels (covering the range of 0.025 to 6.25 ng/mL) were analysed by LC/MS/MS. The ratio of organic to plasma was never more than 5 % organic when creating the plasma spiked samples.

3.3 Results and Discussion

3.3.1 Development of a sub-2 µm LC method versus the standard HPLC method

The development of a LC/MS/MS method requires the selection and optimization of the mass spectrometry and chromatography conditions. Alprazolam is a benzodiazepine class of compound having an elemental composition of $C_{17}H_{13}ClN_4$, giving a monoisotopic molecular mass of 308.0829. Alprazolam was found to give the best response in positive ion mode using electrospray ionization mode. The cone voltage, collision energy and most selective and sensitive fragment ion were optimised using both manual and automatic tuning. The best fragment transition in terms of specificity and sensitivity was determined to be m/z 309.2 > 281.0 using a cone voltage of 40 V and a collision energy of 25 eV.

The chromatographic conditions were developed using a reversed-phase chromatography system, using sub 2 µm particle LC. Previous work by many authors has illustrated the benefits of these sub 2 µm particle LC materials [4-6]. Most importantly this extra resolution allows for greater confidence in resolution of the analyte of interest from the endogenous components in the sample and from the metabolites of the dosed candidate pharmaceutical. The LC/MS chromatographic method for alprazolam in rat plasma was developed to address the following major factors; resolution of the analyte from endogenous components in plasma, resolution from associated metabolites, sensitivity of the assay to lower than 50 pg/mL, and high throughput.

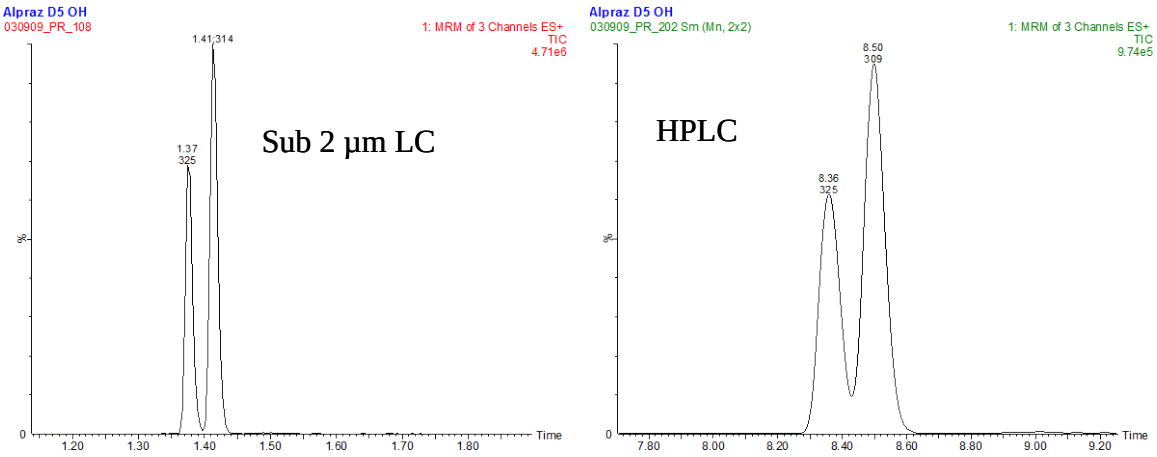


Figure 3.3.1 Increase in the resolution of analytes separated under sub 2 µm UPLC conditions compared to standard 5 µm HPLC. Both separations carried out under gradient conditions and at optimum linear velocity (sub 2 µm = 0.6 mL/min and 5 µm = 0.2 mL/min) Equivelent column volumes of mobile phase were calculated and reflected in the total run time of the analysis ie the HPLC analysis run time was 3 X longer.

The data in **Figure 3.3.1** shows an expanded view of the resolution between alprazolam and the hydroxyl metabolite acquired utilizing sub 2 µm LC and traditional HPLC. The resolution value measured at peak base produced by the sub 2 µm LC separation was 1.5 compared with only partial resolution value of 0.7, for the HPLC separation. This result is of particular importance due to the fact that some drug metabolites such as N-oxides and glucuronides, if unresolved from their parent drug, may decompose in the source of the mass spectrometer back to the parent drug structure [7]. This can result in the over estimation of parent drug concentration and the subsequent erroneous pharmacokinetic plots.

Chromatographic peak widths were also measured at peak base, for alprazolam, hydroxyl alprazolam and deuterated alprazolam separated under each chromatographic condition are displayed in **Figure 3.3.2**. The data in this figure show that peak widths obtained under HPLC conditions are in the order of three times larger than when the same analytes are separated under sub 2 µm LC conditions. The narrower peaks generated from the sub 2 µm LC separation gave a peak capacity, measured at peak base, in 1.5 minutes, of 29.3. This value is 16 percent higher than the peak capacity for the HPLC separation, measured in the same manner, in 5 minutes, of 24.6. This result indicates that a greater number of analytes can be separated in less time with the sub-2 µm LC separation compared to the conventional HPLC separation. The increase in the peak capacity produced by the sub 2 µm LC can be of particular importance in the reduction of co-elution of target analytes from endogenous components present in biofluid matrices. As stated in Chapter 1, co-elution of target analytes with matrix components can produce a phenomenon of matrix effects when MS detection is utilized [8].

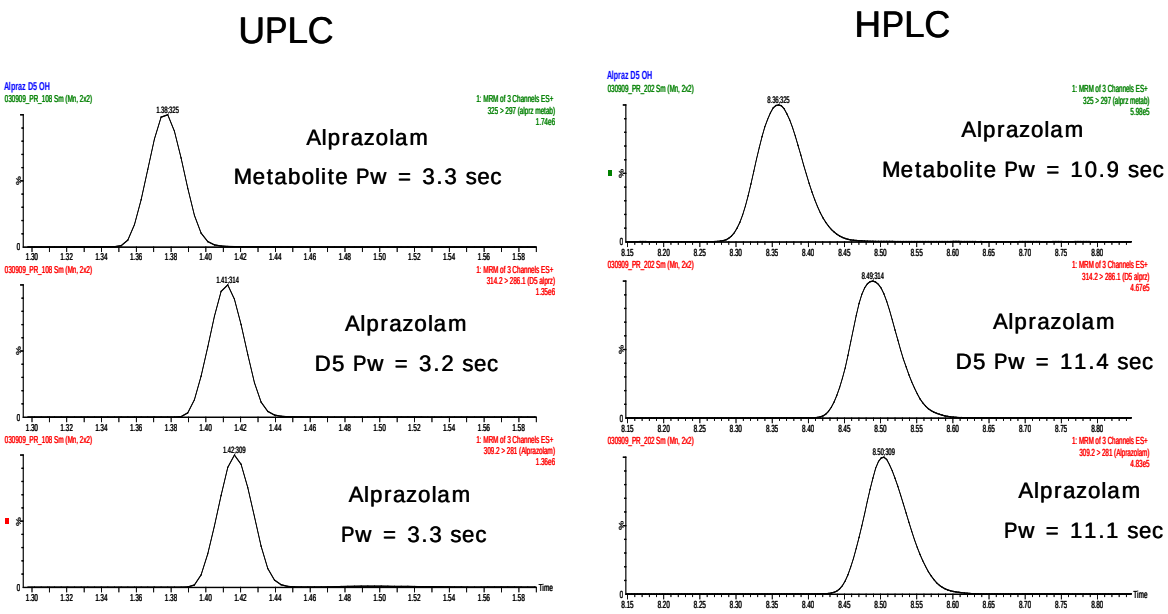


Figure 3.3.2 Comparison of peak widths using sub 2 µm LC and HPLC conditions.

The observed narrow chromatographic peak widths observed for the 2 µm LC separation enables greater peak height to be obtained, therefore increasing the signal of an analyte. This has the effect of lowering the limits of quantification and detection of analytes. **Figure 3.3.3** shows the increase in peak height for equal injection volume of the analytes on both the sub 2 µm LC and HPLC systems. A 4.5 fold increase in signal is observed with sub 2 µm LC versus HPLC conditions while maintaining baseline resolution.

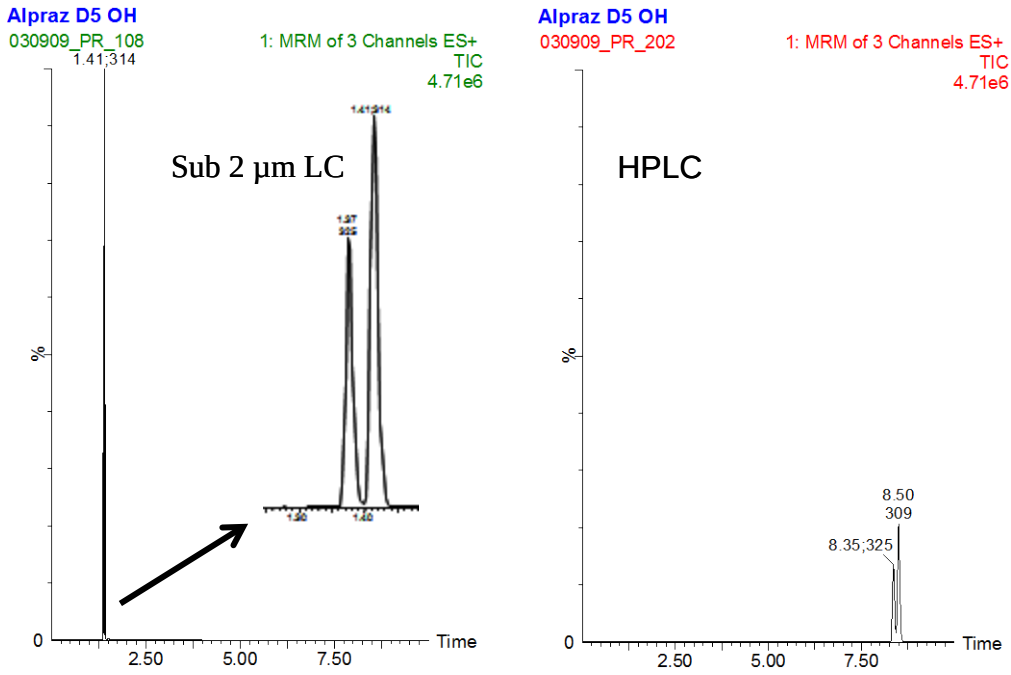


Figure 3.3.3 Comparison in sensitivity of analytes under sub 2 µm LC and HPLC conditions.

The retention of alprazolam and its resolution from hydroxyl-alprazolam was evaluated on various organo-silica hybrid materials including: BEH C_8, BEH C_{18}, and polar embedded C_{18} column (BEH RP C_{18}) as well as a high strength silica C_{18} column (HSS T3), **Figure 3.3.4**. The chromatography was performed using 2.1 x 50 mm columns to facilitate short analysis times and hence rapid evaluation. A generic 5-95 % gradient over 2 minutes was utilized with mobile phase A comprised of 0.1 % v/v formic acid in water and mobile phase B comprised of acetonitrile. The data generated showed that the silica-based C_{18} column gave the greatest retention and resolution of the compound and metabolite.

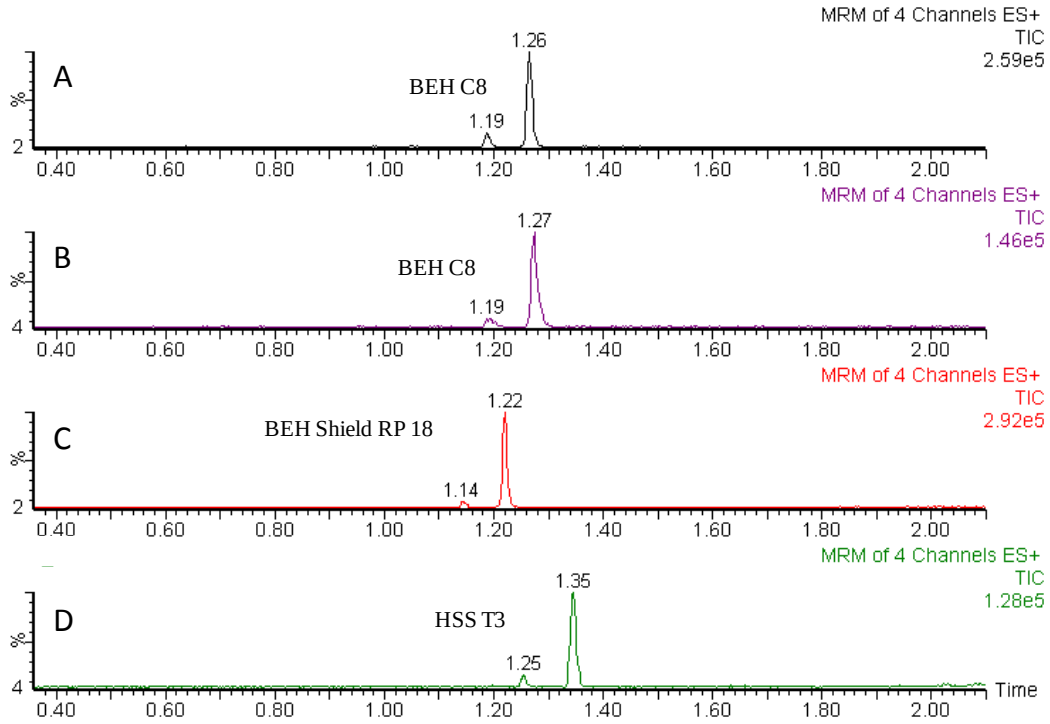


Figure 3.3.4 Comparison of the separation of alprazolam and hydroxyl- alprazolam on (A) BEH C_8, (B) BEH C_{18}, (C) BEH Shield RP_{18}, and (D) HSS T3.

The BEH C_8 gave good resolution between alprazolam and hydroxyl-alprazolam as well as better peak shape for the metabolite compared to the BEH C_{18}. Although the BEH RP C_{18} gave sufficient resolution between the alprazolam and hydroxyl-alprazolam as well as good peak shape for both analytes it was not chosen due to potential bleed issues that could cause potential matrix effects in the MS detector. Therefore the best columns to progress the method development process were the organo-silica hybrid BEH C_8 and the high strength silica C_{18} material (HSS T3).

3.3.2 Optimization of mobile phase composition

As previously stated in Chapter 2, the acidity or alkalinity of the mobile phase will change the ionization state of the analytes, *e.g*. acids will be in their ionic form with high pH mobile phases whereas they will be neutral at acidic pH. This change in ionization state will directly affect the chromatography; in reversed phase mode neutral compounds will be retained longer and generate less peak tailing. Thus operation at a pH where the analytes are in their neutral form is preferred from a chromatographic point of view, whereas from an MS point of view it is typically beneficial to have the analyte in a charged state as it will enhance the sensitivity. A comparison of MS sensitivity for alprazolam utilizing the two different pH values (< 3 and >10 pH units) was carried out and the data is shown in **Figure 3.3.5**. The data displayed in this figure, was obtained for alprazolam analyzed with positive mode electrospray ionization. The data shows a 5 fold increase in sensitivity with the basic mobile phase compared with the traditional acidic mobile phase was obtained for alprazolam analyzed with positive mode electrospray ionization.

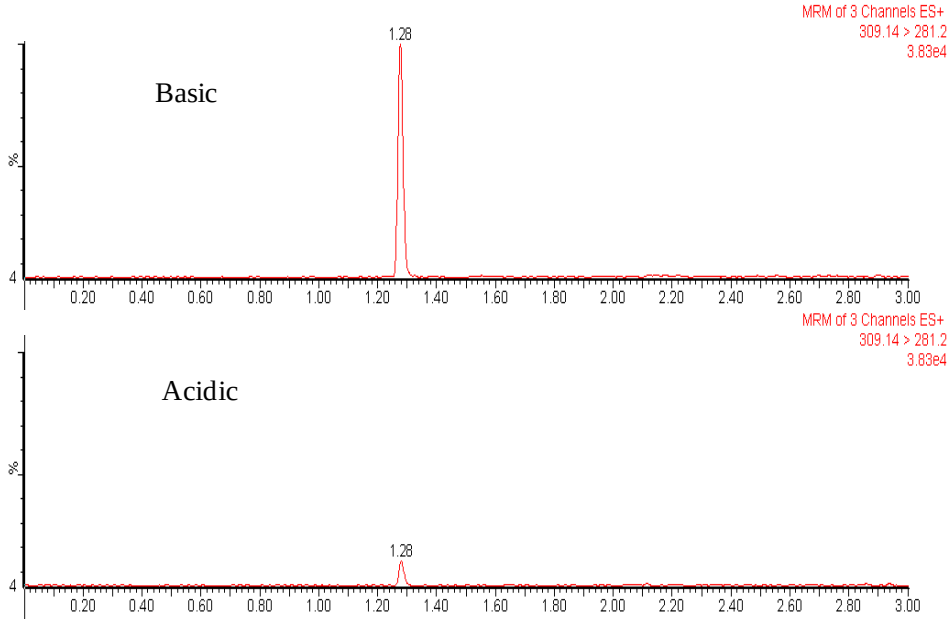


Figure 3.3.5 Comparison of MS peak response for alprazolam in basic (top) and acidic (bottom) mobile phases.

The initial chromatographic evaluation of alprazolam was performed using acetonitrile as the organic modifier and the effect of changing the organic modifier to methanol on the retention time and MS detector response is displayed in **Figure 3.3.6**. The data acquired showed that, when using methanol, the retention time of alprazolam was increased from 1.28 minutes to 1.63 minutes, and the signal response increased by a factor of 2.5.

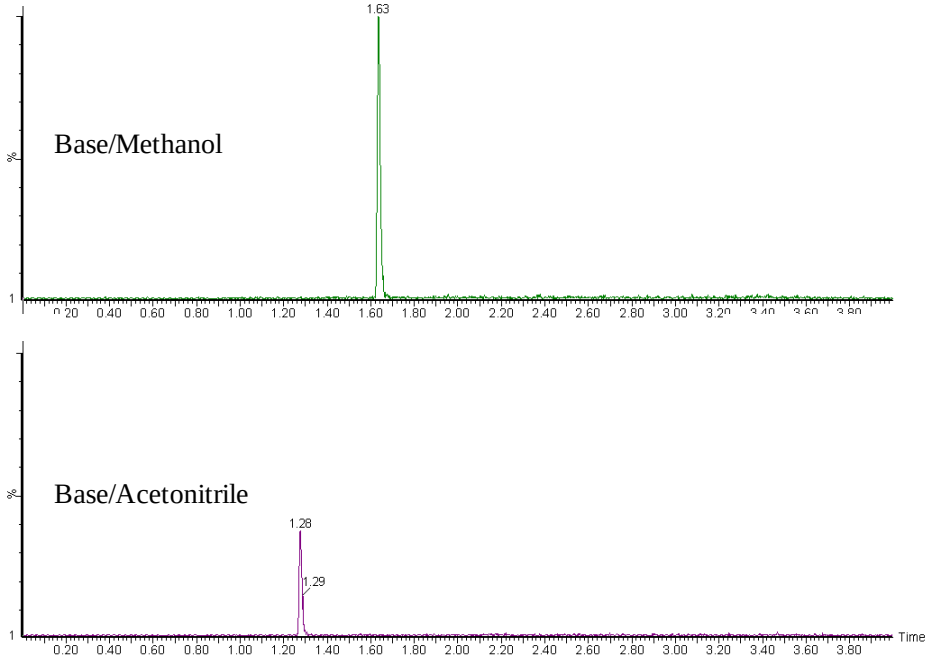


Figure 3.3.6 Comparison of MS peak response for alprazolam using basic/methanol (top) and basic/acetonitrile (bottom) mobile phases.

As the sensitivity was substantially greater with the aqueous high basic pH/ methanol mobile phase, this was therefore selected for further studies. This selection meant that the high strength silica column (HSS T3) could not be used as it is not chemically stable at this high pH. However the chemical stability of the organo-silica hybrid BEH C_8 column allows this column to be operated at high pH and thus this column was selected for this analysis.

3.3.3 Partial method validation

Using protein precipitation as the sample preparation technique the basic mobile phase assay was shown to be linear (using a weighting factor of 1/x) over the range of 0.01-10 ng/mL. To demonstrate the robustness of the methodology the assay was

subjected to partial validation over the concentration range. The calculated concentration RSD of 6 replicate analysis of the QC samples at levels 0.025, 0.250, 0.625, 2.50 and 6.25 ng/mL is displayed in **Table 3.3.1**.

Blank

0.01 ng/mL

Figure 3.3.7 Standard curve, 0.01 ng/mL alprazolam LLOQ, and matrix blank.

QC Level	Actual (ng/mL)	Average Measured (ng/mL)	Accuracy	Precison
QC 1	0.025	0.023	8.00	3.01
QC 2	0.25	0.24	4.00	5.29
QC 3	0.63	0.60	4.76	2.09
QC 4	2.50	2.40	4.00	2.46
QC 5	6.25	6.11	2.24	2.00

Table 3.3.1 QC accuracy and precision data for alprazolam assay run with basic mobile phase obtained from 6 replicates.

3.3.4 Sub 2 µm LC method robustness evaluation

Assay robustness is an issue for methods that are to be utilized for routine analysis. This is the case for many bioanalytical methods. These methods must be able to quantify drug molecules from thousands of samples during clinical trials. Column plugging can result in system overpressure and poor peak shape due to build-up on the inlet frit. Protein precipitated plasma represents one of the most problematic biological samples due to the limited sample cleanup the methods offer. It is however, one of the most utilized methods for sample preparation of plasma due to the ease to perform, speed, and low cost of the technique.

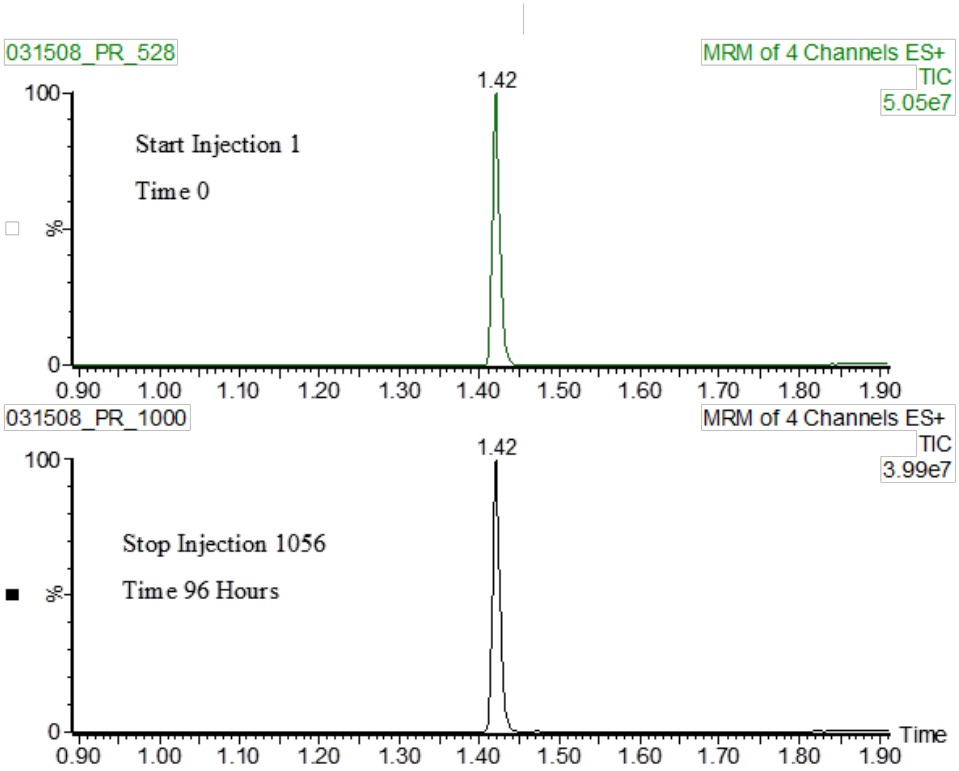


Figure 3.3.8 Chromatographic comparison between first injection and last injection after 96 hours.

The use of sub 2 µm chromatographic particles required the incorporation of smaller porosity frits compared to those utilized with conventional HPLC columns and therefore could be the site of blockage. Therefore a robustness test was carried out utilizing a protein-precipitated plasma sample spiked with alprazolam.

Figure 3.3.8 shows the alprazolam peak at the first injection and after 4 days (96 hours) of continuous analytical runs. Here we can see that after this length of time the chromatographic performance is maintained for the sub 2 µm LC separation as the retention time and peak width for the analyte is unchanged.

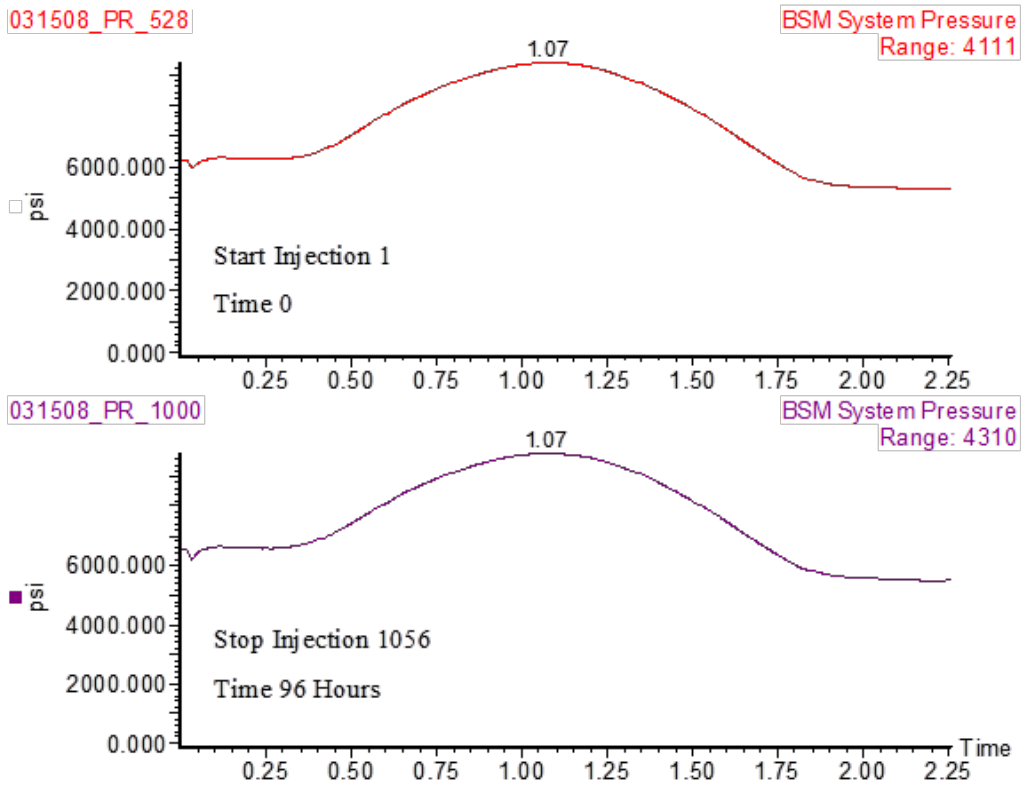


Figure 3.3.9 Comparison of system back pressure comparison between first injection and last injection after 96 hours.

Figure 3.3.9 shows the pressure trace at the start and end of the column lifetime experiment. The pressure trace and the end of the experiment shows no increase compared to the pressure trace at the beginning. This would indicate that the inlet frit or other column parts have not plugged as the pressure traces are virtually identical.

3.4 Conclusion

The data shown here demonstrates the advantages of narrow bore columns packed with sub 2 µm particles run on a low volume, high pressure system under gradient conditions over that of 5 µm particles run using a conventional HPLC system. Greater resolution between alprazolam and the associated hydroxyl metabolite was observed. Reduction in analysis time and gains in sensitivity were also obtained with the sub 2 µm LC assay. A comparison of a basic aqueous mobile phase showed a fivefold increase in the MS response for alprazolam compared to the traditional acidic aqueous mobile phase used with positive ion mode ESI MS. In addition to the increase in sensitivity that the base modified aqueous mobile phase produced, methanol provided a further increase in the MS signal for alprazolam. These conditions were then utilized to produce a simple, sensitive and specific LC/MS assay for alprazolam in rat plasma. The limit of quantification using protein precipitation was determined to 0.01 ng/mL. The precision of the protein precipitation assay was determined to be less than 6 % and below calculated via multiple QC sample levels. The assay was shown to be linear over a range of 0.01 to 10 ng/mL. The system and column were also shown to be robust as it ran continuously over 4 days and 1000 injections of protein precipitated plasma samples without any deterioration of chromatographic peak shape or increase in system backpressure.

3.5 References

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2. Kinani, S., et al., *A sensitive and selective method for the detection of diazepam and its main metabolites in urine by gas chromatography–tandem mass spectrometry.* Journal of Chromatography A, 2007. **1141**(1): p. 131-137.
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Chapter 4

Investigation of Basic and Acidic LC Mobile phases with Positive Mode ESI Mass Spectrometry in Biomarker Discovery

This chapter is based on the following publication:

Investigation of Basic Mobile Phases with Positive ESI LC/MS for Metabonomics Studies

Paul D Rainville, Norman W Smith, David Cowan, Jeremy K Nicholson, J P Shockcor, St John Skilton and Robert S Plumb

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Every addition to true knowledge is an addition

to human power.-Horace Mann

4.1 Introduction

Metabonomics has been defined as “Quantitative measurement of multivariate metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification” and has been primarily utilized to provide information for the wide-spread understanding of global metabolic regulation and biological system failure [1]. Initial work in the area of metabonomics was focused upon understanding the mechanisms of drug toxicity in mammalian system [2-5].

The main aim of these studies was to collate information on biomarkers of nephrotoxicity, and hepatotoxicity. This information was subsequently used to build statistical models to monitor early safety assessment studies as a means to eliminate potentially undesirable compounds earlier in the drug development process [6-8]. As part of these early studies it was clear that the use of spectroscopic and spectrometric profiling of biological fluids could yield data that could result in the ability to discriminate between gender, age, and diurnal variations [9, 10].

As a result of these promising early studies more elaborate studies where undertaken such as the Consortium on Metabolism and Toxicology (COMET I&II) to understand and build statistical models of toxicity [11, 12]. These promising initial studies resulted in a dramatic increase in the interest and use of the metabonomic approach to investigate disease models and dietary effects [13-16]. More recently the use of data from high resolution NMR and MS has aided decision making in both surgical and clinical settings [17, 18].

Much of the initial metabonomics research employed either NMR or GC-MS as the primary platforms to acquire analytical data [19-21]. Although NMR and LC/NMR are used in drug metabolism studies, LC/MS is by far the dominant analytical platform for the acquisition of qualitative and quantitative in drug metabolism and pharmacokinetics (DMPK) studies because of the inherent sensitivity, speed and throughput of the technique. The application of LC/MS to metabonomics studies has provided complementary data to that obtained by NMR, often producing assays of greater sensitivity [22-24].

The vast majority of the LC/MS metabonomics methodologies employed the use of formic acid based organo-aqueous gradient approaches [25-27], primarily due to the fact that it is most common mobile phase combination used in LC/MS when the ionization mode utilized is positive ESI. This could have been further compounded by the dearth of chromatographic stationary phases that were chemically and mechanically stable in basic pH environments prior to the late 1990s. In Chapter 3, the sensitivity benefits of using basic pH mobile phases for the analysis of the benzodiazepine drug alprazolam analyzed under positive ESI mode [28], and in Chapter 2 a subsequent larger bioanalytical study for a significant number of compounds varying in structure, molecular weight, pKa, and hydrophobicity [29].

In this study, the first known investigation into the utilization of basic pH mobile phase with reversed-phase chromatography coupled with positive ESI LC/MS for metabonomic analysis is presented. Urine samples from rats treated with the model hepatotoxin, hydrazine, were utilized to determine the benefit derived from this non-traditional mobile phase combination for positive ESI LC/MS. Chromatographic resolution, analyte response and retention were all compared to that obtained when using the same column and MS conditions with traditional acidic mobile phase. An

additional goal of this study was to compare the differences in the detection of analytes between the traditional acidic mobile phase LC/MS analysis with the non-traditional basic mobile phase LC/MS analysis. Furthermore due to the great interest in retention and subsequent detection of highly polar analytes that elute in the void volume of an LC/MS/MS analysis, part of the study was to evaluate the effectiveness of high pH mobile phases to better retain the polar components that elute in the chromatographic void when using traditional acidic based mobile phases.

4.2 Materials and Methods

4.2.1 Chemicals and Materials

Optima grade acetonitrile (HPLC grade) was purchased from Fisher Scientific (Hampton, NH, USA), formic acid (spectroscopic grade) and 0.1 M ammonium hydroxide solution were purchased from Sigma/Aldrich (MO, USA). Purified water was generated purified in-house using a MilliQ system Millipore (MA, USA).

4.2.2 Animal Studies

Rat urine samples were obtained from a study of hydrazine toxicity conducted as part of a major toxicology project[30]. All animal experiments were conducted according to appropriate national guidelines. In this study, male Sprague-Dawley rats were randomly allocated to groups control, low and high-dose (n = 10 per group). Here we only consider the control and high-dose groups. The high-dose group received hydrazine hydrochloride in 0.9 % saline administered orally at 90 mg/kg. Dose vehicles alone were administered to matched control animals. Rats were housed in individual metabolic cages under controlled temperature, humidity, and light cycles. Urine samples were collected at times from -16 h pre-dosing to 168 h post-dosing. Data for the high-dose group were obtained from 58 samples, of which 20 corresponded to pre-dose time points (showing no toxic response) and 38 for time points known to demonstrate a high toxic response. Data for the control group were obtained from 54 control samples.

4.2.3 Chromatography

The separations were performed on a 2.1 x 100 mm ACQUITY UPLC 1.7 µm BEH C_{18} column using an ACQUITY Ultra Performance LC Chromatography™ (UPLC) System (Waters® Corporation, MA, USA). The column was maintained at 40 ºC and eluted with a linear acetonitrile – aqueous gradient over 10 minutes at 500 µL/min, starting at 5 % acetonitrile and rising to 95 % over the course of the gradient. The aqueous mobile phase was either 0.1 % v/v formic acid or 0.1 % v/v ammonium hydroxide. The column eluent was directed to the mass spectrometer for analysis.

4.2.4 Mass Spectrometry

Mass spectrometry was performed on a Waters® Micromass® Xevo™ G2 QTof mass spectrometer (Waters Micromass, Manchester, UK) operated in positive ESI mode with “W-Optics” , the collision cell was set to alternate between a collision energy of 5 eV and 25 eV every 60 mSec with a 20 mSec inter scan delay. The instrument was operated with Lockspray™ frequency of 11 with a scans to average set to 5. Leucine enkephalin was employed as the Lockspray™ solution at a concentration of 200 ng/mL.

4.2.5 Statistical Analysis

Data were analysed using the MarkerLynx XS™ applications manager (Waters, UK); this application manager integrates peaks in the LC/MS data by using automated ApexTrack™ peak detection algorithm. The LC/MS data were peak-detected and noise-reduced in both the LC and MS domains such that only true analytical peaks were further processed by the software (e.g., noise spikes are rejected). A list of the intensities of the peaks detected was then generated for the first sample, using the retention time (RT) and m/z data pairs as the identifier for each peak. An arbitrary number is then assigned to each of these RT-m/z pairs in order of elution, (1,2,3,4,….etc). This process was repeated for each run. Once this was completed the data from each LC/MS analysis in the batch were then sorted such that the correct peak intensity data for each RT-m/z pair was aligned in the final data table. The resulting 3 dimensional data, peak number (RT – m/z pair), sample name, and ion intensity were analysed by Principal Components Analysis (PCA). The PCA was performed in the MarkerLynx application software where it is automatically applied while processing the data.

4.3 Results and Discussion

4.3.1 Basic pH mobile phase with positive electrospray MS

The benefits of basic pH mobile phases in reversed-phase chromatography are well known for compounds containing basic groups. Operation at a pH above the pKa of these compounds allows them to be chromatographed as neutral species, resulting in greater retention, and more symmetrical peak shapes. Further, the use of basic pH helps to overcome the effect of peak tailing.

The routine application of basic pH mobile phase was made possible by the development of organo-silica based stationary phases in the late 1990s [31]. These new phases relied on the incorporation of a methyl group into the core silica structure, reducing phase dissolution and allowing operation in the range 9-10 pH units. More recently the development of a bridged-ethyl silica hybrid stationary phase has resulted in greater phase stability allowing continual operation at pH 10-11 [32] .

As mentioned in Chapter 2, Delatour and Leclercq showed the potential for incorporating basic pH mobile phases for use in pharmaceutical analysis by positive ESI mode LC/MS in 2005 [33]. Following in 2008, Farkas and Peng reported the first in-depth study utilizing basic pH mobile phases for the analysis of over forty basic drug substances analyzed with positive ESI mode LC/MS. In this work, the authors reported actual increases in MS response for the basic probe pharmaceutical compounds [34].

4.3.2 Application to metabonomic study

Plasma and urine contain a significant proportion of basic compounds [35], so the ability to improve retention and separation of these compounds in reversed-phase LC/MS would be of significant benefit. Greater separation of analytes from the components eluting in the void of the column is also likely to reduce ion suppression and improved assay reproducibility. This is critical in metabonomics studies as any variation in analyte response could be interpreted as the result of a toxicological or disease state response. The use of other forms of chromatography such as hydrophilic interaction liquid chromatography (HILIC) has yielded promising results in this area[35]. However, this form of chromatography is less effective than reversed-phase at dealing with the broad range of analyte polarities present in biological fluids.

The data displayed in **Figure 4.3.1** illustrates two chromatograms obtained for the analysis of control rat urine, using either acidic or basic mobile phase. In these two examples, the gradient duration, organic solvent and gradient steepness has been kept constant. The top chromatogram represents the acidic mobile phase separation and the bottom that obtained from a basic mobile phase separation. A qualitative review of the data shows that there is a significant difference in the elution profile across the whole of the chromatogram.

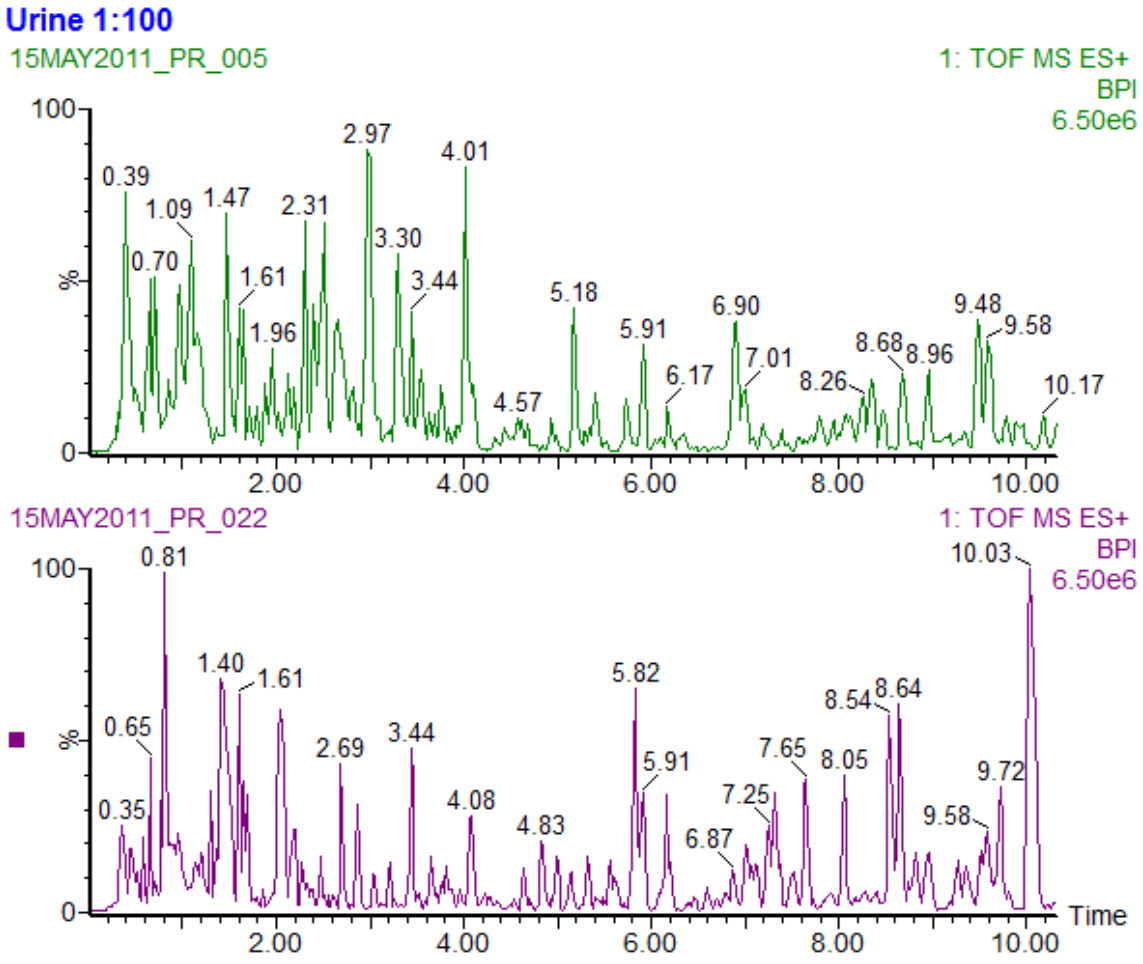

Figure 4.3.1 Positive ESI LC/MS chromatogram of control rat urine using an acidic (top chromatogram) and basic mobile phase (bottom chromatogram).

The use of the basic mobile phase not only increased analyte retention but also improved analyte sensitivity and peak resolution. The data displayed in **Figure 4.3.2** illustrates the increased analyte response obtained from the two ions detected within the mass window 150.077 +/- 50mDa.

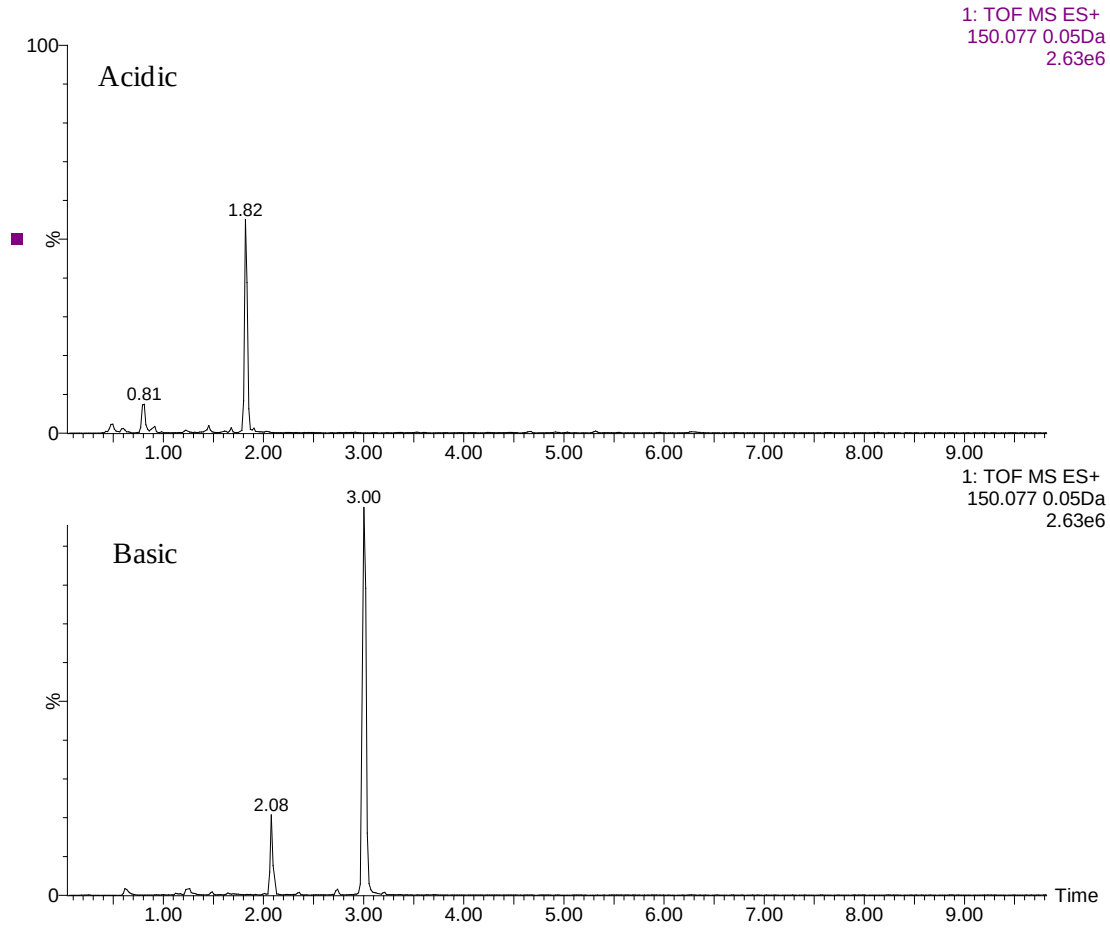

Figure 4.3.2 Influence of basic mobile phase pH on urinary analyte with m/z 150.077.

The top chromatogram is the separation with the acidic mobile phase and the bottom chromatogram that obtained from the basic mobile phase. The two chromatograms axes are linked for intensity. The peak eluting with a retention time of 3.00 minutes in the basic separation shows a response almost 50 % greater than the same analyte in the acidic separation, where it elutes with a retention time of 1.82 minutes.

The same phenomenon is also observed for the smaller peak in the two chromatograms eluting with a retention time of 2.08 and 0.81 min in the basic and acidic mobile phase systems respectively. Due to the fact that many analytes in biological fluids are basic in nature, upon exposure to an acidic mobile phase these analytes will be present in a charged state. This charged state will in effect make the

analyte more polar and therefore less likely to retain on a reversed-phase column. However, when basic analytes are exposed to a basic mobile phase, they exist in a neutral state and are more likely to be retained by reversed-phase.

The use of a basic mobile phase also, in some cases, provided enhanced resolution. This fact is illustrated in **Figure 4.3.3**. In this example the single peak eluting with a retention time of 10.96 minutes in the acidic system (top chromatogram) is resolved into two discrete peaks eluting at 9.72 and 10.03 minutes in the basic system. The accurate mass of the two peaks detected, m/z = 358.269 was subjected to a search on multiple databases but did not return any potential compounds. The data shown in **Figure 4.3.3** clearly illustrates that the spectra

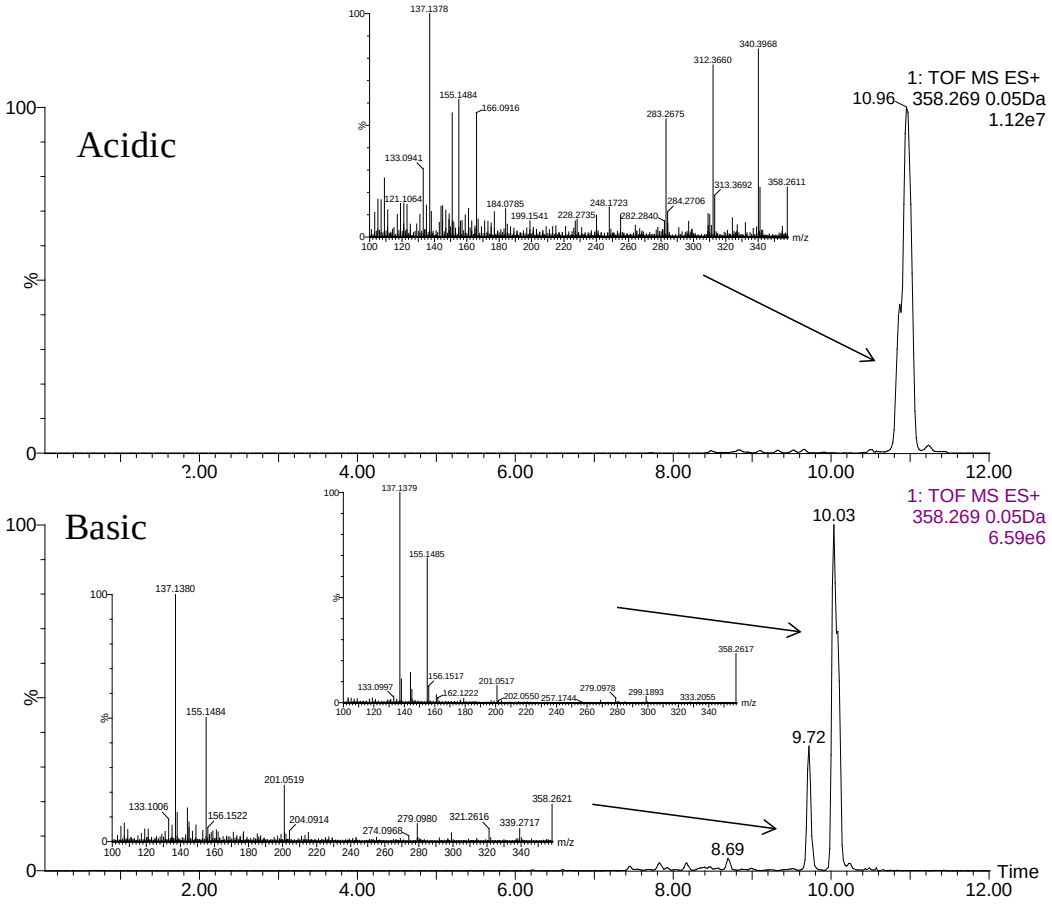

Figure 4.3.3 Increase in chromatographic resolution of analytes analyzed with basic pH mobile phase.

from the two discrete peaks from the basic separation were significantly cleaner whereas the spectra obtained from the acidic separation was less informative. The extra resolution is a result of extra selectivity imparted onto the separation system as a result of employing a basic pH mobile phase. This improved resolution can be beneficial not only in metabonomic studies but also in metabolite identification studies especially in the light of the recent Metabolites in Safety Testing (MIST) guidelines which emphasize the need to study toxic species [36].

4.3.3 Model toxin study samples

The effect of utilizing basic mobile phase combinations in an LC/MS based metabonomics study was investigated using urine samples obtained from a toxicology study with a model toxin, hydrazine, dosed to rats. The analysis of these samples was previously reported by Crockford *et al*.[30] In that paper the authors describe the use of a high resolution chromatography, accurate mass QTof MS system combined with advanced correlation statistical analysis to obtain structural, as well as higher level biological, information relating to changes in metabolic pathway activity and connectivity.

For the present study we have reanalyzed a subset of these samples using both the acidic and basic aqueous-organic reversed-phase chromatography systems. Again as with the previous control urine, the samples were analysed using high-resolution sub 2 μm porous particle LC/MS with QTof detection in positive ion mode. The data displayed in **Figure 4.3.4** illustrates the separations obtained from the LC/MS analysis of the resulting urine samples from the hydrazine study for control animal number 20

pre-dose. The top chromatogram illustrates the data obtained from the acidic separation and the bottom chromatogram that obtained from the basic LC/MS analysis.

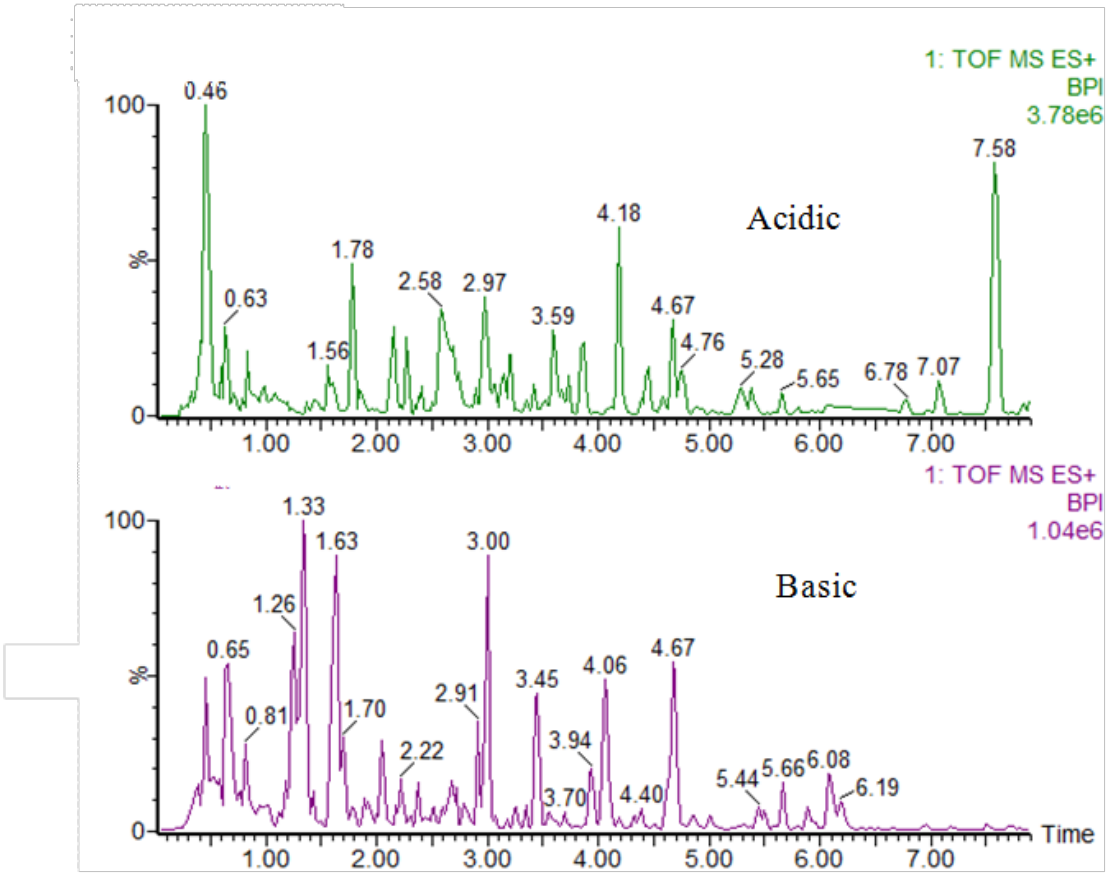

Figure 4.3.4 Positive ESI LC/MS chromatogram of rat urine following the oral administration of hydrazine at 60 mg/kg, using an acidic (top chromatogram) and basic (bottom chromatogram) mobile phase.

The samples were analysed by the PCA method and using the OPLS class model described by Shockcor *et al*.[20] The data shown in **Figure 4.3.5** shows the PCA plots for the analysis of the 0 hr and 48 Hr samples using either the acid mobile phase (**Fig. 4.3.5a**) or basic mobile phase (**Fig. 4.3.5b**). The statistical analysis of the two data sets showed that both systems clearly separated the 0 Hr samples from the 48 Hr samples with a very similar pattern.

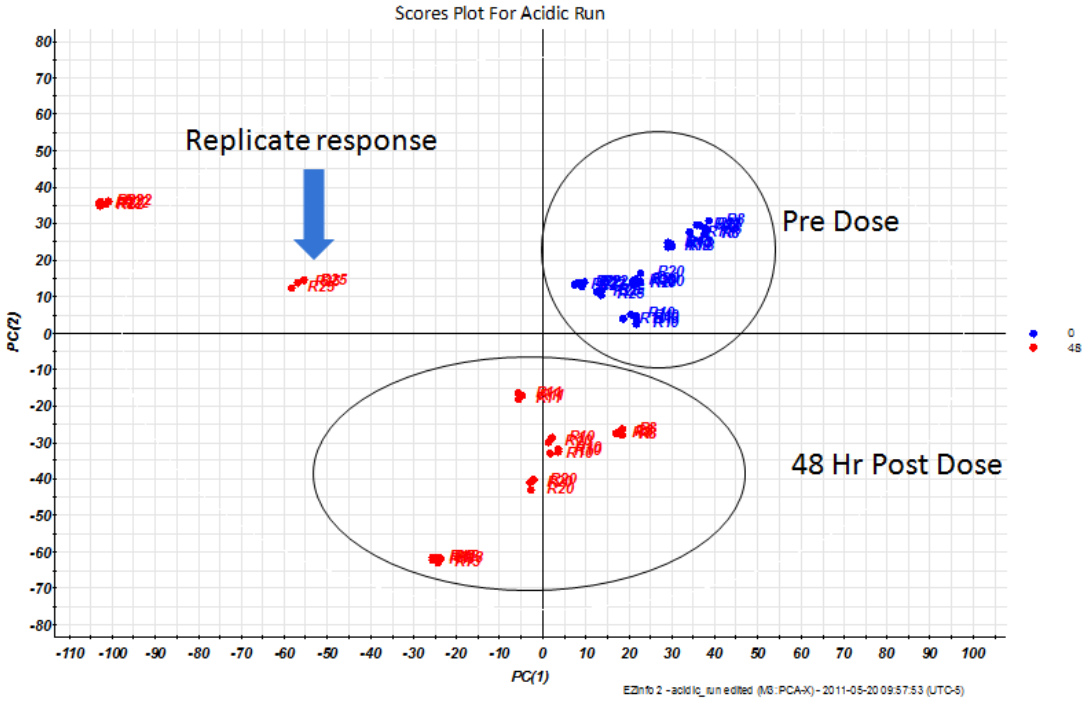

Figure 4.3.5a Principal components analysis of positive ion LC/MS analysis of rat urine following the oral administration of hydrazine at 60 mg/kg with acidic mobile phase.

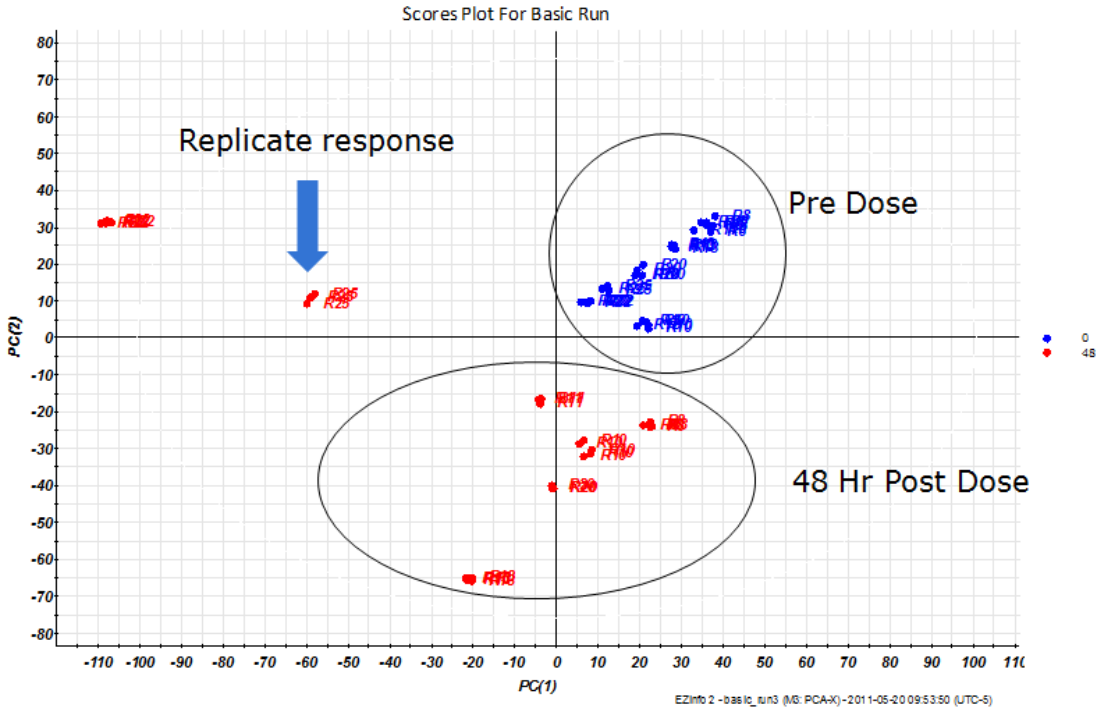

Figure 4.3.5b Principal components analysis of positive ion LC/MS analysis of rat urine following the oral administration of hydrazine at 60 mg/kg with basic mobile phase.

However it is clear from the data analysis, that the ions showing the greatest contribution to the variance in the data were different between the two chromatographic systems, irrespective of the actual retention time. The data displayed in **Table 4.3.1** list the top ten ions contributing to variation in the data for both the 0 hr and 48 hr samples. It is likely that the different datasets would provide different complementary data.

Mobile phase pH 2.5, 0 Hr	Mobile phase pH 2.5, 48 Hr	Mobile phase pH 10, 0 Hr	Mobile phase pH 10, 48 Hr
m/z	**m/z**	**m/z**	**m/z**
287.863	431.688	284.948	309.044
162.660	295.731	215.744	267.815
144.131	160.858	182.489	234.191
144.044	141.272	167.834	211.589
139.497	129.423	163.578	156.260
117.805	123.879	160.262	143.760
117.787	113.722	160.199	140.175
114.180	109.387	111.560	137.354
104.861	100.739	110.745	131.268
287.863	431.688	106.291	309.044

Table 4.3.1 Comparison of the LC/MS marker ions detected with acidic and basic mobile phase in the analysis of hydrazine dosed rats.

This point can be further illustrated graphically in **Figure 4.3.6**. In this data we can see the acid and basic separations as well as a trend plot one of the ions. From the data we can see that with the acid separation the ion m/z = 962.3001 is not detected but is well detected in the basic separation.

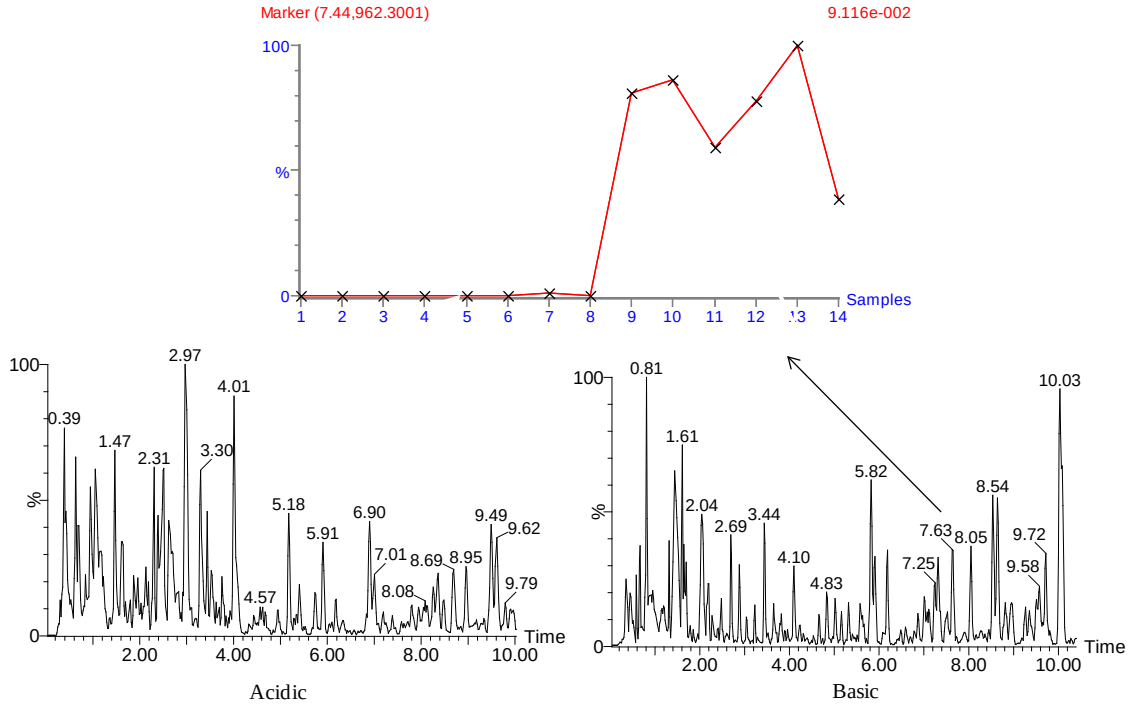

Figure 4.3.6 Positive ion LC/MS analysis of rat urine using acidic and basic mobile phases and trend plot for the marker ion detected eluting at 7.44 min, m/z = 962.3001.

The data displayed in **Table 4.3.2** compares the retention time and signal intensity obtained for a select set of extracted signals (ions) previously identified in the work of Crockford *et al.* on hydrazine toxicity as well as selected other commonly reported marker of toxicity compounds, in both the acidic and basic mobile phases. As can be seen from the data, the retention time was increased for some of the marker ions under basic conditions and the signal intensity increased for fifty percent of ions under basic conditions (all measurements were made under the same positive ESI conditions). Not unsurprisingly the acidic compounds such as hippuric acid and pantothenic acid were retained to a greater extent under acidic conditions, however even when the retention (k_g) did not increase under basic conditions the signal response often did. For example,

biomarker candidate m/z 170.06 had a reduced retention time under basic conditions but the signal intensity increased by a factor of 5.

			Signal intensity		k_g	
	Compound	m/z	Acid	Base	Acid	Base
1	Biomarker candidate	127.04	1	0.5	1.7	4.2
2	Biomarker candidate	149.04	1	ND*	1.7	ND*
3	Biomarker candidate	156.03	1	0.7	0.1	0.3
4	Biomarker candidate	156.06	1	0.4	0.9	0.3
5	Biomarker candidate	170.06	1	5.0	8.5	7.7
6	Pantothenic acid	220.12	1	1.1	6.0	4.8
7	Hippuric acid	180.06	1	1.5	7.5	3.6
8	Hydrazine toxicity marker	402.1	1	2.7	7.0	6.3
9	Hydrazine toxicity marker	265.05	1	2.9	0.3	0.3
10	Hydrazine toxicity marker	218.11	1	0.2	10.6	4.7

Not Detected*

Table 4.3.2 Comparison of the retention (k_g) of reported biomarkers of toxicity under acidic and basic LC/MS conditions.

4.3.4 Effect of basic mobile phase pH on poorly retained compounds

As mentioned previously, it is of great interest to retain highly polar analytes that elute early near or in the void volume of an LC/MS/MS analysis. Therefore part of the study was to evaluate the effectiveness of utilizing high pH mobile phases to better retain the polar components that elute early in the chromatographic run when using traditional acidic mobile phases. Due to the fact that many analytes in biological fluids are basic in nature, upon exposure to an acidic mobile phase, these analytes will be present in a charged state. This charged state will in effect make the analyte more polar and therefore less likely to retain on a reversed-phase column.

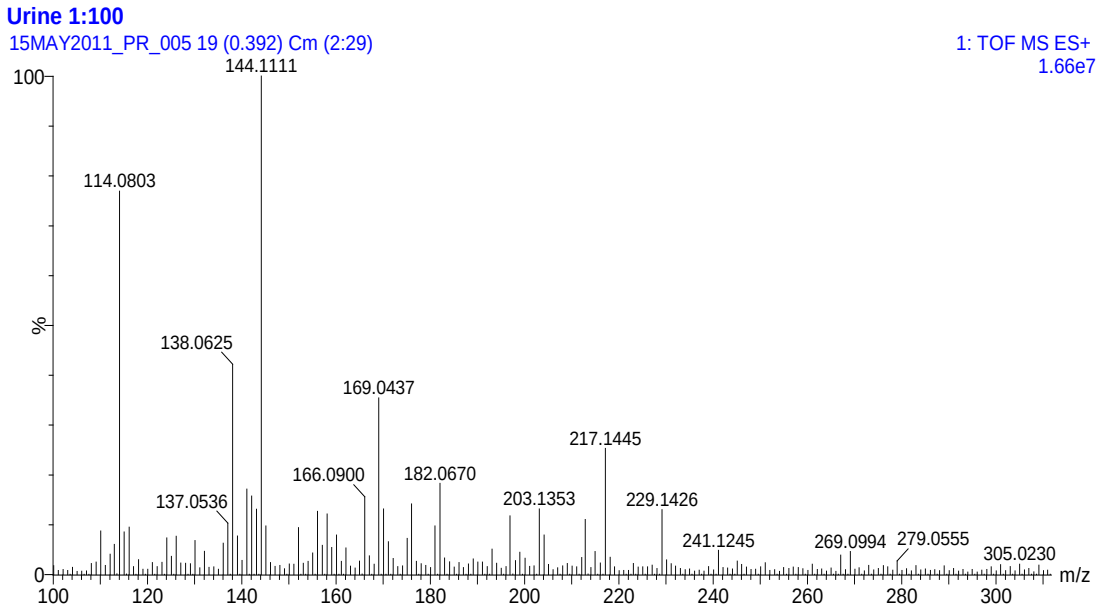

Figure 4.3.7 Summed MS spectra of first two minutes of human urine separated by LC/MS with traditional acidic mobile phase modifier.

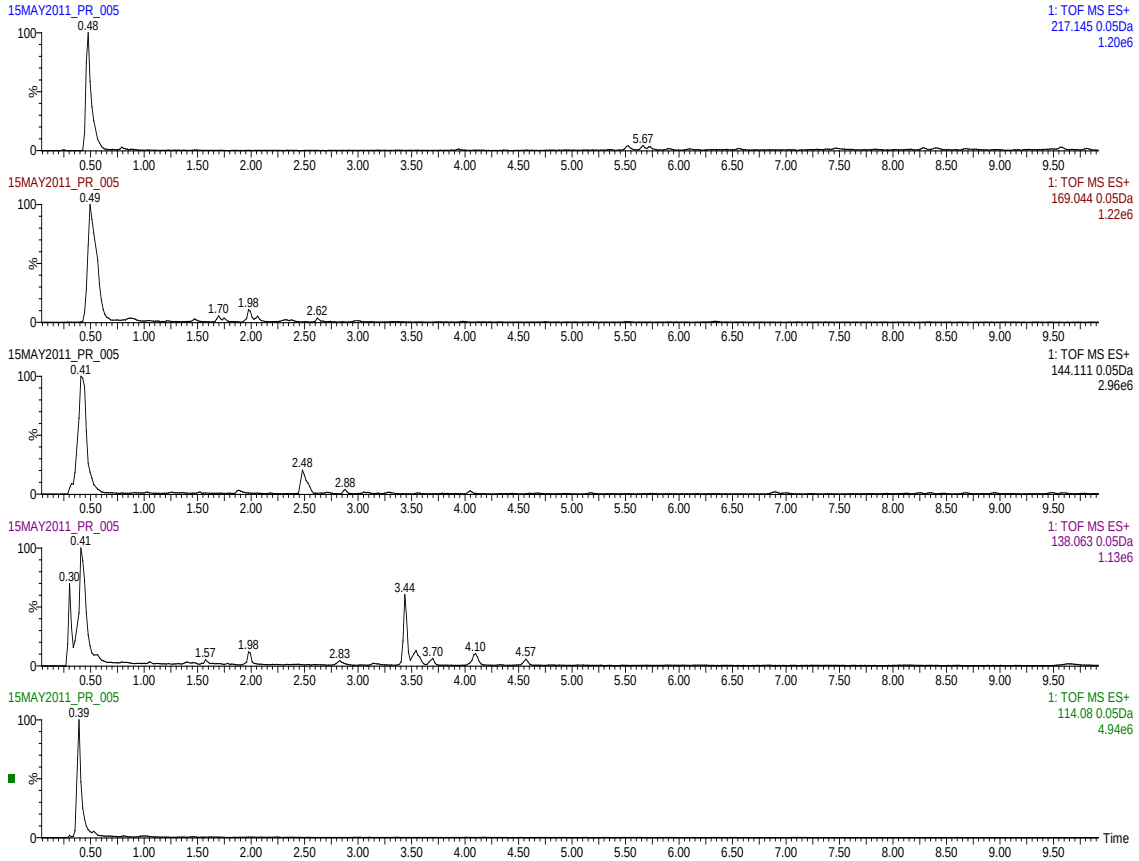

Figure 4.3.8 Extracted Ion Chromatogram (XIC) of top five m/z detected in first two minute separation of human urine by LC/MS with traditional acidic mobile phase modifier.

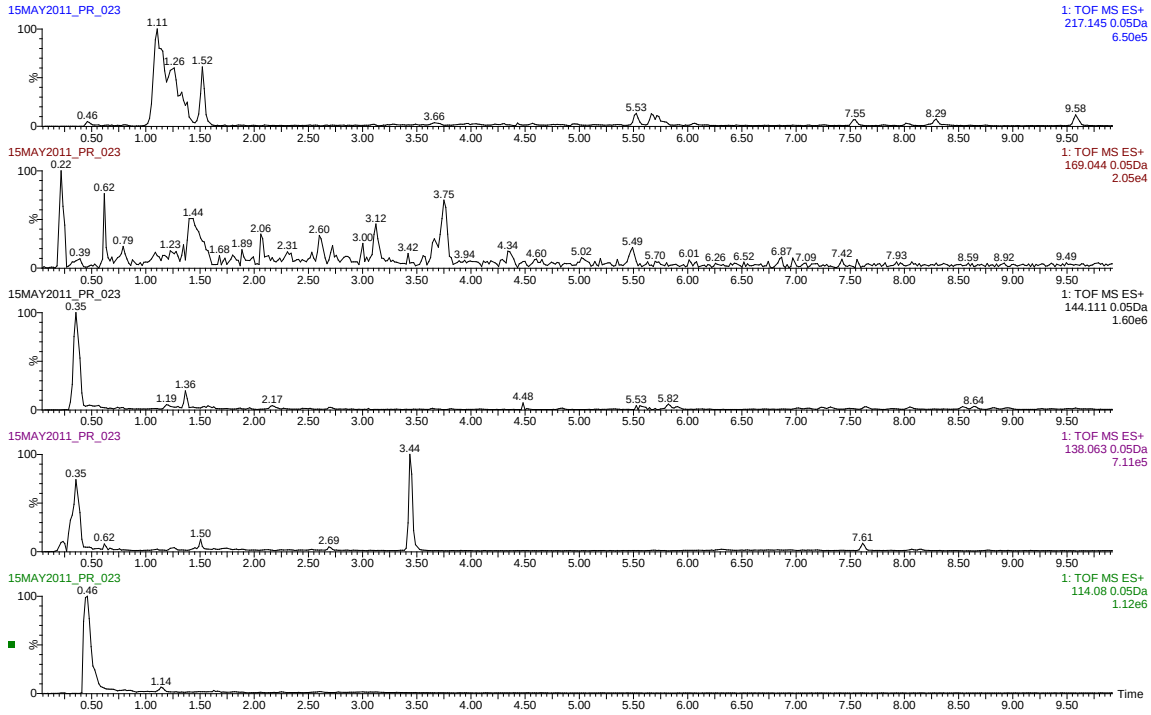

Figure 4.3.9 XIC of top five m/z detected in first two minute separation of human urine by LC/MS with basic mobile phase modifier.

However, when basic analytes are exposed to a basic mobile phase, they exist in a neutral state and may be more likely to be retained by a reversed-phase system. The data displayed in **Figure 4.3.1** showed significant increase in retention for the analyte with m/z 150.077. These observations lead to the targeted analysis of the analytes eluting in the void time of the column. **Figure 4.3.7** showed the summed MS spectra for the first two minutes of the LC/MS separation of human urine carried out with traditional acidic mobile phase. An analysis of the top five most abundant ions was then carried out to determine the applicability of utilizing basic mobile phase to increase the retention of analytes eluting in the void under traditional acidic mobile phase conditions. As can be seen in comparing the XIC for the top five abundant ions in **Figures 4.3.8** and **4.3.9**

there was little effect in the increase in retention for these particular analytes in this experiment.

4.4 Conclusion

The application of basic mobile phases coupled with positive ESI LC/MS to the analysis of biological samples in metabonomic studies shows great promise. It provides complementary data to that obtained with traditional acidic based mobile phases and results in an increased number of ions detected overall. For many analytes, the use of a basic mobile phase increases analyte retention and results in enhanced response in the mass spectrometer. This increase in analyte retention did not occur with many highly polar compounds that eluted in the column void when chromatographed with the basic mobile phase conditions. During this study, it was also noted that by changing the pH to a basic system it was possible to resolve compounds that had previously co-eluted on an acidic reversed-phase system. The analysis of a real sample set of urines from control rats and rats dosed with hydrazine revealed that both the acidic and basic system were capable of differentiating between the 0-hr and 48 h-hr time points.

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Chapter 5

Development of a Ceramic Microfluidic Separations LC/MS Platform for DMPK and Biomarker Discovery Studies

This chapter is based on the following publication:

Microfluidic LC–MS for analysis of small-volume biofluid samples: where we have been and where we need to go

Paul D. Rainville

Bioanalysis Volume 3, Number 1, January 2011, Pages: 1-3.

Many things difficult to design prove easy to performance

Samuel Johnson

5.1 Introduction

The use of micro-bore i.d. chromatographic columns is not a new concept, as the separation and detection of analytes by micro-column liquid chromatography can be traced as far back as the late 1960s by Horvath and contributors [1]. These researchers used 1.0 mm i.d. columns packed with pellicular ion exchange sorbents to separate nucleotides. During the mid 1970s, Novotny, Scott and Ishii were all working on various aspects of micro-column separations from the miniaturization of LC systems to the development of columns [2-4]. These early researchers identified that by reducing the i.d. of a chromatographic column, benefits could be realized in sensitivity, reduction in the consumption of mobile phases, and the use of smaller sample volumes. The work performed by these various researchers during this period is considered to be the foundation and premier references for micro-column separations.

The coupling of LC to MS was first accomplished by Victor Talrose and followed by MacLaffety and others [5] Some of these early LC/MS interfaces were composed of wires or belts whereby the liquid from the chromatographic columns was directed, subsequently evaporated, and the sample then introduced into the MS [3, 6]. This approach was common with the early MS interfaces which had a limited capability to cope with the water content utilized by reversed-phase chromatography, and the inadequacies of the vacuum pumping systems made it impossible to introduce the entire LC eluent into the MS.

Prior to the development of interfaces that could introduce LC column effluent directly into a MS, individual chromatographic peaks had to be trapped or isolated, concentrated and then introduced into the MS via a probe [3]. This could become a very time consuming process particularly when the analysis involved the isolation of many

analytes from a complex sample since multiple fractions would need to be collected and processed.

Therefore a more efficient approach for this type of analysis would be the direct linking of the liquid chromatography system with the MS. This process of coupling these two powerful analytical techniques however was impeded for a period of time by the unavailability of vacuum systems and interfaces that were capable of coping with the LC column effluent.

The use of capillary columns as a means to overcome some of the potential issues of direct on-line sampling was noted by many researchers including Novotny [7, 8]. This idea was introduced as the reduced flow rates required for these columns was much smaller compared to conventional columns using flow rates in the range of 0.5 – 2 mL/min. This therefore made it more amenable for direct on-line coupling with MS [9]. This utilization of micro-scale columns coupled with MS was further investigated and utilized by researchers such as J. Henion, and others to solve real analytical problems.[10, 11] Throughout the 1980s there were many advances with respect to how the LC effluent could be directly introduced into the MS and in the development of ionization source [12, 13] . Further in the late 1990s and into the early 2000s much work was done in the development of 2.1 mm i.d. columns as a means to directly couple LC with MS. Prior to this the use of 4.6 mm i.d. columns were utilized. Due to the required flow rates of 1.4 mL/min or higher, and the inability of MS sources to cope with this volume of liquid the flow had to be split before introduction into the MS. Current LC/MS systems with ESI sources are now fully capable of handling flow rates of up to 1 mL/min.

The implementation and wide-spread use of nano and capillary scale LC with MS detection occurred during the last decade and was a result of the explosion of work in the field of proteomics. Here, 75 -150 µm i.d. columns coupled with MS became the workhorse for exploring and identifying the protein component in living organisms [14, 15]. The primary reason for the use of micro-scale columns for this type of chromatography was that the sample size available for the analysis was greatly limited, and the increased sensitivity offered by the technique was both necessary and enabling.

During the late 1990s, Fraser, Dear *et. al*. described the first uses of commercial capillary LC/MS systems for use in DMPK studies [16, 17]. This work highlighted the benefits of utilizing capillary LC coupled with mass spectrometry for both qualitative and quantitative bioanalysis. The authors showed the quantification of drugs present in biofluid using a minimal sample volume (1 µL of extracted plasma). This technique was further utilized to detect low level metabolites in complex biofluids [18].

Of the benefits previously listed earlier in this introduction, increased sensitivity has the greatest impact in DMPK and biomarker studies. This increase in sensitivity could mean the difference in detecting a critical, low level, metabolite or correctly defining the pharmacokinetic (PK) parameters of a dosed medicine. Although interesting, the reduced consumption of mobile phases is purely an economic benefit, but could allow for the use of costly, exotic mobile phases such as deuterated acetonitrile or methanol, as well as chiral additives. The ability to address very small samples has great importance in the bioanalytical field. It can facilitate the analysis of serial sampling from tail bled animals. Small sample volumes allows for the use of expensive animal models such as genetically engineered mice, where an individual animal can cost thousands of dollars. In this case, one does not wish to quickly sacrifice the animal for data, but instead use the model to obtain data for longer periods of time due to the

inherent cost associated with the animals. An added benefit of smaller sample volumes may be observed in the clinic where fewer traumas will be inflicted during the course of a study. With less trauma inflicted, the metabolic profile and body functions will more closely resemble the actual healthy or disease state of the subject rather than a stressed state. It may also lead to better patient recruitment.

Although microfluidics and capillary scale formats are currently utilized in the bioanalytical community, they are still not as widely accepted as their larger i.d. column counterparts. There are a few reasons for this. First, it is difficult to make dispersion-free fluidic connections using this scale of chromatography. The cutting and making of connections with glass capillary tubing requires an experienced analyst in order to get the best possible connection, often requiring both patience and time. Second, smaller i.d. columns and tubing can plug easier when sampling complex biofluids, which makes the columns less robust. Third, the speed in which analysis can be carried out. The use of smaller i.d. columns equates to a lower flow rate being employed and therefore longer time to overcome system volumes from injection to detection. Finally the development of MS sources that can effectively operate with the coupled LC to exploit optimal performance of the MS.

In an attempt to overcome these problems and to simplify the operation of nano/capillary LC/MS many researchers have investigated the use of fully integrated microfluidic devices, often described as “chips” as a means to increase sensitivity and reduce sample consumption. [19] Many of these early chip-based separations were performed by researchers such as Ramsey with electrophoretic separations [20, 21]. However the concept quickly migrated from electrophoresis to devices aimed to improve the spray/tip connection such as the nanomate™ [22, 23] to LC-based separation devices where the injector, column and emitter are located all on one device

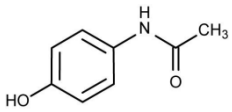
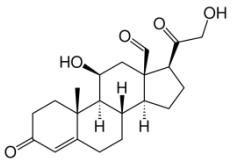
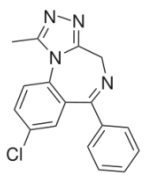
[24]. Most of the work directed with these devices has been focused on protein, peptides and proteomic based research [24-26], however there has also been work carried out with small molecule drugs [27].

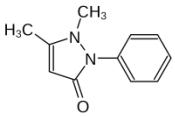
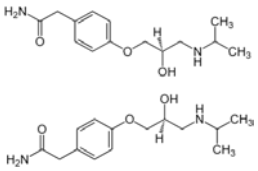
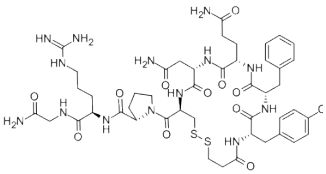
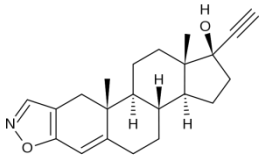
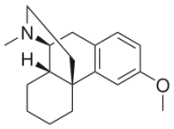
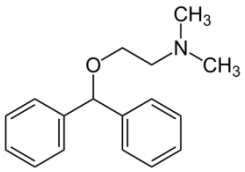
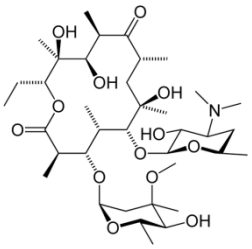
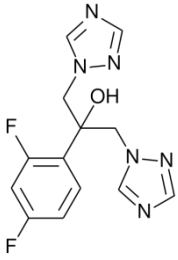
In this chapter the development of a 300 μm i.d. ceramic microfluidic device packed with sub 2 μm alkyl-bonded porous hybrid – silica particles for the analysis of small molecules and peptide analytes in solvent and biological samples is discussed. This chapter will demonstrate the chromatographic performance, robustness, and sensitivity benefits, of such a device. The chapter will further describe the development of a closed ESI MS source capable of working at a wide range of capillary voltages and within the operating flow rates of the separations device. Finally the performance of this device compared to standard analytical UPLC will be presented.

5.2 Materials and Methods

5.2.1 Chemicals and Materials

Acetaminophen, antipyrine, atenolol, danazol, desmopressin, dextromethorphan, diphenhydramine, erythromycin, fluconazol, propanolol, pentamidine, metoptolol, nefazadone, and ammonium acetate (LC/MS grade) were purchased from Sigma Chemical Co. (St. Louis, MO., USA). Their structures are shown in **Table 5.2.1** Acetonitrile, ammonium hydroxide, formic acid, and methanol were all purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Alprazolam, and the hydroxyl metabolite were obtained from Cerilliant (Round Rock, TX, USA). Salmeterol was purchased from Toronto Research (Toronto, Canada) Rat plasma was obtained from Equitech-Bio (Kerrville, TX, USA). Water was obtained from an in-house milli Q filtration system.

Number	Compound	Structure	Class	Indication
1	Acetaminophen		• Analgesic	Pain reliever Antipyretic
2	Aldosterone		• Steroid hormone	Biomarker
3	Alprazolam		• Benzodiazepines	Anti anxiety and sedative-hypnotic actions

4	Antipyrine		• Pyrazolones • Benzene and Derivatives • Anilines	Analgesic
5	Atenolol		• Phenols and Derivatives • Ethers • Phenethylamines • Anisoles	Cardioselective beta-adrenergic blocker
6	Desmopressin		• Peptide	Diabetes Nocturia
7	Danazol		• Steroids • Steroid Derivatives	Treatment of endometriosis and some benign breast disorders
8	Dextromethorphan		• Morphinans • Benzylisoquinoline	Antitussives
9	Diphenhydramine		• Benzyl alcohol dereivatives	Antihistamine
10	Erythromycin		• Macrolides	Macrolide antibiotic
11	Fluconazole		• Benzyl Alcohols and Derivatives • Cumenes and Derivatives • Phenethylamines	Antifungal

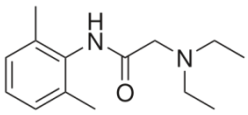
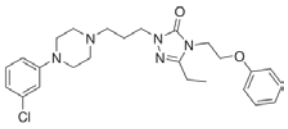
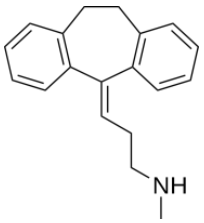
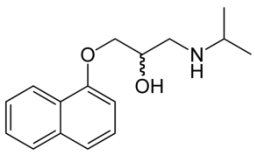
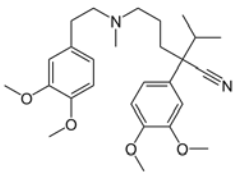
12	Lidocaine		• Acetanilides	Local anesthetic and cardiac depressant
13	Nefazodone		• Phenols and Derivatives • Ethers • Halobenzenes • Anisoles • Phenyl Esters • Anilines	Antidepressant
14	Nortriptlyine		• Dibenzocyclohepte -nes	Antidepressant
15	Propranolol		• Naphthalenes	acute myocardial infarction, arrhythmias, angina pectoris, hypertension
16	Verapamil		• Catecholamines and Derivatives	Anti-arrhythmia agent

Table 5.2.1 Pharmaceutical compounds utilized in this study.

5.2.2 Preparation of gradient test mix

Atenolol, pentamidine, metoprolol, antipyrine, dextromethorphan, diphenhydramine, erythromycin, and danazol were dissolved in methanol at a concentration of 1 mg/mL to make stock solutions. The individual analytes were then combined and diluted with a solution of 95:5 warter:methanol to produce a dilution series from 0.1 ng/mL to 1000 ng/mL.

5.2.3 Preparation of alprazolam test mix

Alprazolam, and hydroxyl-alprazolam metabolite were dissolved in 50:50 methanol: water and further diluted with 95:5 water:methanol to produce spiking standards. The spiking standards were then diluted in rat plasma to produce a dilution series. A simple protein crash was then performed using a 2:1, acetonitrile:plasma mix. The samples were vortex mixed and then centrifuged at 13,000 RCF for 10 minutes. The supernatant was then removed and diluted 50:50 with H_2O.

5.2.4 Preparation of prototype MS source desolvation gas/temperature test

Acetaminophen, dextromethorphan, fluconazole, and verapamil were dissolved in methanol at a concentration of 1 mg/mL. The samples were then diluted with a solution of 50:50 methanol: water with 0.1 % formic acid (v/v) to a concentration of 50 ng.mL. All compounds were infused at their optimal capillary and cone voltages at 10 µL/min.

5.2.5 Preparation of standards for LC platform comparison

Alprazolam, hydroxyl alprazolam, fluconazole, and nefazodone were dissolved in 100 % methanol to make a 1 mg/mL stock solution. Samples where then diluted with

95:5 (water:acetonitrile) to make a concentration series from 0.01 ng.mL to 1000 ng/mL. Desmopressin was dissolved in 95:5 (water with 0.1%formic acid:acetonitrile). Samples were then diluted with the same solution to make a concentration series from1000 ng/mL to 0.1 ng/mL.

5.2.6 300 µm i.d. Chromatography

Chromatography was carried out on a modified nanoACQUITY™ UPLC system (Waters Corporation, Milford, MA, USA) equipped with a prototype ceramic microfluidic device (CMFD) with a 0.3 mm x 100 mm channel packed with 1.7 µm BEH C_{18}. The CMFD was thermostatically controlled at 45 °C. Control of device temperature was accomplished via the use of insulating material and an electrical heater controlled via resistance adjustment. The mobile phase consisted of A: 0.1% formic acid, or 0.1 % ammonium hydroxide, or 10 mM ammonium acetate, adjusted to pH 3.0 or 10.0 and B: acetonitrile. The analytes were eluted under a linear gradient from 5 – 95 % B at a flow rate of 10 – 12 µL/min on the UPLC system The column effluent from the system was routed into a Xevo TQ mass spectrometer operated in electrospray positive ionization mode and in SRM mode acquisition.

5.2.7 Analytical Scale Chromatography

1 µL of each sample was injected onto an ACQUITY® Ultra Performance LC® separations module (Waters Corporation, MA, USA). Chromatography was performed on a 2.1 x 50 mm 1.7 µm ACQUITY BEH C_{18} column. Mobile phase A consisted of water with formic acid (0.1% v/v) while mobile phase B consisted of acetonitrile. The column was maintained at 45 °C and eluted under linear gradient conditions from 5 to 95 %B over 2.0 minutes at a flow rate of 0.6 mL/min.

5.2.8 Mass Spectrometry

Mass spectrometry was performed on a Waters Xevo™ TQ mass spectrometer (Waters Corporation, Manchester, UK) equipped with a traditional high flow source modified with a narrow bore (75 µm) steel capillary and a prototype modified electrospray interface. Capillary, cone and collision energies were optimized for each compound. The mass spectrometer was operated in SRM mode. **Table 5.2.2** lists the transitions and conditions utilized for each of the compounds analyzed in this work. Desolvation gas flow and temperature were also optimized.

Compound Name	Cone (V)	Collision Energy (eV)	SRM
Alprazolam	40	25	309.2 > 281.0
Alprazolam OH	40	25	325.0 > 297.0
Antipyrine	36	26	189.3 > 106.3
Atenolol	30	24	267.2 > 144.9
Danazol	30	24	338.3 > 148.0
Desmopressin	18	16	535.5 > 328.2
Dextromethorphan	40	38	272.3 > 171.0
Diphenhydramine	14	12	256.3 > 167.1
Erythromycin	28	32	716.8 > 158.1
Fluconazole	28	16	307.2 > 220.0
Nefazodone	48	34	470.3 > 246.1
Metoprolol	24	14	262.2 > 233.0
Pentamidine	40	38	341.3 > 120.0

Table 5.2.2 SRM transitions and MS conditions utilized for each compound.

5.2.9 Converting a nanoACQUITY UPLC system for non proteomics based application work

The Waters nanoACQUITY UPLC system as designed by K. Fadgen *et. al.* was designed as proteomics based nano to micro-bore scale LC system to interface with MS, primarily, Q-TOF based mass analyzers. In order to optimize the system for drug metabolism and bioanalytical work several attributes of the system had to be addressed in order for the system to accommodate the sub 2 µm chromatographic particle CMFD.

The first of these was to extend the upper limit of the operating pressure range of the system. This was achieved through the use of an algorithm that enables the system to operate above the previous upper pressure limit of 5000 psi up to a limit of 15,000 psi. In addition to this change, tubing(s), and flow sensors needed to be replaced with higher pressure tolerant variants.

In addition to enabling the increased upper operating pressure limits of the LC system the removal of system pressure ramping was also addressed. The use of pressure ramps for standard proteomic applications is based on the fact that in most proteomic analysis a two dimensional chromatographic plumbing configuration aims first of all to decrease sample loading time and then provide increased resolving power by increasing chromatographic selectivity and the means to efficiently separate complex proteomic samples. The pressure ramps were originally instituted in an order to remove any issues with flow sensor compensation created by the pressure differences related to column lengths, i.d.s or particle size or any further configuration of these combinations. Therefore this ramping capability was removed from the software to provide for the delivery of constant pressure during analysis.

The last major modification that was carried out was the installation of fittings and tubings that could withstand high pH, *i.e.* mobile phase pH values greater than eight. The impetus for the desire to make these changes in the LC system in order to operate at higher pH levels was shown previously in Chapters 2 through 4. This modification was accomplished through the use of highly polished silica-capillary tubing. This highly polished silica-capillary tubing impedes dissolution of the silica material by limiting the exposure of the basic solution to the silica tubing surface by decreasing the surface area interactions due to the nature of the highly polished surface.

5.3 Results and Discussion

5.3.1 Construction of the CMFD

High-temperature co-fired ceramics (HTCC) technology has been widely used in the production of multilayered electronic circuits. Unlike common printed circuit board (PCB) technology the HTCC fabrication process enables passive electronic components to be embedded with multiple layers on ceramic material resulting in a high density complex board in a very small scale. This same principle has been implemented to create a microfluidic device. Instead of using a multi-layered technique to embed electronic components, fluidic channels are created within the device. Thereafter the device was packed with chromatography media at high pressure creating a compact efficient chromatographic column.

The manufacturing process begins with a raw material called “green” tape it is referred to as “green” because of its virgin quality as it has not been manipulated in manufacturing process(s). **Figure 5.3.1** shows an example of the “green” tape before the CMFD manufacturing process. The tape consists of ceramic material and organic binder.

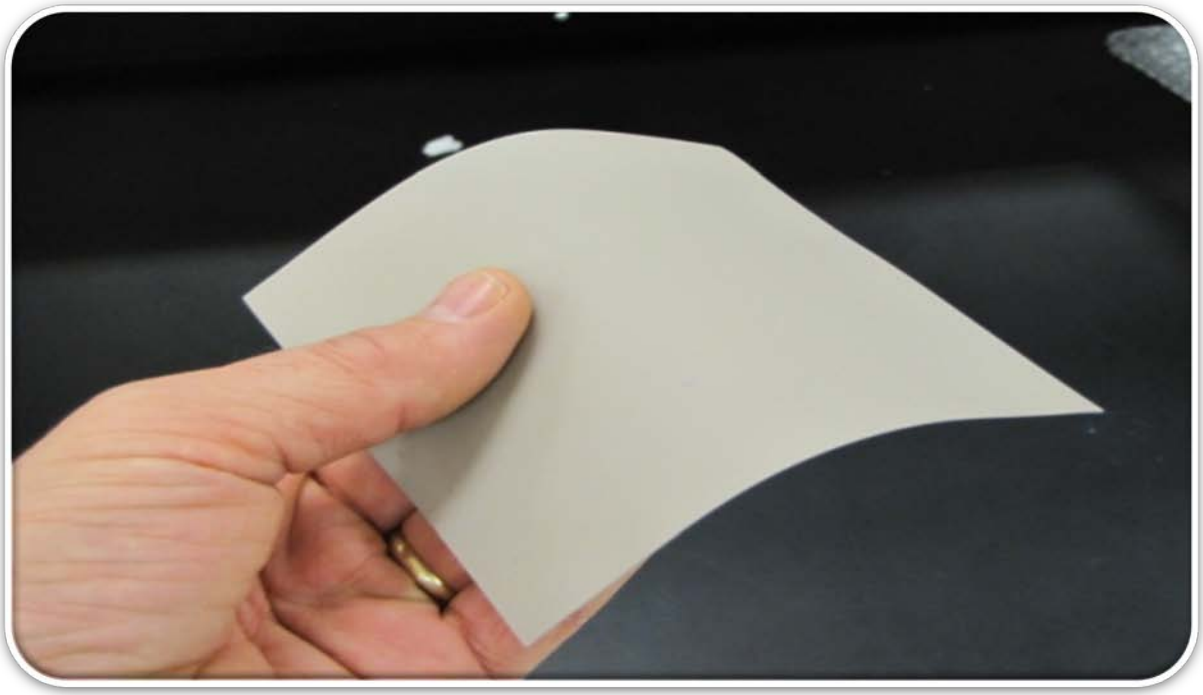


Figure 5.3.1 Picture of green tape before development process. Figure courtenay of Waters Corporation

A precision UV micromachining system is used to create a microfluidic channel on a single sheet of tape. The thickness of the tape defines the ultimate height of the microfluidic channel, the width of the laser cut determines the channel width. The centre layer is sandwiched by two pairs of sheets closing off the channel creating either a square or rectangular cross section. Vias are placed through the top two layers of green tape above the origin and the terminal end of the channel to serve as ports to direct fluid into and out of the device.

The five-layered stack of green tape is laminated under isostatic pressure and elevated temperature ensuring that all layers are in direct contact prior to firing. The firing process occurs in two steps: An initial low-temperature organic burnout is used to remove the organic binder. Following this stage, the furnace temperature is slowly ramped to the sintering temperature of the ceramic. During the sintering process the

ceramic glass particles begin to melt and flow while the structure of the device and internal features are maintained. This process is shown in **Figure 5.3.2.** The densification which accompanies the sintering process results in a monolithic structure with an embedded channel able to withstand high pressures. In this prototype device a 0.3 mm i.d. channel was capable of 13 kpsi. At this stage the device can be packed with chromatographic material as shown in **Figures 5.3.3** and **5.3.4**.

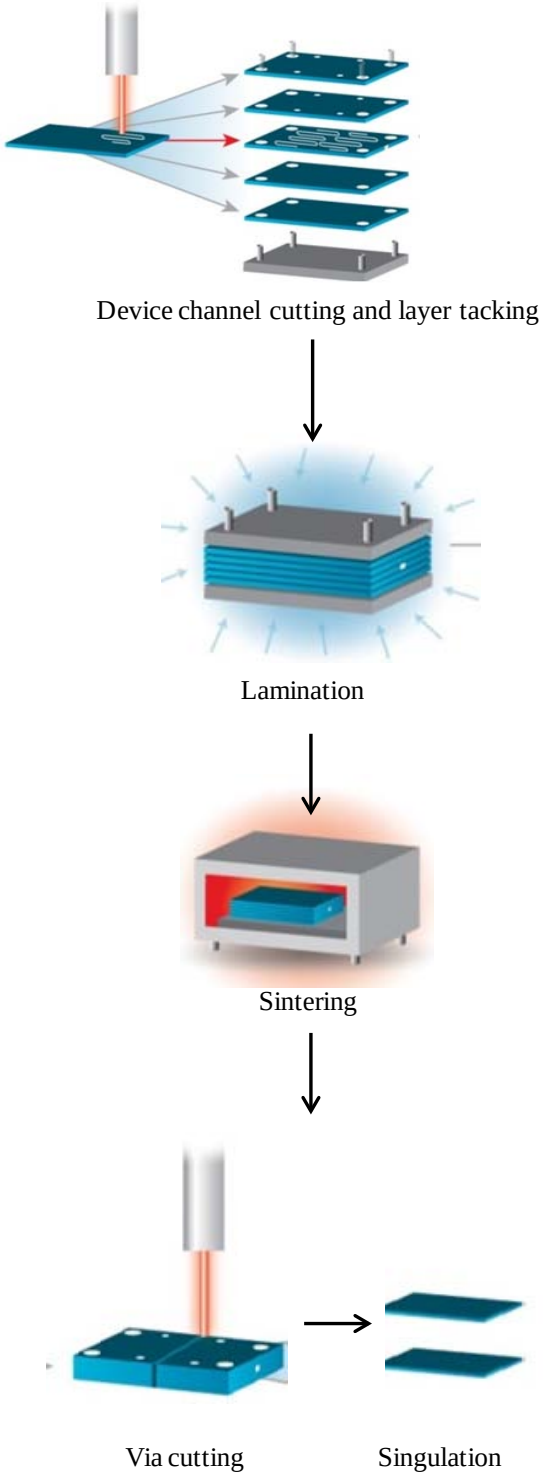
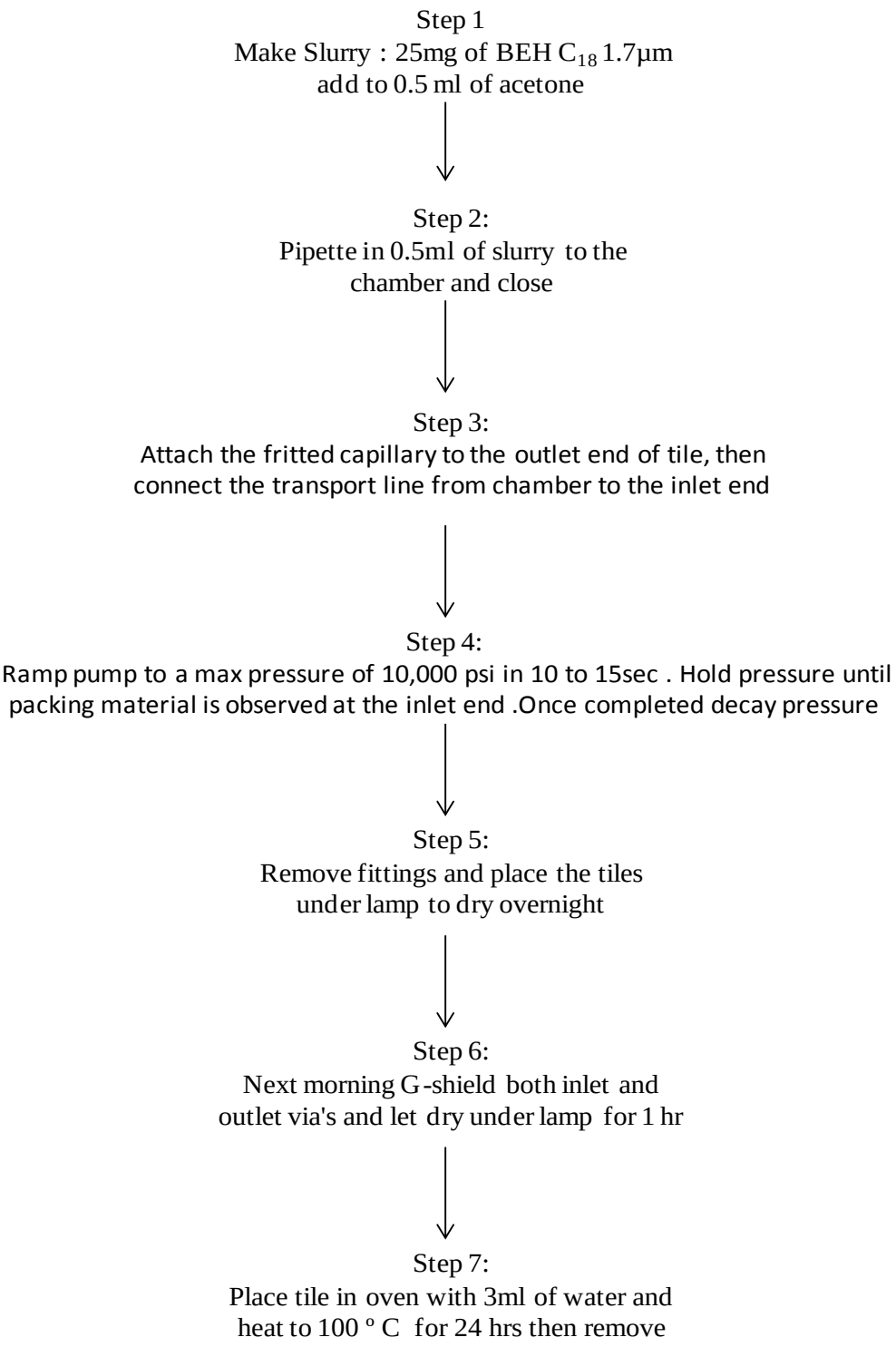


Figure 5.3.2 Manufacturing process of CMFD. Figure courtesy of Waters Corporation.



5.3.3 CMFD packing procedure.

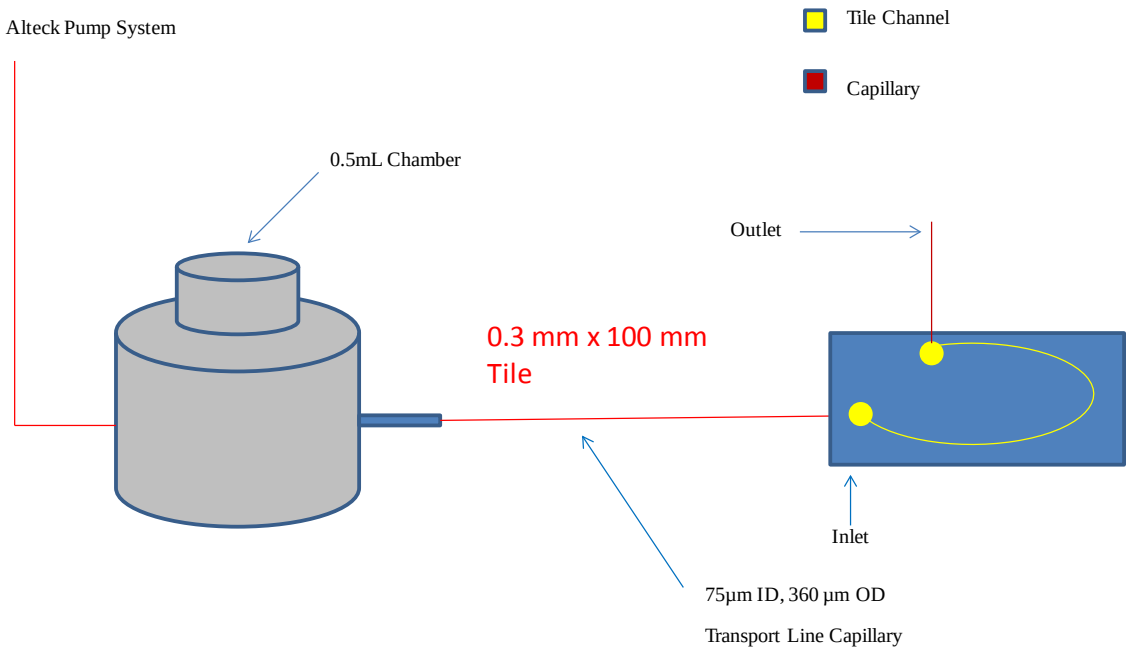


Figure 5.3.4 Packing bomb with CMFD. Figure Courtesy of R. Collamati Water Corporation

5.3.2 Chromatographic performance and efficiency

One of the most important factors to be considered when evaluating a new separations device format is the chromatographic performance. The performance of the system was tested in both isocratic and gradient mode. This approach would allow for a true evaluation of the separation performance of the system across all applications areas. The chromatographic performance of the prototype device, **Figure 5.3.5** and **5.3.6**, was compared to that of a 0.3 mm capillary column packed with identical separation media, 1.7um C_{18} bonded bridged ethyl-hybrid silica material. The isocratic performance efficiency for a 0.3 mm x 100 mm packed column was determined to average 10,219 plates for n=6 and that of the prototype microfluidic device was measured to be 9038 plates for n=6. **Figure 5.3.7** shows the results from testing of one of the CMFDs

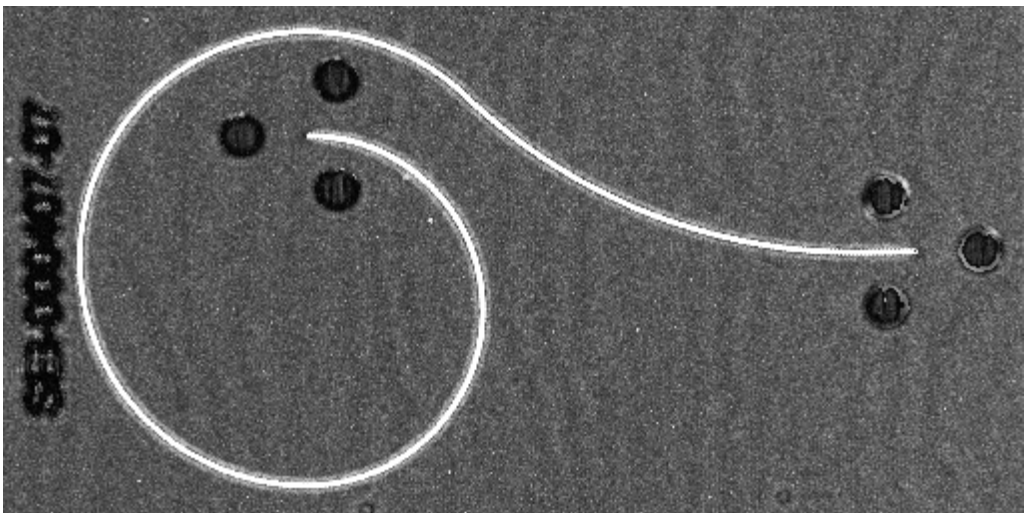


Figure 5.3.5 Sonogram of prototype “question mark” fluidic path CMFD 0.3 x 100 mm.

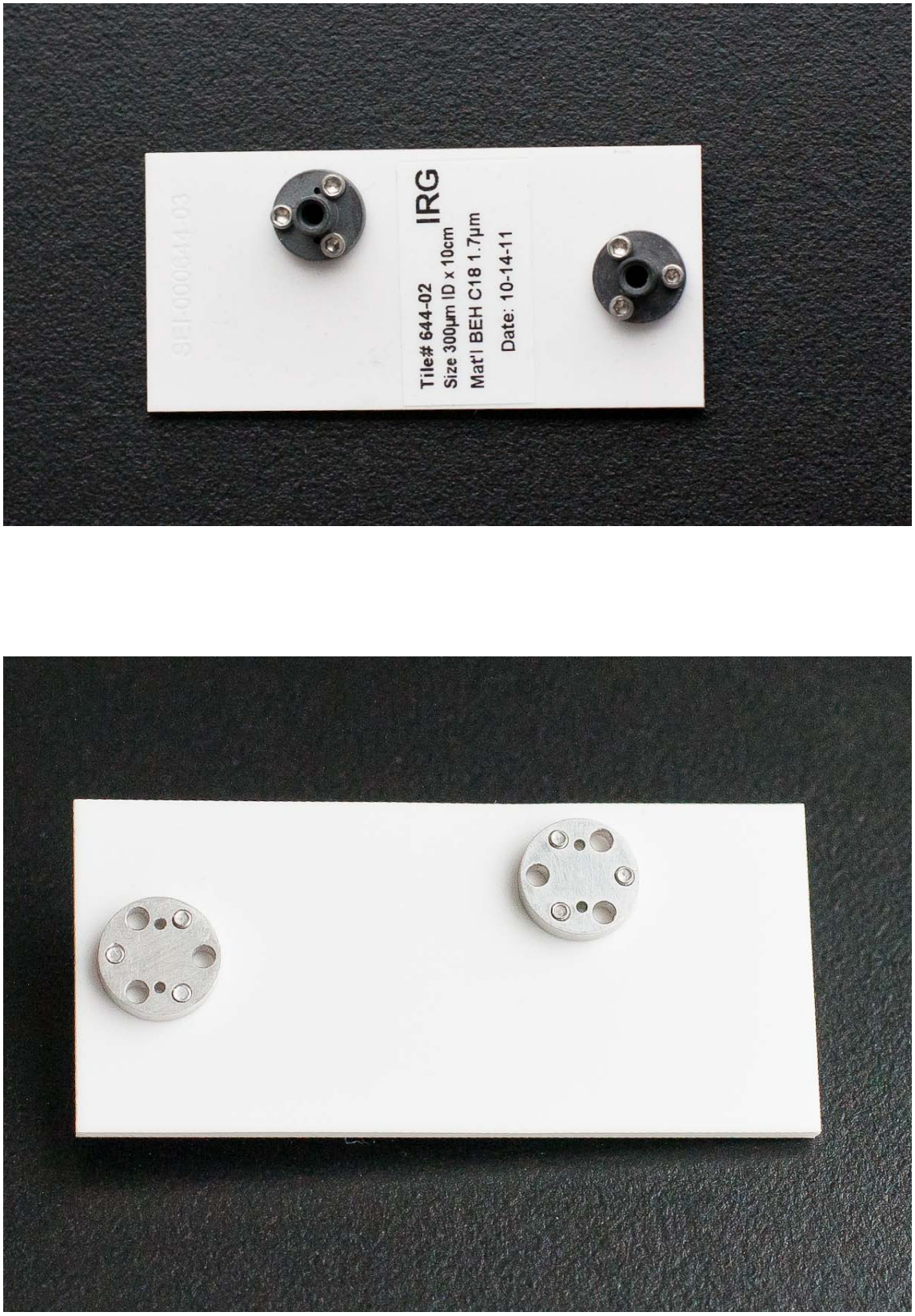


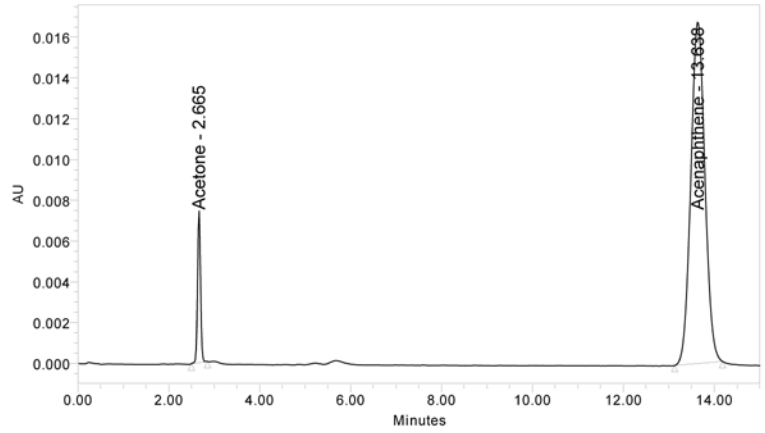
Figure 5.3.6 Front (top) and back (bottom) view of prototype CMFD 0.3 x 100 mm.

300µm x 100 TL Report

Reported by User: System Project Name: HTCC

SAMPLE INFORMATION

Sample Name:	HTCC 311-06 300x100mm	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	4/15/2009 1:49:22 PM EDT
Vial:	1	Acq. Method Set:	HTCC MS
Injection #:	1	Date Processed:	4/24/2009 9:37:42 AM EDT
Injection Volume:	5.00 ul	Processing Method	HTCC Tile Process
Run Time:	15.0 Minutes	Channel Name:	ACQUITY TUV ChA
Sample Set Name:		Proc. Chnl. Descr.:	ACQUITY TUV ChA 254nm



	Peak Name	RT	Area	% Area	Height	K Prime	Width @ 4.4%	USP Tailing	USP Plate Count
1	Acetone	2.665	33943	8.60	7313	1.66	0.1573	1.022	7510
2	Acenaphthene	13.638	360753	91.40	16758	12.64	0.7304	1.043	9114

Label

Material

Packing_Pressure 5500

Testing_Pressure_Tile_Only

Report Method: 300µm x 100 TL Report Printed 11:28:51 AM 1/29/2013 Page: 1 of 1

Figure 5.3.7 Example packing efficiency test for CMFD.

	Capillary column	Ceramic microfluidic
1	8287	8689
2	10329	8892
3	11813	8995
4	11747	7289
5	8640	11250
6	10500	9114
Average N	10219	9038
Stdev	1496	1273
% RSD	15	14

Table 5.3.1 Plate count (N) for multiple standard capillary columns and CMFDs.

The reversed-phased gradient performance of the system was investigated using a test mix modeled on samples previously developed by Astra Zeneca (AZ) for testing of capillary LC performance. A linear aqueous ammonium acetate pH 3.0 and acetonitrile gradient of 5-95% over 6 minutes at a flow rate of 10 µL/min was employed for the analysis. The tile was maintained at 45 °C. The analyte detection was performed on a tandem quadrupole mass spectrometer equipped with a low volume, 75 um internal diameter, and stainless-steel capillary to limit chromatographic peak dispersion. The result of this test can be seen in **Figure 5.3.8**. The data observed for the CMFD showed gaussian peak shape for all analytes within the test mixture and a average peak width between 4 and 7 seconds, measured at peak base, producing a peak capacity of 55 for this separation of six minutes. In addition the chromatographic performance; rudimentary four point calibration of each of the components, dissolved in 95:5 water:ACN, was carried out over a range of 0.1 ng/mL to 100 ng/mL. The data in **Figure 5.3.9** shows the calibration line for atenolol. Here we observe good linearity of the compound over the previously described concentration range, with a linear, 1/x fit producing a R^2 value of 0,99375. The results of these experiments are further shown in

Table 5.3.2. for the additional compound in the AZ test mix. Again good linearity was obtained for the rest of the compounds with the exception of erythromycin due to the detection of multiple peak under the conditions utilized during the test.

Figure 5.3.8 Separation of AZ test mix.

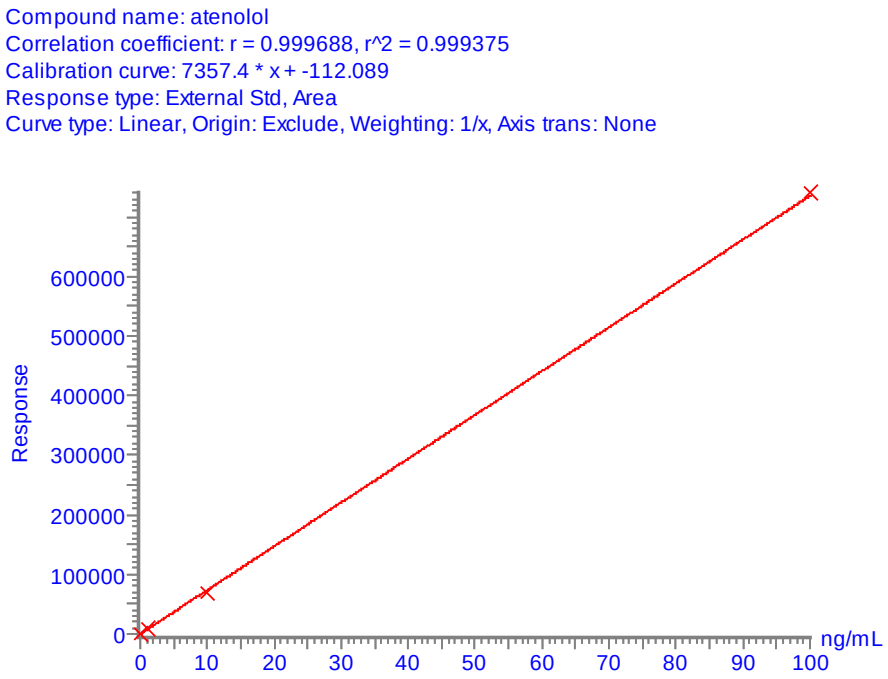


Figure 5.3.9 Four point calibration line of atenolol solvent standard from 0.1 ng/mL – 100 ng/mL.

Compound	Curve type	Weighting	Correlation coefficient
antipyrine	linear	1/x	0.991152
danazol	linear	1/x	0.998556
dextromethorphan	linear	1/x	0.999312
diphenhydramine	linear	1/x	0.998637
erythromycin			
metoprolol	linear	1/x	0.999817
pentamidine	linear	1/x	0.998515

Table 5.3.2 AZ test mix calibration line weighting and correlation coefficients for individual solvent standards.

5.3.3 Ceramic microfluidic device reproducibility

Reproducibility of a bioanalytical separation is a key aspect that must be assessed during method development in order for the method to be successfully implemented for validation and subsequent use by the analytical laboratory for the analysis of clinical samples. Maintenance of chromatographic resolution between parent drugs and their associated metabolites is especially important as conversion of drug metabolites may convert back to their parent form in the source of the MS [28]. The result of this in-source conversion process is that the levels of the parent drug may be overestimated leading to erroneous results being reported by the analytical team to the clinicians. Therefore and evaluation of four prototype 0.3 mm x 100 mm CMFDs was carried out utilizing the probe pharmaceutical and the hydroxylated metabolite as a critical resolution pair to assess the reproducibility between different ceramic tiles. The chromatogram displayed in **Figure 5.3.10** illustrates the separation between parent drug and metabolite.

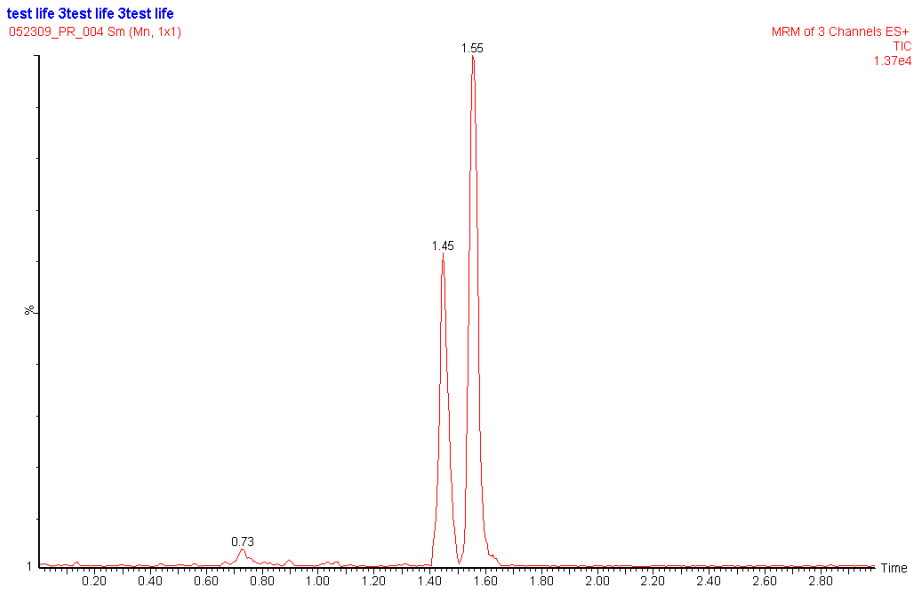


Figure 5.3.10 Separation of alprazolam and hydroxyl metabolite utilized to assess device to device reproducibility**.**

The separation utilized a fast three minute gradient at a flow rate of 12 µL/min with the tile thermostatically controlled at 45 °C. 1 µL injection volume of the compound solvent standards was utilized. **Table 5.3.3** shows the data for four different 0.3 x 100 mm ceramic tiles.

n=6 injection	Device 1	Device 2	Device 3	Device 4
Retention time Alprazolam	1.55	1.57	1.56	1.56
% RSD	0.33	0.00	0.94	0.35
Retention time OH Alprazolam	1.45	1.48	1.46	1.46
% RSD	0.28	0.55	0.80	0.27
Rs at 5% height	1.50	1.20	1.33	1.17

Table 5.3.3 Reproducibility between four 0.3 x 100 mm CMFDs.

The data shown in **Table 5.3.3** indicates that the ceramic tiles showed very reproducible separations of alprazolam and hydroxyl-alprazolam metabolite for four different ceramic tiles. This indicates that the packing procedure is capable and sufficient for these types of analyses. The data further indicates that the analytical system as a whole, *ie* autosampler, gradient delivery pumps, and temperature control can provide reproducible results while being run at this scale of chromatography.

5.3.4 Loading capacity example

When performing any form of LC/MS analysis the sensitivity of the system is partly dependent on the mass of material loaded onto the column. For a fixed concentration of analyte introduced onto the system, the greater the injection volume, the greater the

peak intensity. This will be true as long as the injection volume does not adversely affect the chromatographic peak shape. Change in peak shape manifest itself in one of two ways: mass overload, or volume overload [29]. In mass overload conditions the retention time of the peak is gradually reduced, and the peak takes on a characteristic shark-fin shape. In volume overload conditions the centre of the peak remains unchanged and the peak broadens in both directions. Both manifestations of chromatographic overload reduce the performance and usability of the system. To evaluate the maximum injection volume and loading capacity of the microfluidic system, two real situations were mimicked; the first was the loading of a model pharmaceutical (alprazolam) in an aqueous solution, the second was a bioanalytical analysis.

In the second case, a solution of alprazolam was prepared in a 60:40 acetonitrile:aqueous solution and diluted 1:1 with water. This organo-aqueous mixture replicates the situation encountered in drug discovery and some areas of drug development where simple protein precipitation with organic solvent is employed. The injection volume was gradually increased from 0.1 µL to 2 µL and the MS response and chromatographic peak shape was monitored (**Figures 5.3.11 and 5.3.12**). From this data we can see that for the aqueous solution the peak shape remained stable and the change in MS response with injection volume was linear up to an injection volume of 2 uL, whereas with the organo-aqueous solution the maximum loading volume was 1.2 uL before the peak became distorted. As the injection is increased beyond 1.2 uL with the organo-aqueous solution we see that there is evidence of severe peak fronting/shouldering and even the formation of two peaks. This is due to the high volume and organic modifier from the injection loading acting as a mobile phase and

carrying the drug analyte through the column. Thus for this application the maximum usable injection volume for a bioanalysis / DMPK type application would be 1 – 1.2 uL.

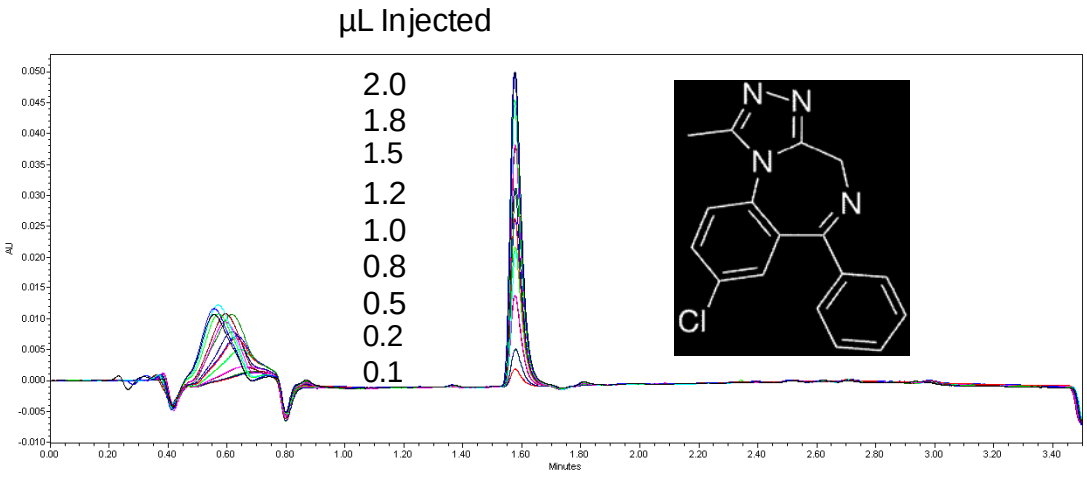


Figure 5.3.11 Injection series for alprazolam (1000 ng/mL) dissolved in 95:5 H2O:CH_3CN performed under gradient conditions.

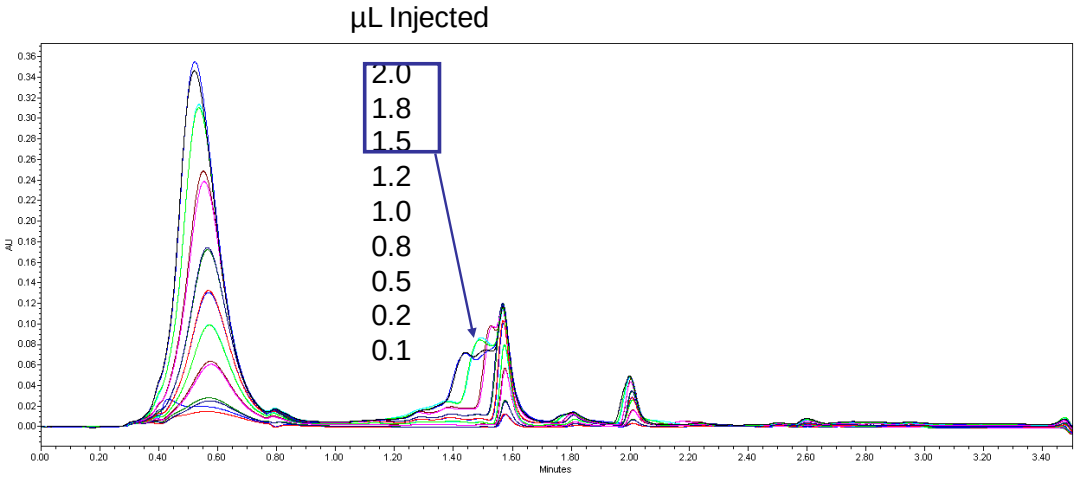


Figure 5.3.12 Injection series for alprazolam (1000 ng/mL) prepared in 33.3:66.6 H2O: CH_3CN performed under gradient conditions.

The injection volume of 1-1.2 uL compares favorably with the bed volume of 7uL of the micro-fabricated device. As with any form of compound analyzed by reversed-phase chromatography the more hydrophobic the compound, the greater the elution

strength required to elute the compound. Hence for these compounds, if injected in a lower strength solvent than the starting conditions of the mobile phase, a greater mass can be injected onto the column as they can be focused on the head of the column.

5.3.5 Tolerance towards plasma derived samples

The sensitivity increase of the micro–scale separation observed in this is attractive to the bioanalyst particularly for low volume samples, such as those from tail bled mice or genetically modified rodents. However in order to be an effective analytical technique the system must be robust towards the injection of plasma extract on the chromatographic system, allowing for routine day-to-day operation. The majority of bioanalytical assays require the analysis of seventy to one hundred samples in a single batch with the majority of bioanalyst expecting to obtain five hundred to one thousand injections per column. In order to evaluate the robustness of the CMFD towards plasma derived samples rat plasma was spiked with alprazolam and hydroxyl alprazolam metabolite at concentrations of 1000 pg/mL. The several replicate plasma samples were processed by protein precipitation with acetonitrile; the resulting supernatant was transferred to glass autosampler vials for analysis. A 1 μL aliquot of the resulting solution was injected onto a CMFD that contained a 0.3 x 100 mm column packed with 1.7 μm C_{18} organo-hybrid material. The sample was eluted with a 5-95% reversed phase acetonitrile – 0.1% aqueous formic acid gradient over three minutes at 12 μL/min. The samples were injected in batches of 100 injections after which the column performance was evaluated with the check standard. The data displayed in **Figure 5.3.13** shows the overlaid injection of the first and one thousandth injection. The column proved to be robust and reproducible over the injection of plasma derived

samples, with little to no reduction in column performance or increase in column backpressure.

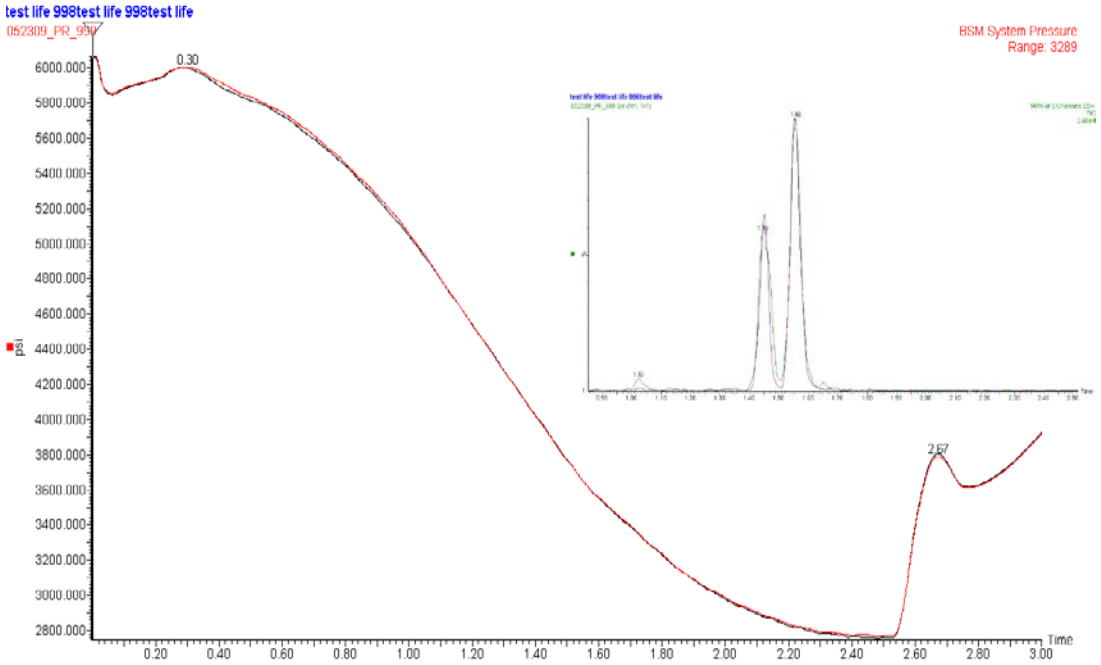


Figure 5.3.13 Overlay of pressure traces and separations of alprazolam and hydroxyl alprazolam at injection 1 and injection 1000 prepared in protein precipitated plasma.

5.3.6 Operation of system under high basic conditions and ESI + MS

The previous three chapters focused on the utilization and benefits of high pH (> 10 pH units) mobile phases with ESI + MS detection. Based on the results generated from these studies the desire to reproduce these types of conditions and to incorporate them with methods utilized with the ceramic tile and nanoscale LC/MS configuration described in this chapter would therefore be highly advantageous. Therefore testing was carried out to determine the feasibility of utilizing extremely high pH conditions with the ceramic tile. **Figures 5.3.14 and 5.3.15** show magnified sonogram images of the

crude ceramic material utilized in the building of the ceramic separations device. Incubation of the material was performed in pH 10.5 environment. This was accomplished by the addition of 1 mL of ammonium hydroxide to one liter of water. Both of the images show that there appears to be no dissolution of the material and the morphology of the material appears to be similar after incubation of the ceramic material in pH 10.5 solution. The BEH chromatographic particles were not a concern as they have been previously demonstrated to be capability to operate at basic pH [30]. In addition the LC system was modified as described in the experimental section in order that the capillary tubing and other fitting would be capable of withstanding prolonged exposure to these conditions.

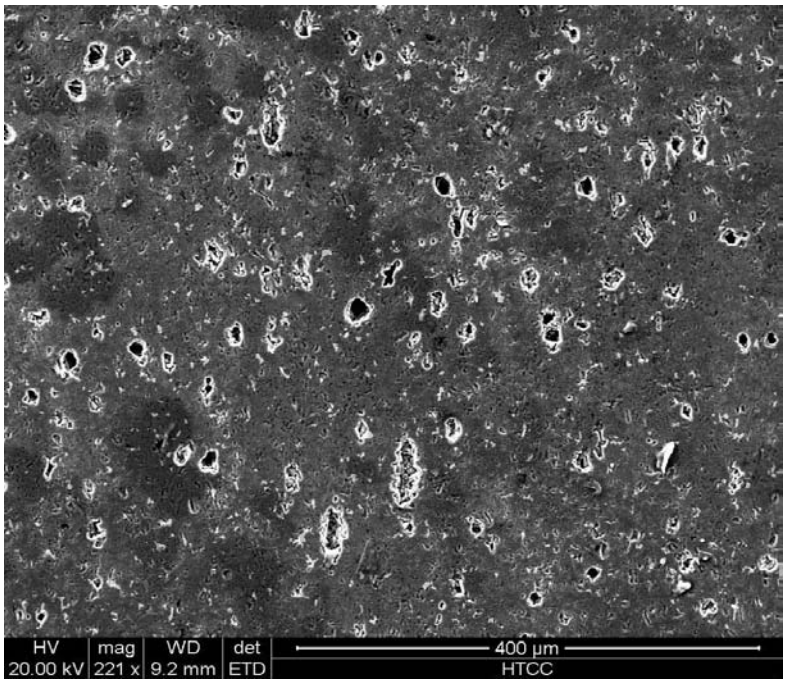


Figure 5.3.14 Magnified sonogram of ceramic material before incubation in pH 10.5.

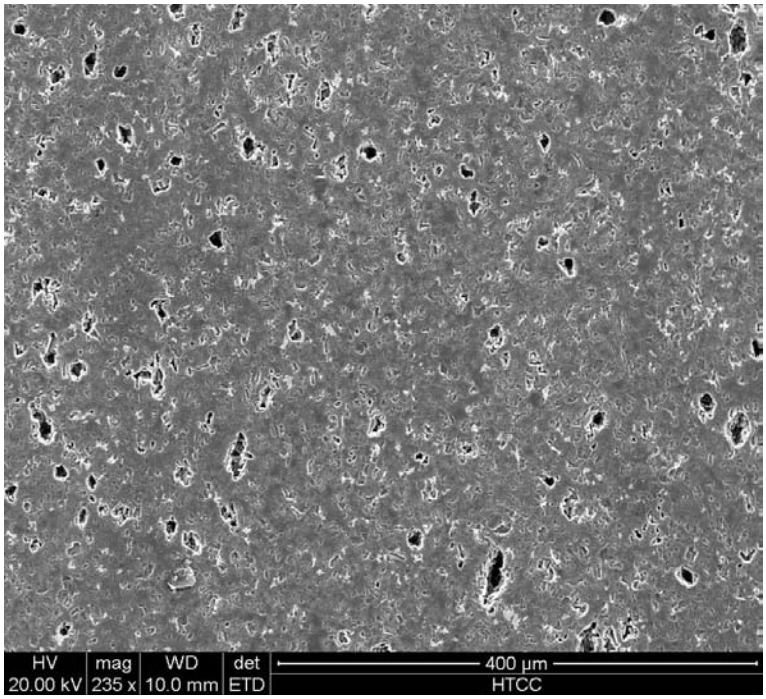


Figure 5.3.15 Magnified sonogram of ceramic material after 24 hours of incubation in pH 10.5.

However the modification of the typical silica-based tubing with high-polished silica tubing did not withstand the extreme of pH 10.5. The system resulted in over pressure in a time period of roughly 10 samples, one hour of constant run time. Examination of in line frits and filters resulted in the determination that the high-polished silica was dissolving when exposed to pH 10.5. Therefore the system was replumbed with the high-polished silica tubing and cleaned througly. High pH mobile phases was then remade with a pH of 10 utilizing a buffer of ammonium acetate. The data produced with this mobile phase combination yielded stable system back pressure and chromatographic sepersations. **Figure 5.3.16** below shows an example of alprazolam run with the pH 10 aqeous mobile phase.

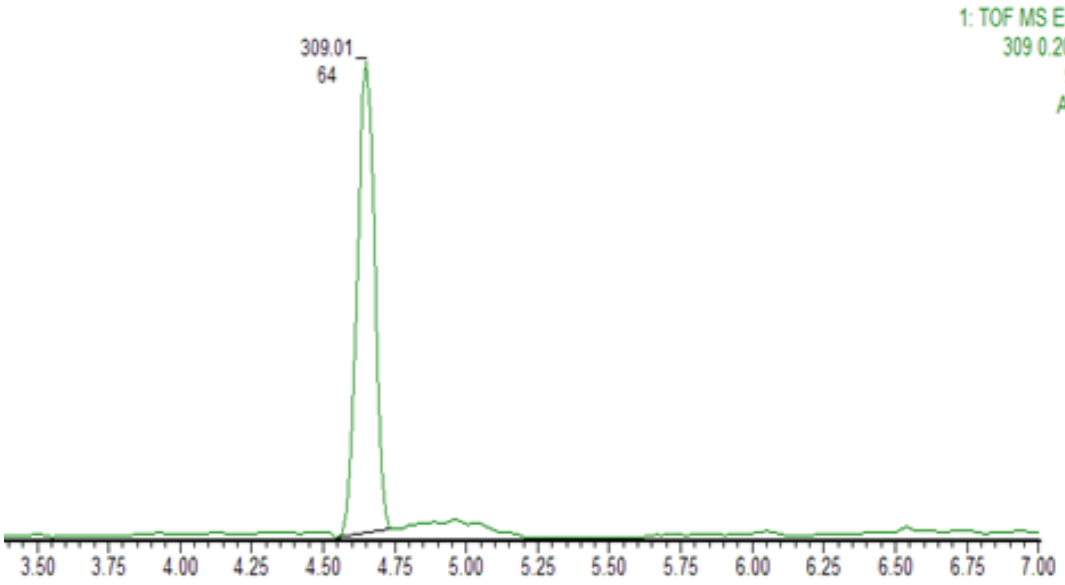


Figure 5.3.16 Separation of alprazolam under pH 10 on ceramic microfliudic device and modified nano LC system.

The chromatogram produced in Figure 5.3.14 had an average peak width of 7 seconds at base +/- 0.08 seconds over a period of six injections and a peak area of 61 area count +/- 2 area counts

The system was then run continuously over a 7 day period the pressure trace is shown in **Figure 5.3.17** is an overlay of the system pressure taken at the beginning of the day. As can be seen the system pressure is stable and no plugging of any frits, filters or tubings were observed over the test period.

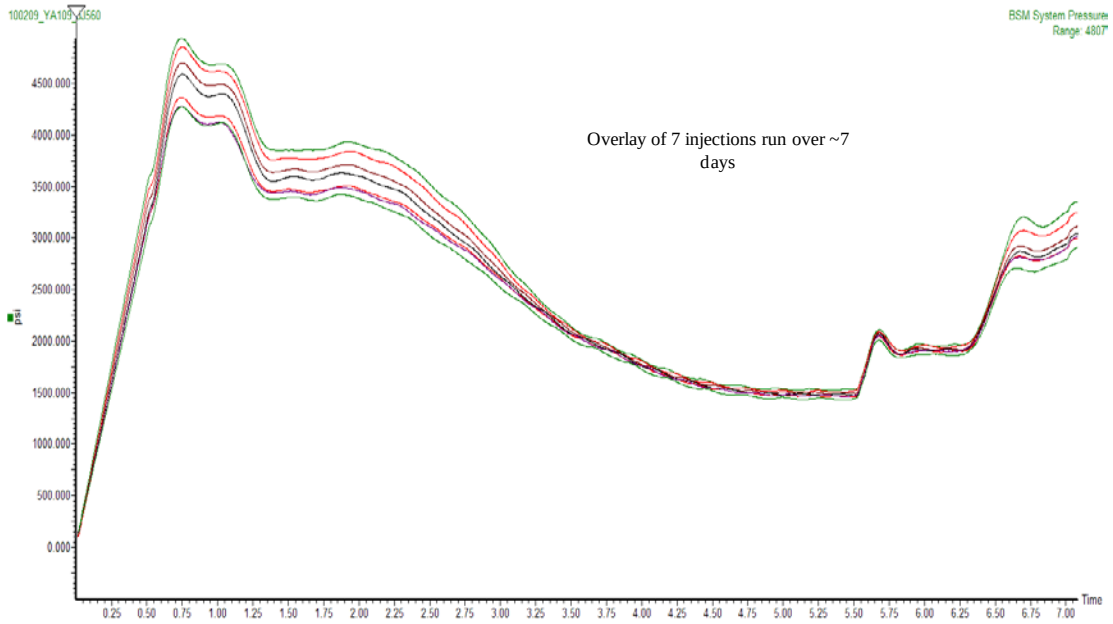


Figure 5.3.17 System pressure trace over a seven day period run under pH ten conditions.

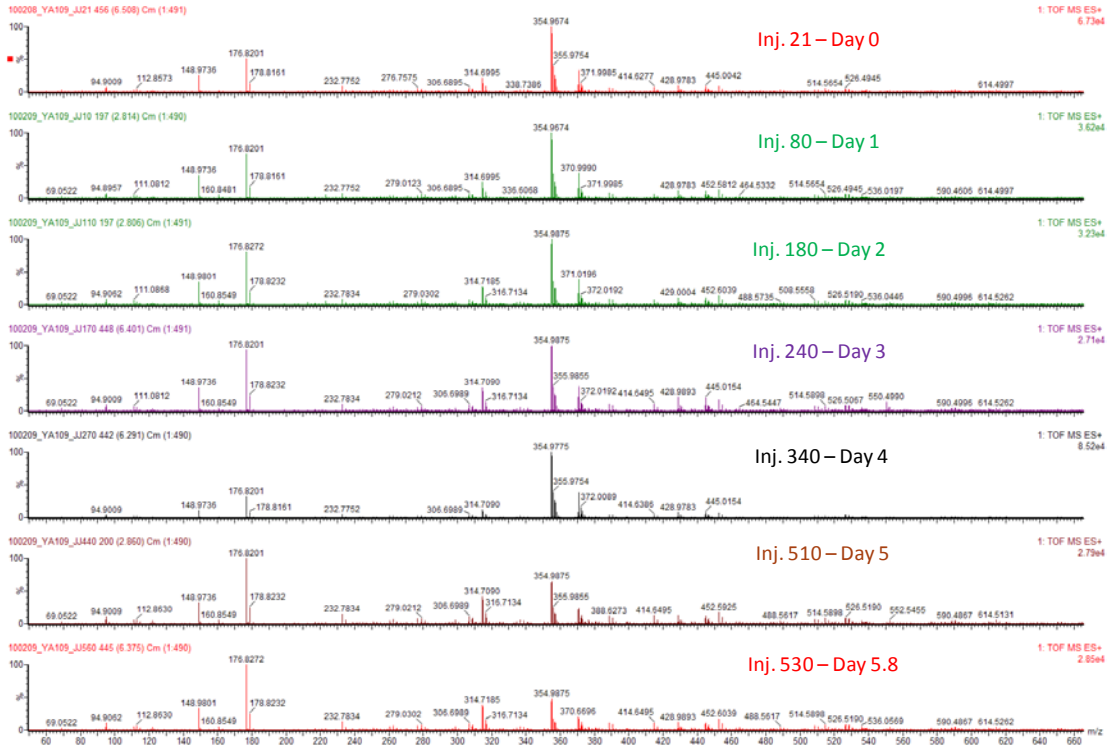


Figure 5.3.18 Overlay of summed spectra over gradient period of six days.

The MS background was further monitored over a six day period with the CMFD in line to determine if any chemical bleed or appearance of new additional ions were detected over the test period. This study was carried out to ensure an increase in chemical noise would not be produced by these method conditions as any increase could be indicative of system degradation and lead to contamination of the MS source. However, as the data from **Figure 5.3.18** shows there was no data to indicate that there was any degradation of the system and CMFD under these conditions and for the designated test period.

5.3.7 MS Source Optimization

5.3.7.1 Open versus closed MS Source(s)

Nano and capillary scale LC has been predominantly exploited in the field of proteomics for the detection and characterization of peptides and intact small molecular weight proteins. The majority of the MS sources employed in for these applications can be classified as “open-sources” and are not isolated from the general laboratory atmosphere. This is in contrast to the ion-spray sources (electrospray or turbo ion-spray) that are employed with conventional scale chromatography where the source is isolated from the laboratory environment. This “open-source” construction allows easy adjustment of spray position and optimization of response by the scientist, the higher mass-to-charge values monitored in proteomics, typically 600 m/z and above ensures that the majority of the chemicals present the laboratory atmosphere do not interfere with the experiment being undertaken. However with small molecule analysis these low molecular weight compounds present in the laboratory atmosphere can significantly impact assay performance. A comparison of the background signal obtained with both

the “open-source” construction and “closed-source” construction are shown in **Figure 5.3.19.**

These results show that the “open-source” possessed significantly more low m/z background signal than the “closed-source” construction. In the closed source there a relatively few ions above m/z = 300 with the m/z = 218 ion being the dominant ion, where as with the open source the m/z = 148.9 and m/z = 95 ion show a significant response as does the m/z = 354 and m/z = 370.9. While operation in SRM acquisition mode on a tandem quadrupole may eliminate or limit any interfering chemical noise from the ions illustrated in **Figure 5.3.19** there still exists the possibility of interference that could influence overall assay sensitivity via ion suppression.

This incidence of interference would be exasperated in full scan or SRM mode. This could become very problematic when carrying out full scan type work which is often the mode of operation when conducting biomarker discovery or “omics” types of experiments. As a result of this all of the experimentation conducted in this study was performed utilizing a prototype closed style source described later in this chapter.

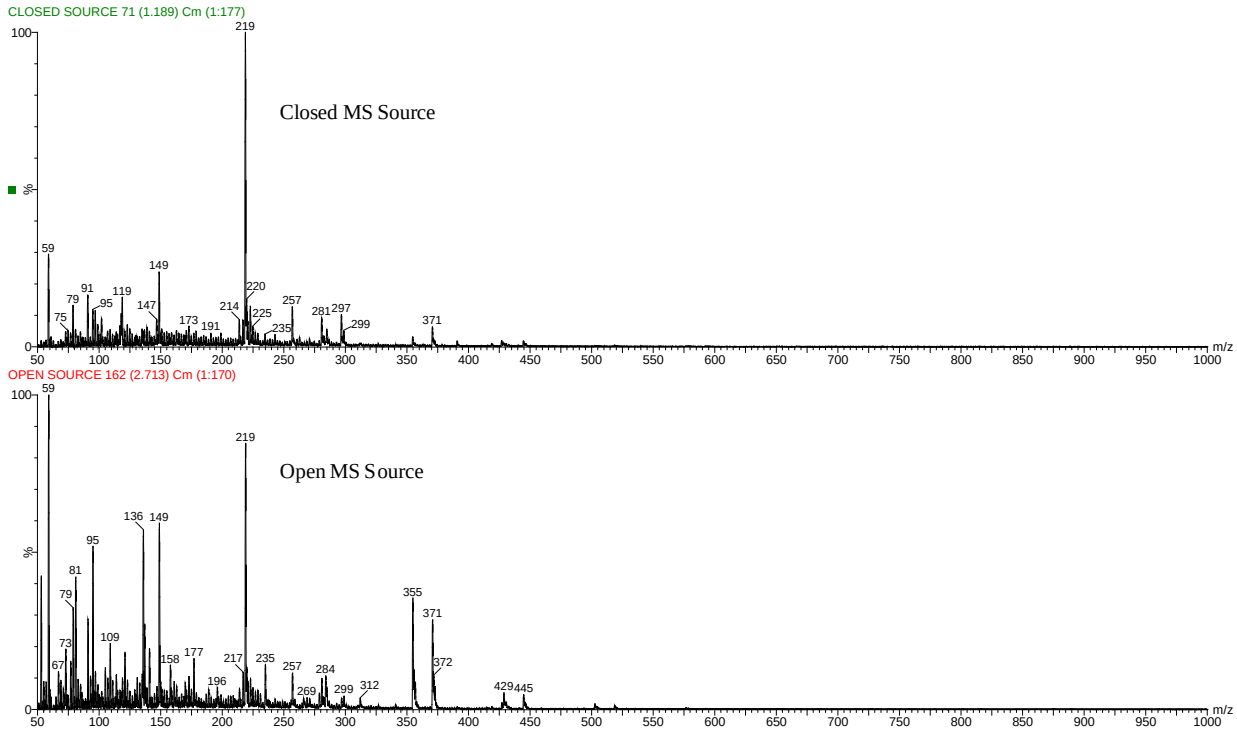


Figure 5.3.19 Summed spectra from blank chromatographic run on closed an open MS source.

5.3.7.2 Capillary voltage

In nano spray LC/MS the column flow rate is in the two hundred to four hundred nanolitres per minute and electrospray ionization process is assisted by the application of a capillary voltage (usually 3kV) to the capillary or spray tip. However, with capillary LC the column flow rate in the order of 3 to 10 µL per minute the column flow rate is too high for the column effluent to be nebulised by voltage alone. Thus for the capillary scale analysis a heated nebulising gas was added co-axially to the column spray tip to assist in the nebulisation process. There is an additional beneficial factor from the use of a nebulising gas and this is analyte response. The affect of capillary voltage on signal response is illustrated in **Figure 5.3.20** for the analysis of alprazolam operated at five different capillary voltages.

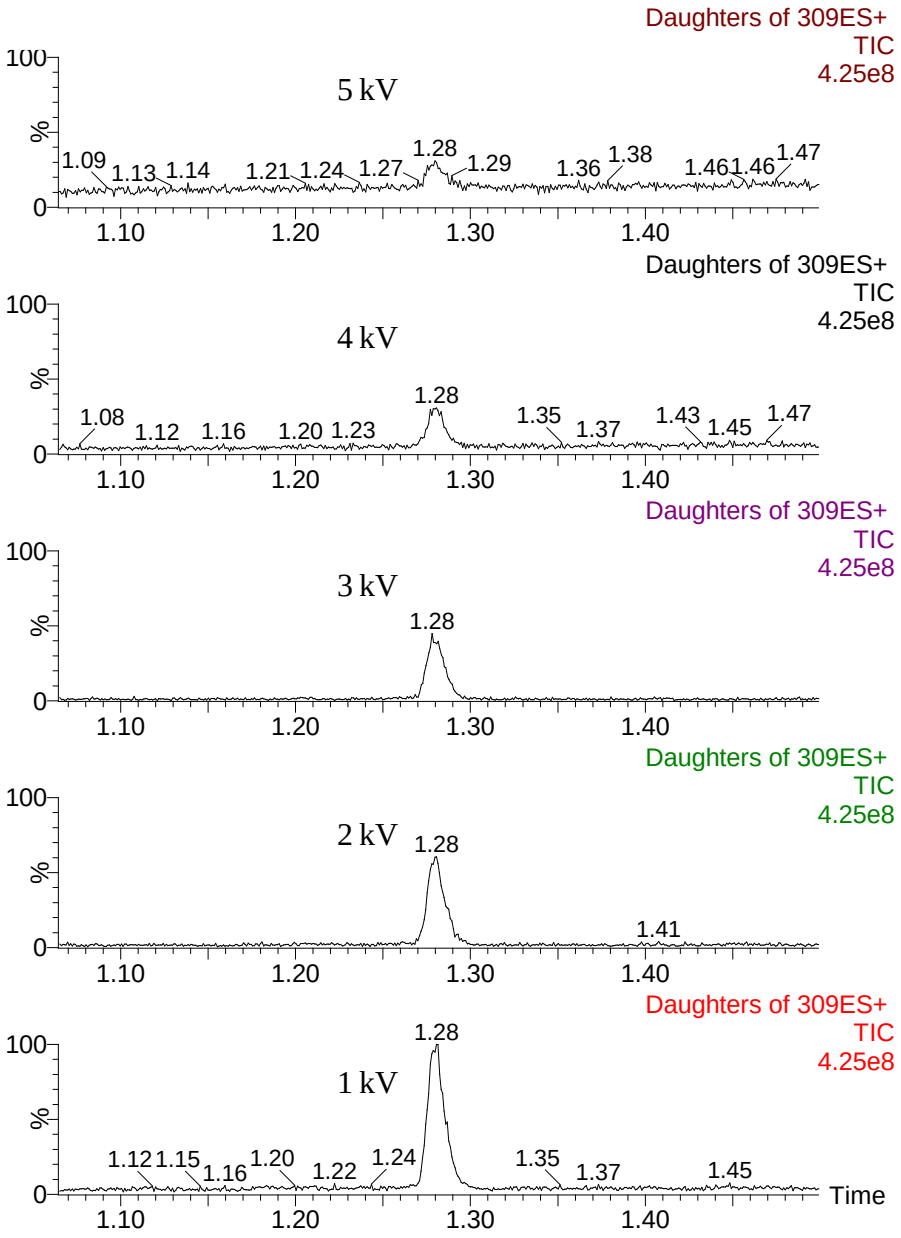


Figure 5.3.20 Influence of capillary voltage on alprazolam.

It can be clearly seen from this data that the use of a 1.0 kV value produces the greatest signal response. An additional drawback to using just the capillary voltage to effect ionization is that at low organic solvent compositions the mobile phase becomes increasingly difficult to ionize resulting in poor droplet formation at the end of the spray tip. This deleteriously affects the sensitivity of the system for the more polar analytes,

which elute with a low k′ value. These previous statements suggest that, at the 300 μm capillary scale, the use of capillary voltage alone is not ideal to promote ionization and nebulisation. As can be seen in **Table 5.3.4** many different molecules behave much like the alprazolam example in **Figure 5.3.18**. Here in this table we see that a vast majority, 80 percent of the molecules tested ionize best below the 3.0 kV setting. In Figures **5.3.21** and **5.3.22** the influence on the spray is seen at with a capillary voltage of 3 kV (**Figure 5.3.22**) and with a capillary voltage of 1 kV (**Figure 5.3.21**).

Compound	Class	Capillary Voltage (kV)
Aldosterone	Steroid Biomarker	2.5
Alprazolam	Benzodiazepine Sedative	1.0
Lidocaine	Amide Anesthetic	2.1
Fluconazole	Trazole Antifungal	0.5
Propranolol	Beta Blocker	3.2
Verapamil	Calcium Channel Blocker	2.0
Danazol	Steroid	1.7
Nortriptyline	Tricylic Antidepresent	2.0
Acetaminophen	Aniline Analgesics	0.5
Desmopressin	Peptide Hormone	3.0

Table 5.3.4 Optimal capillary voltages for various pharmaceuticals.

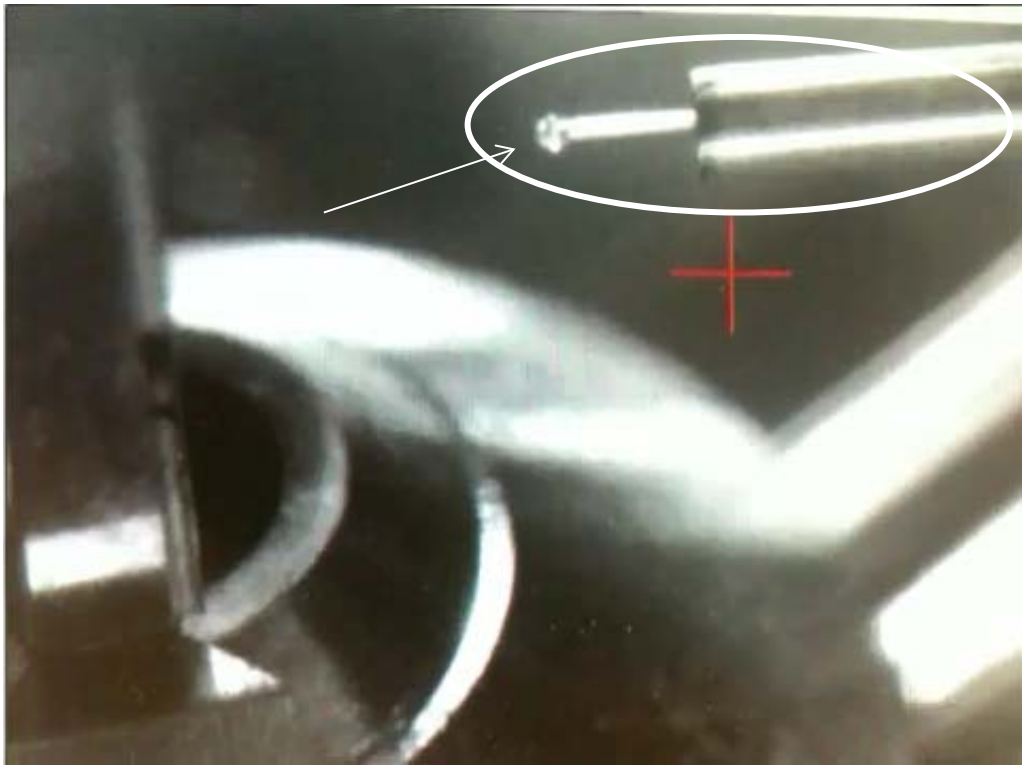


Figure 5.3.21 Column effluent exiting MS capillary probe with 1 kV applied.

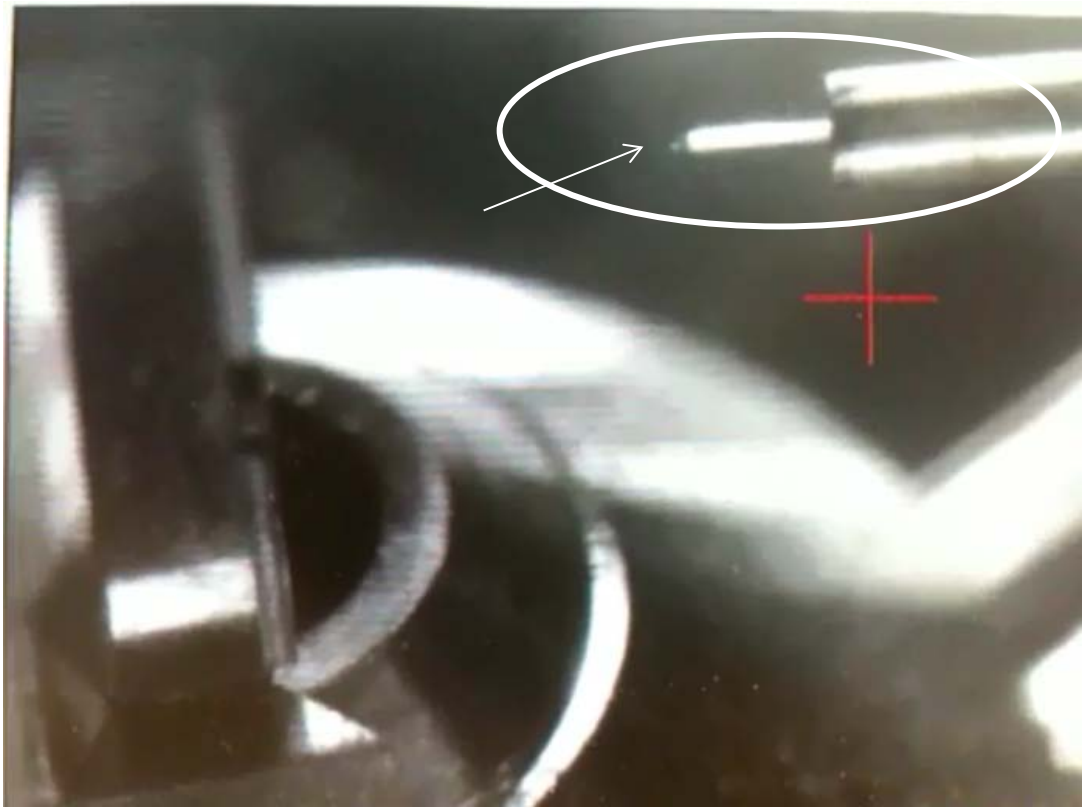


Figure 5.3.22 Column effluent exiting MS capillary probe with 3 kV applied.

5.3.8 Development of the Prototype MS Source

The previously discussed points regarding the need for a closed source to the laboratory environment and the desire to run lower than 3.0 kV capillary voltage setting for small molecule pharmaceutical compounds was the impetus for the development of an ESI source that could operate with these conditions at flow rates in the range of 9 to 16 µL/min. Therefore a collaborative effort was made with Michael Tomany, Principal Engineer, Instrument Research Group, Waters Corporation to assist in the creation of such a device. **Figure 5.3.23** below shows iteration on the development of the Prototype MS source.

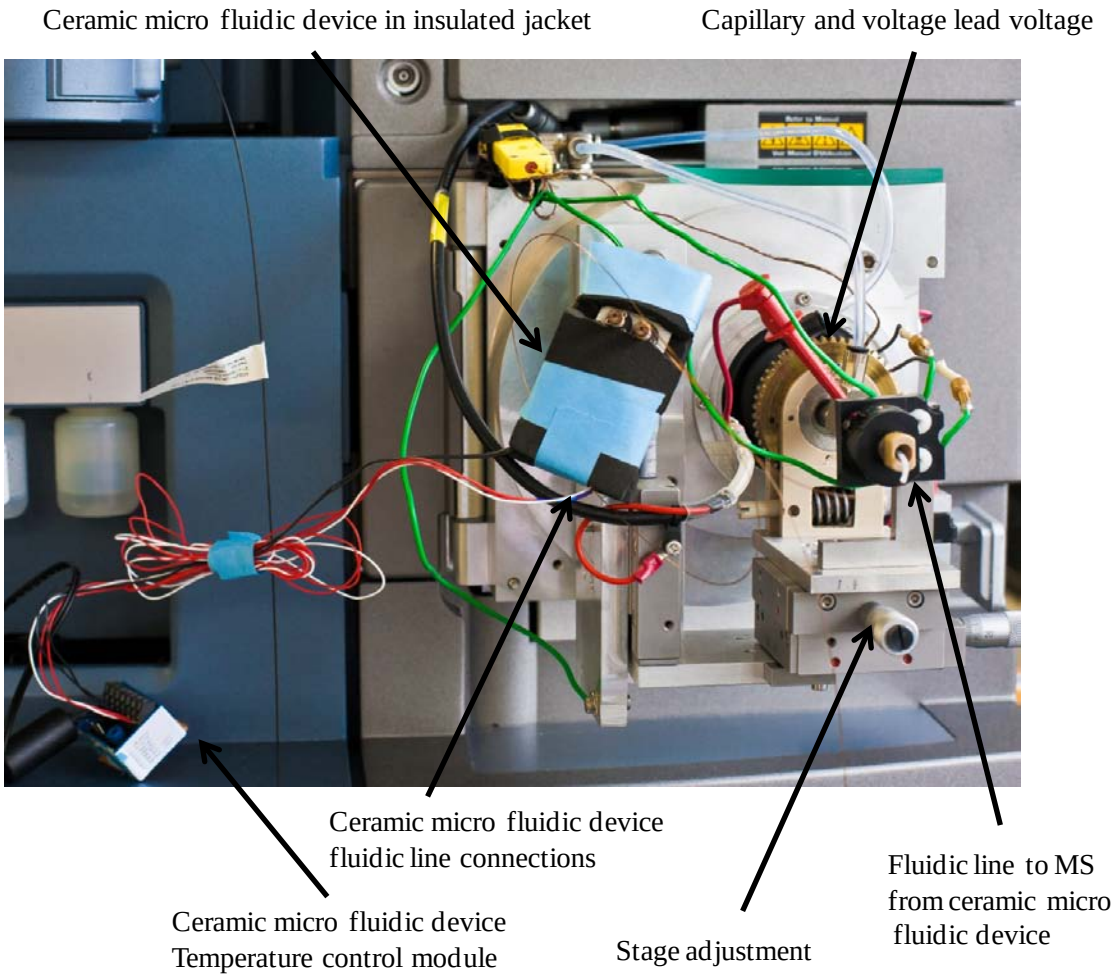


Figure 5.3.23 Prototype MS source utilized on tandem quadrupole MS.

The prototype source consisted on an aluminum block modified from a regular high flow source. The introduction of the column effluent was directed from the front (horizontal) side of the housing in contrast to the top position (vertical) as is normal for analytical scale LC. This was done as a manner to reduce the capillary length and thus post column dispersion. **Figure 5.3.23** shows the prototype MS source utilized to gather all of the data within this chapter. Capillary voltage was applied via the jumper clip shown in the previous figure and was controlled via the software. The source contained adjustment of the capillary position via a x and y axis stage device with independent adjustments for each axis as a means to locate the optimal position with respect to the entrance cone of the mass spectrometer. The ceramic microfluidic device was connected to the source capillary via highly polished fused silica tubing with standard peek fittings and polymer sleeves. A further difference with this source design as compared to traditional nano flow sources is the ability to have and control desolvation gas flow and temperature, a requirement due to the flow rates required to operate the 0.3 mm i.d. ceramic microfluidic device under optimal chromatographic conditions.

The following Figure (**5.3.24a** through **5.3.24d)** show the signal intensity as a function of both desolvation gas temperature and flow rate for acetaminophen, dextromethorphan, alprazolam, and verapamil. These compounds were chosen due to their range in elution order and thus hydrophobic nature, under reversed-phase conditions. Acetaminophen being the least hydrophobic and verapamil being the most hydrophobic of the series tested here.

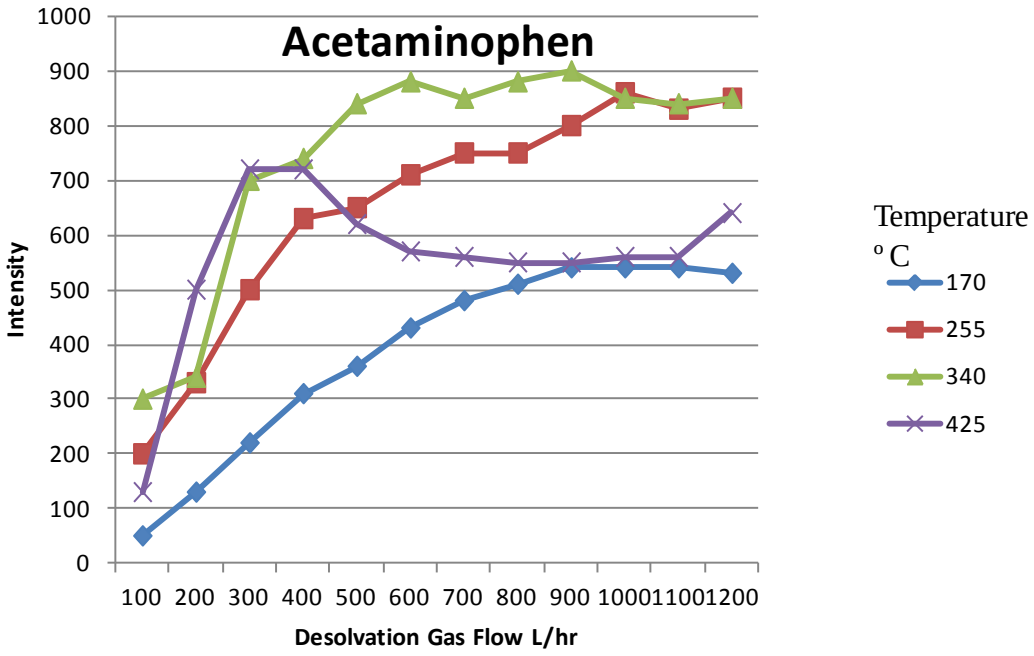


Figure 5.3.24a Influence of desolvation gas flow and temperature on MS response for acetaminophen.

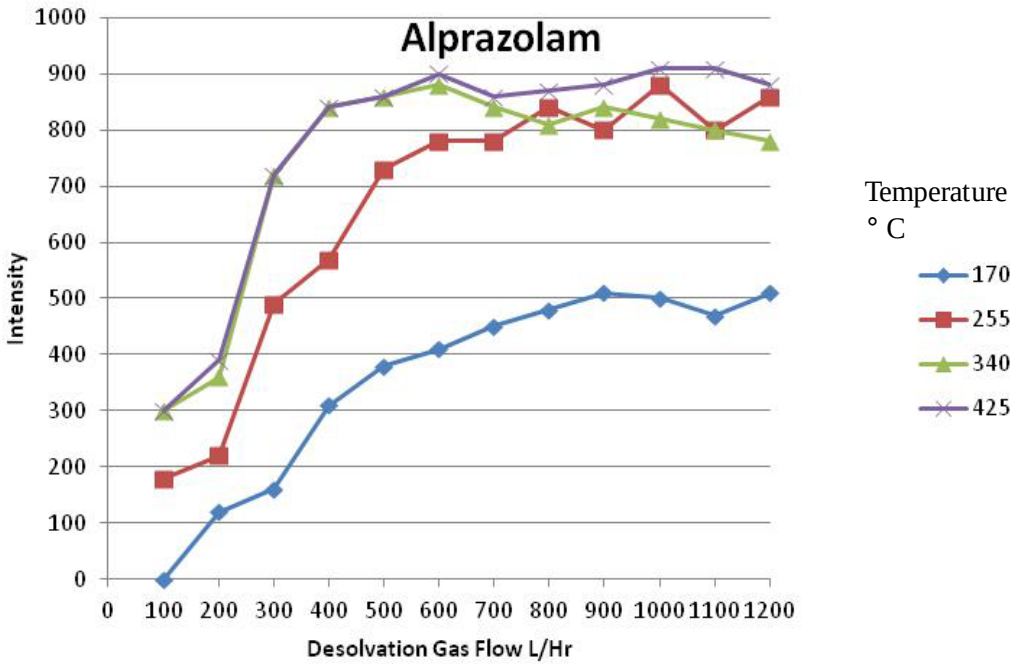


Figure 5.3.24b Influence of desolvation gas flow and temperature on MS response for alprazolam.

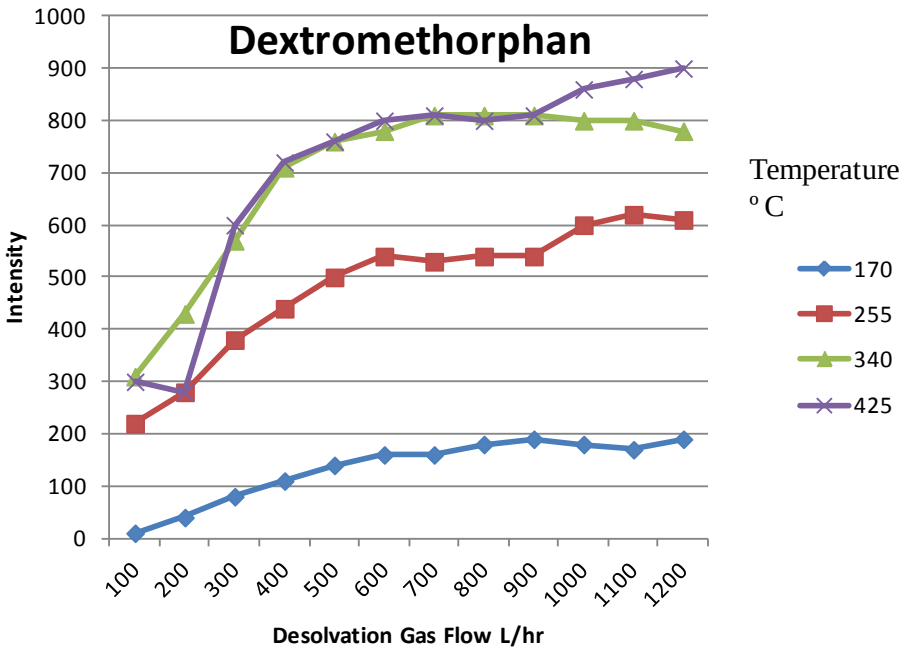


Figure 5.3.24c Influence of desolvation gas flow and temperature on MS response for dextromethorphan.

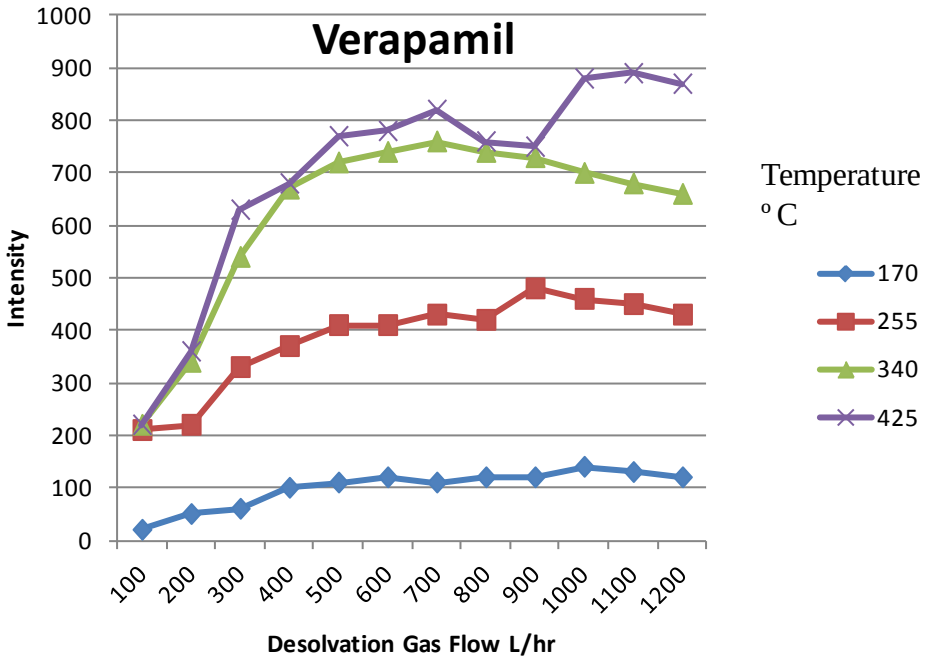


Figure 5.3.24d Influence of desolvation gas flow and temperature on MS response for verapamil.

These results show the different responses for these four compunds have to the change desolvation gas flow and temperature. It can be seen however that at a temperature of 340 °C and gas flow of 600 L/hr or greater high signal intensities were obtained for all compounds regardlesss of where they eluted durting the reversed-phase gradient.

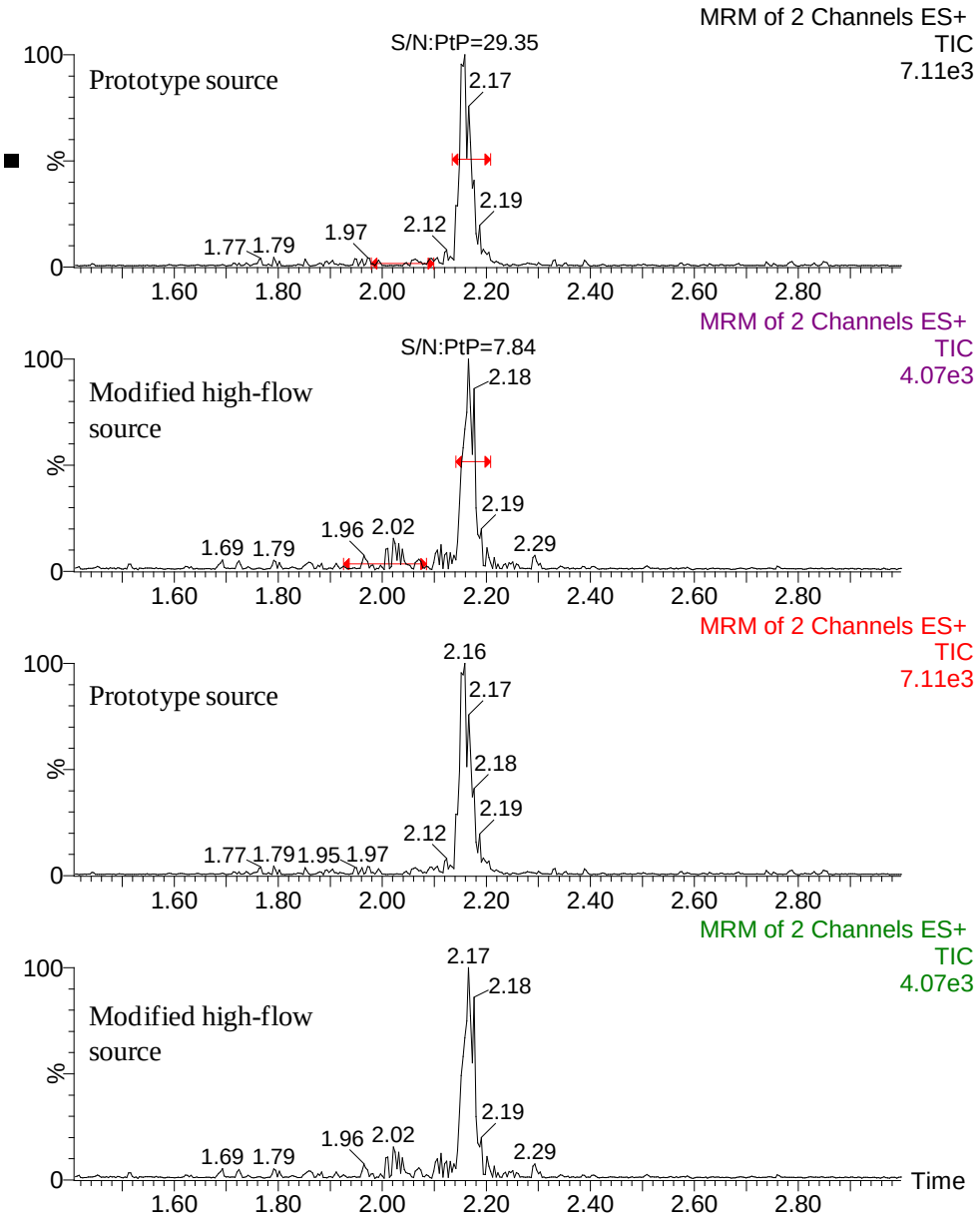


Figure 5.3.25 Comparison of signal and background noise for alprazolam on prototype MS source and conventional high flow source with narrow bore capillary installed.

Once the optimal position, desolvation temperature and gas flow were determined for optimal operation of the prototype source a comparison in overall performance based on overall signal response and signal to noise was carried out to compare it to a standard high flow source modified with the narrow bore capillary. The data shown in **Figure 5.3.25** shows the increase in signal that was achievable with the prototype source compared to the modified high flow MS source. The high flow source was modified replacing the standard 120 µm i.d. capillary with a 75 µm i.d. capillary as a means to control post column peak dispersion which is pronounced as the diameter of a column is reduced. An important note to make again is the chromatographic performance of the system. Exhibited here is a peak width measured at peak base of 3.6 seconds with a 1 µL injection. This comparison of the two MS sources for the analyte alprazolam produced a result of a 1.7 fold increase in sensitivity and a 3.7 fold increase in the signal to noise for the prototype source as compared to the modified high flow source.

5.3.9 Sensitivity improvement

Once the MS source was optimized for operation an investigation into the increase in area counts, overall MS signal and signal to noise was carried out using multiple injections of standards in solvent on both the ceramic microfluidic/MS platform and a standard UPLC/MS run on a 2.1 x 50 mm i.d. column. **Figure 5.3.26** shows the measurement of these parameters from a single 1 µL injection of the nefazodone standard (0.01 ng/mL). In the data from **Figure 5.3.26**, shows an increase in area counts from 329 to 2066, an increase in MS signal from 1.66e4 to 8.01e4, and an increase in signal to noise from 12.8 to 158.1 when analyzed on the ceramic microfluidic/MS platform versus the traditional UPLC/MS system. Similar results were observed for the other small molecule pharmaceuticals tested in the study. The

combined results of these comparisons are shown in **Tables 5.3.5** and the individual compound results from three consecutive injections are shown in **Tables**: **5.3.6**, **5.3.7**, **5.3.8**, and **5.3.10**.

The interest in peptide and protein drugs has seen an increase in recent years from pharmaceutical companies. Therefore the inclusion of a peptide drug desmopressin was carried out to evaluate the effect of analyzing this class of molecule on each of the analytical platforms. The results of this test can be viewed in **Figure 5.3.27**. Here we observe for a single 1 µL injection of desmopressin solvent standard that the ceramic microfluidic/MS system yielding an increase in area counts from 250 to 15201, an increase in signal to noise from 40 to 1748, and finally an increase in MS signal from 1.66 e4 to 5.35 e5 compared to the results generated on the traditional UPLC/MS system. The results of repeated consecutive injections are shown in Table **5.3.9**.

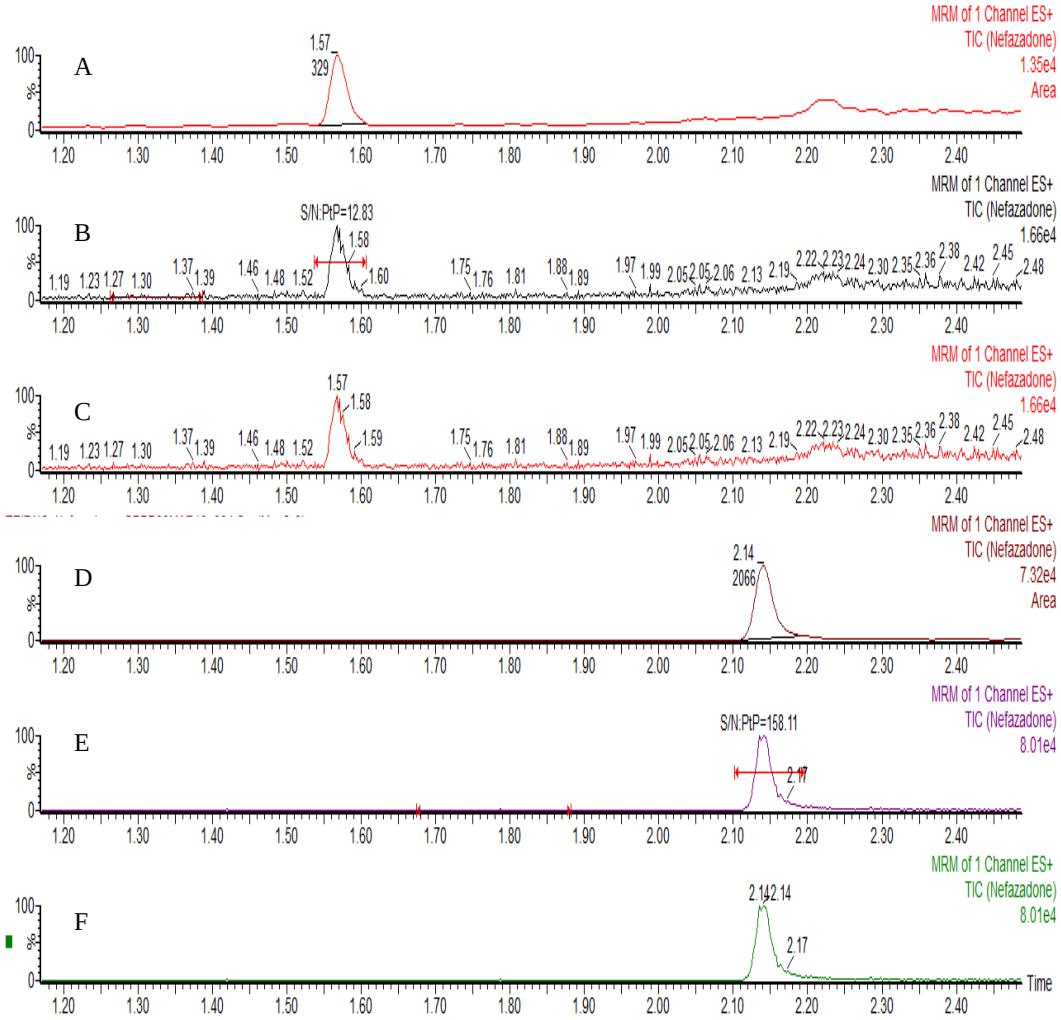


Figure 5.3.26 A) area counts B) signal to noise C) signal for nefazodone separated on a conventional 2.1 mm i.d. UPLC/MS configuration. D) area counts E) signal to noise F) signal for nefazodone separated on the CMFD and prototype MS source configuration.

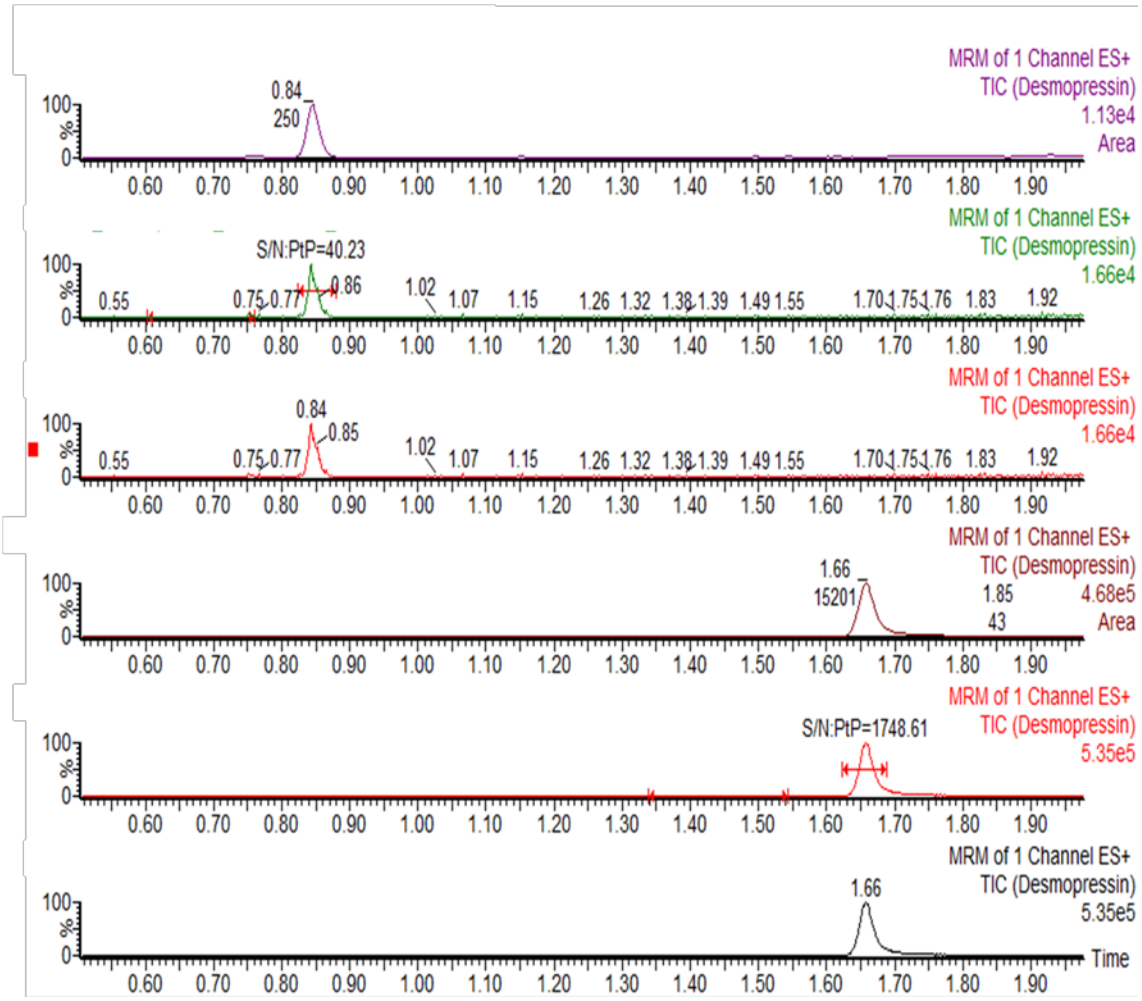


Figure 5.3.27 A) area counts B) signal to noise C) signal for desmopressin separated on a conventional 2.1 mm i.d. UPLC/MS configuration. D) area counts E) signal to noise F) signal for desmopressin separated on the CMFD and prototype MS source configuration.

	Area	Signal	Signal to noise
Alprazolam	10.1	4.7	8.3
Alprazolam OH	16.4	6.3	15.5
Desmopressin	66.6	38.2	38.6
Fluconazole	12.1	4.7	10.5
Nefazodone	6.3	5.2	13.5

Table 5.3.5 Combined average area counts, signal, and signal to noise increases for small molecule pharmaceuticals analyzed on the ceramic microfluidic/MS system compared with traditional UPLC/MS for alprazolam.

Compound name	alprazolam		area counts	signal	signal to noise
Configuration	UPLC/MS		377	27000	44
			372	28300	35
			353	28500	42
		AVG	367	27933	40
		STDEV	13	814	5
		%RSD	3	3	12

Compound name	alprazolam		area counts	signal	signal to noise
Configuration	CMFD/prototype MS		3805	127000	363
			3589	125700	330
			3641	132000	362
		AVG	3678	128233	352
		STDEV	113	3326	19
		%RSD	3	3	5

Table 5.3.6 Area counts, signal and signal to noise increases for ceramic microfluidic LC/MS system compared with traditional UPLC/MS for alprazolam.

Compound name	alprazolam OH		area counts	signal	signal to noise
Configuration	UPLC/MS		72	6930	9
			74	7910	11
			66	7690	11
		AVG	71	7510	10
		STDEV	4	514	1
		%RSD	6	7	11

Compound name	alprazolam OH		area counts	signal	signal to noise
Configuration	CMFD/prototype MS		1182	44000	223
			1260	42800	226
			1256	42500	220
		AVG	1233	43100	223
		STDEV	44	794	3
		%RSD	4	2	1

Table 5.3.7 Area counts, signal and signal to noise increases for ceramic microfluidic LC/MS system compared with traditional UPLC/MS for hydroxyl alprazolam.

Compound name	nefazodone		area counts	signal	signal to noise
Configuration	UPLC/MS		337	18600	10
			329	16600	13
			357	15900	11
		AVG	341	16250	11
		STDEV	14	495	2
		%RSD	4	3	13

Compound name	nefazodone		area counts	signal	signal to noise
Configuration	CMFD/prototype MS		2231	87100	162
			2066	80100	158
			2106	88500	140
		AVG	2134	84300	153
		STDEV	86	5940	12
		%RSD	4	7	8

Table 5.3.8 Area counts, signal and signal to noise increases for ceramic microfluidic LC/MS system compared with traditional UPLC/MS for nefazodone.

Compound name	desmopressin		area counts	signal	signal to noise
Configuration	UPLC/MS		223	14000	40
			219	12800	50
			250	16600	54
		AVG	231	14467	48
		STDEV	17	1943	7
		%RSD	7	13	14

Compound name	desmopressin		area counts	signal	signal to noise
Configuration	CMFD/prototype MS		15391	568000	2005
			15505	556000	1834
			15201	535000	1748
		AVG	15366	553000	1920
		STDEV	154	16703	131
		%RSD	1	3	7

Table 5.3.9 Area counts, signal and signal to noise increases for ceramic microfluidic LC/MS system compared with traditional UPLC/MS for desmopressin.

Compound name	fluconazole		area counts	signal	signal to noise
Configuration	UPLC/MS		676	31000	39
			584	28300	45
			615	33500	36
		AVG	625	30933	40
		STDEV	47	2601	5
		%RSD	7	8	11

Compound name	fluconazole		area counts	signal	signal to noise
Configuration	CMFD/prototype MS		7805	140000	464
			7583	145000	430
			7241	155000	362
		AVG	7543	146667	419
		STDEV	284	7638	52
		%RSD	4	5	12

Table 5.3.10 Area counts, signal and signal to noise increases for the ceramic microfluidic LC/MS system compared with traditional UPLC/MS for fluconazole.

5.4 Conclusions

A 0.3 x 100 mm i.d. CMFD and compatible MS source was successfully designed for the analysis of biological samples for use in DMPK laboratories. The device showed average chromatographic efficiencies of 9038 plates compared to 10219 plates for standard silica capillary columns. Gradient performance utilizing a diverse mix of compounds yielded a peak capacity of 55 as the average peak widths for all analytes was 0.11 minutes for a 6 minute separation.

Resolution of the probe pharmaceutical alprazolam and associated hydroxyl metabolite was maintained between 1.2 and 1.5 for four different devices. Testing of the device with plasma samples prepared by protein precipitation resulted in over 1000 injections being carried out over approximately a one week period while maintaining resolution of alprazolam and associated hydroxyl metabolite. Furthermore no dramatic increase in system back pressure was observed over this test period.

Sample volume loading studies resulted in the successful loading of 2 µL of alprazolam in a 95:5 H2O:CH_3CN solution. This volume was approximately 30 percent of the open channel volume, and is equivalent to an injection volume of 98 µL on a 2.1 mm i.d. column. Loading of the same analyte from acetonitrile protein precipitated plasma, yielded a top injection volume of 1.2 µL before significant peak fronting was observed. The volume represents a 60 µL injection on a 2.1 mm i.d. column.

The system was unable to withstand the high pH (10.5) utilized in the previous chapters. This was most likely due to the dissolution of the silica-tubing resulting in line plugging and system overpressure. However the ceramic material appeared stable with incubation with the 10.5 pH solution as the morphology and appearance was observed to be similar before and after incubation.

MS source optimization studies indicated the requirements for operation of lower capillary voltages for many of the small molecules tested which cannot be achieved with conventional nano-spray source which typically requires 3 kV to produce a stable spray. Further a closed source resulted in less background noise observed. The resulting MS source built to operate under these conditions and with the flow rates required to operate the 0.3 x 100 mm CMFD yielded increases in signal-to-noise in the range of 8.3 to 38.6 compared to traditional 2.1 mm i.d. column UPLC/MS.

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Chapter 6

Applications using a Ceramic Microfluidic Device coupled with Mass Spectrometry for DMPK and Metabonomic Studies

This chapter is based partially on the following publication:

Addressing the challenge of limited sample volumes in *in vitro* studies with capillary-scale micro-fluidic LC–MS/MS

Paul D. Rainville, Norman W. Smith, Ian D. Wilson, Jeremy K. Nicholson, Robert S. Plumb

Bioanalysis Volume 3, Number 8, April 2011, Pages: 873-882.

You must cut your coat according to your cloth

6.1 Introduction

Advances in the generation of specific model animals through the use of genetics can be used to investigate the potential treatment of a variety of diseases. However, animal models, including genetically modified and “knock-out” mice, more traditional mutation-derived models such as the Zucker rat [1] (a model for type II diabetes) and germ free axenic rats [2]come with a significant economic cost. This, combined with the ethical drive to reduce, replace and refine such experimentation so that a minimum number of animals are used has resulted in a drive to reduce sample volumes to the extent that a complete PK profile is obtained from a single animal, via low volume sampling techniques such as dried blood spots (DBS).

The ability to acquire meaningful PK information from very small sample volumes would allow for serial tail bled sampling [3] and expand the potential of these animal models, while also improving the quality of the data, and also reducing the amount of drug material needed for the study further lowering costs, thus enabling studies to be performed earlier in discovery. The low sample volumes available from such studies require much greater sensitivity from the analytical system compared to those typically obtained for conventional LC/MS/MS analysis (ca. 100-200μL of plasma). Furthermore, the need to extract the maximum information from the previously described animal models biofluids during the drug development process means that multiple assays are generally required. These include analysis by a variety of analytical methods such as bioassays, NMR, LC, and GC and hyphenated MS techniques.

In this chapter these challenges are addressed by a Ceramic Microfluidic Device **(**CMFD) in both quantitative and qualitative DMPK applications. The CMFD/MS system was employed for the quantification of two model pharmaceutical drugs,

alprazolam and sitamaquine. These samples were prepared by protein precipitation and by DBS respectively. Experiments were carried out to determine the feasibility and high-throughput capability of the approach for standard bioananalytical studies. This technique was also utilized to interrogate human liver microsome incubations of propranolol and further in the profiling of axenic rat urine and dog bile for the purposes of metabonomic and biomarker discovery studies. Comparative separations carried out with traditional 2.1 mm i.d. chromatography columns and the CMFD were performed utilizing axenic rat urine in order to determine any increases in sensitivity and signal to noise capability. The combination of basic mobile phases with the CMFD/MS system operating in ESI + was also explored as a strategy for increasing assay sensitivity in biomarker profiling studies.

6.2 Materials and Methods

6.2.1 Chemicals and Materials

Propanolol, monobasic sodium phosphate, dibasic sodium phosphate, and bile acids were purchased from Sigma Chemical Co. (St. Louis, MO., US). Ammonium acetate, ammonium hydroxide, formic acid, methanol and acetonitrile were all purchased from Thermo Fisher Scientific (Pittsburgh, PA, US) Human liver microsomes and co-factors were purchased from BD Biosciences (San Jose, CA, USA) Alprazolam, deuterated internal standard, and the hydroxyl metabolite were obtained from Cerilliant (Round Rock, TX, USA). Rat plasma was obtained from Equitech-Bio (Kerrville, TX, USA). Sitamaquine and sitamaquine D10 were supplied by Dr. Chet Bowen from GlaxoSmithKline Upper Marion PA USA). Dog bile was supplied by Dr. Ian Wilson from Astra Zeneca (Maccelsfield, UK). Axenic rat urine was supplied by Professor Jeremy Nicholson, Imperial College (London, UK). Water was obtained from an in-house milli Q filtration system.

6.2.2 Sample Preparation (alprazolam bioanalytical assay)

Alprazolam, deuterated internal standard (IS), and its hydroxyl metabolite were dissolved in 100 % methanol. Stock solutions were then diluted with 95:5 water:methanol and spiked into rat plasma producing a dilution series ranging from 0.1 ng/mL to 500 ng/mL. A simple protein precipitation was then performed using a 2:1, acetonitrile (with IS): plasma mix. The samples were vortex mixed for one minute and centrifuged at 13,000 RCF for 10 minutes. The supernatant was then removed and diluted 50:50 with water.

6.2.3 CMFD/MS (alprazolam bioanalytical assay)

A 1.2 µL injection of the sample was then made onto the nano ACQUITY UPLC system. The mobile phase consisted of A: 0.1% formic acid and B: acetonitrile. The analytes were eluted under a linear gradient from 5 – 95 % B in 2.0 minutes at a flow rate of 12 µL/min on a modified nanoACQUITY system equipped with a prototype CMFD with a 0.3 mm x 100 mm channel packed with 1.7 µm BEH C_{18} which was thermostatically controlled at 45.0̊ C. The column effluent from the system was routed into a Waters Xevo TQ equipped with a prototype ESI source as described in Chapter 5. The mass spectrometer was operated in electrospray positive and in SRM mode.

Compound	SRM m/z	Cone (V)	Collision Energy (eV)
Alprazolam	309.2 > 281.0	40	25
Alprazolam D5	314.2 > 286.1	40	25
Alprazolam OH	325.0 > 297.0	40	25

Table 6.2.1 SRM transitions and MS conditions utilized for alprazolam assay.

6.2.4 Sample Preparation (metabolic profiling)

Rat liver microsomes were stored at –80.0˚C. They were thawed and re-suspended in a phosphate buffer solution at a ratio of 1:15 (v/v). The co-factor solution was prepared by adding 1.6 mL of cofactor solution A and 0.32 mL of cofactor solution B to 10.9 mL of phosphate buffer. Microsomal incubations were carried out as described in reference [4]. Briefly, 100 µL p450 substrates + 100 µL test compound + 100 µL cofactor solution + 100 µL phosphate buffer were combined and placed into a 2 mL, 96 well plate and heated to 37.0˚C for 10 minutes. Rat liver microsomes were then added in 100 µL increments and the 96 well plate was then incubated at 37 ˚C while shaking for 60 minutes. The reaction was quenched by the addition of 500 µL of ice cold

acetonitrile (4˚C). Controls were included consisting of a quenched incubation at time 0 and an incubation containing no test compounds.

6.2.5 CMFD/MS (metabolic profiling)

A 1 µL injection of the sample was then made onto the nanoACQUITY UPLC system. The mobile phase consisted of A: 0.1% formic acid and B: acetonitrile. The analytes were eluted under a linear gradient from 5 – 95 % B in 10 minutes at a flow rate of 10 µL/min on a modified nano ACQUITY system equipped with a CMFD with a 0.3 mm x 100 mm channel packed with 1.7 µm BEH C_{18} thermostatically controlled at 45.0˚ C. The column effluent from the system was routed into a Waters Xevo TQ equipped with a prototype ESI source as described in Chapter five. The mass spectrometer was operated in electrospray positive and in SRM mode, full scan mode, and parent scan mode.

6.2.6 Sample preparation (bile acid profiling)

Bile acid stock standards where dissolved in 95:5 water:methanol at a concentration of 1 mg/mL Bile acids were then diluted to a concentration of 1 ng/mL. Dog bile was prepared by diluting with water 1:10 (bile:water), then centrifuged at 13,000 RCF for 5 minute.

6.2.7 CMFD/MS (bile acid profiling)

A 0.2 µL injection of bile acid standards was made onto the nano ACQUITY UPLC system. The mobile phase consisted of A: 10 mM ammonium acetate (pH 5) and B: acetonitrile. The analytes were eluted under a linear gradient from 20 – 80 % B in 10 minutes at a flow rate of 10 µL/min on a CMFD with a 0.3 mm x 100 mm channel packed with 1.7 µm BEH C_{18} thermostatically controlled at 45.0C. The column

effluent from the system was routed into a Waters Xevo G2 QTof equipped with a prototype ESI source as described in Chapter 5. The mass spectrometer was operated in electrospray negative ionization and in full scan mode.

6.2.8 Sample preparation (sitamiquine dried blood spot assay)

Sitamaquine is an orally active 8-aminoquinoline analog and is GlaxoSmithKline's (GSK) potential new once-a-day oral treatment for visceral leishmaniasis, a disease caused by protozoan parasites of the *Leishmania* genus. This disease is the second-largest parasitic killer in the world (after malaria), responsible for an estimated 500,000 infections each year worldwide and is usually fatal if untreated Sitamaquine and deuterated internal standard (IS) were dissolved in 100 % methanol. Stock solutions were then diluted with 95:5 water:methanol and spiked into rat blood producing a dilution series ranging from 1 ng/mL to 10,000 ng/mL. Dried blood spot cards (DBS) were prepared as previously described [5]. Briefly, a 20 µL spot of blood was placed on the DBS and left to dry. Spots were then cut out with a 7 mm punch and placed into a tube with methanol containing the IS and vortex mixed for 60 minutes. The samples were then centrifuged at 13,000 RCF for five minutes, and subsequently diluted with water (50:50)

6.2.9 CMFD/MS (sitamiquine dried blood spot assay)

A 1.2 µL injection of the sample was then made onto the nano ACQUITY UPLC system. The mobile phase consisted of A: 0.1% formic acid and B: acetonitrile. The analytes were eluted under a linear gradient from 30 – 70 % B in 2.5 minutes at a flow rate of 12 µL/min on a modified nano ACQUITY system equipped with a prototype

CMFD with a 0.3 mm x 100 mm channel packed with 1.7 µm BEH C_{18} thermostatically controlled at 45.0̊ C. The column effluent from the system was routed into a Waters Xevo TQ equipped with a prototype ESI source as described in Chapter 5. The mass spectrometer was operated in electrospray positive and in SRM mode. These conditions are listed in **Table 6.2.2**.

Compound	SRM	Cone (V)	Collision Energy (eV)
Sitamaquine	344.3 > 271.2	35	27
Sitamaquine D10	354.2 > 271.2	35	27

Table 6.2.2 SRM transitions and MS conditions utilized for sitamaquine assay.

6.2.10 Sample preparation (metabonomic study)

Rat urine was diluted 1:4 (urine:water), vortex mixed and then centrifuged at 13,000 RCF for five minutes.

6.2.11 CMFD/MS (metabonomic study)

A 2 µL injection of the urine sample was then made onto the nano ACQUITY UPLC system. The mobile phase consisted of A: 0.1% formic acid or 10 mM ammonium acetate pH 10 and B: acetonitrile. The analytes were eluted under a linear gradient from 2 –70 % B in 10 minutes at a flow rate of 12 µL/min on a modified nano ACQUITY system equipped with a prototype CMFD with a 0.3 mm x 100 mm channel packed with 1.7 µm BEH C_{18} thermostatically controlled at 45.0̊ C. The column effluent from the system was routed into a Waters Xevo G2 QTof equipped with a prototype ESI source as described in Chapter 5. The mass spectrometer was operated in electrospray positive and in full scan mode.

6.3 Results and Discussion

6.3.1 Quantitative assay for alprazolam derived from plasma and sitamiquine from whole blood.

Previous work by Fraser *et a.l.* and other illustrated the use of capillary scale LC/MS for the analysis of biofluids [3, 6]. In this study the CMFD/MS system was implemented for the quantitative analysis of alprazolam in rat plasma. The study further investigated the compound sitamaquine derived from whole rat blood and prepared from DBS. **Figure 6.3.1** shows the structure of alprazolam. Alprazolam is a short acting benzodiazepine used for the treatment of moderate to severe anxiety disorders, panic attacks, and as an adjunctive treatment for anxiety associated with clinical depression. It was previosly described in Chapter 3 of this thesis.

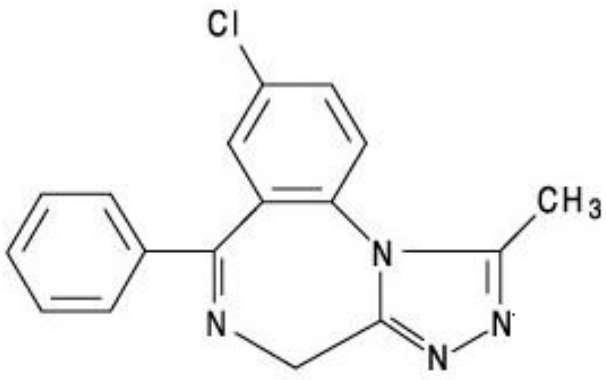


Figure 6.3.1 Structure of alprazolam.

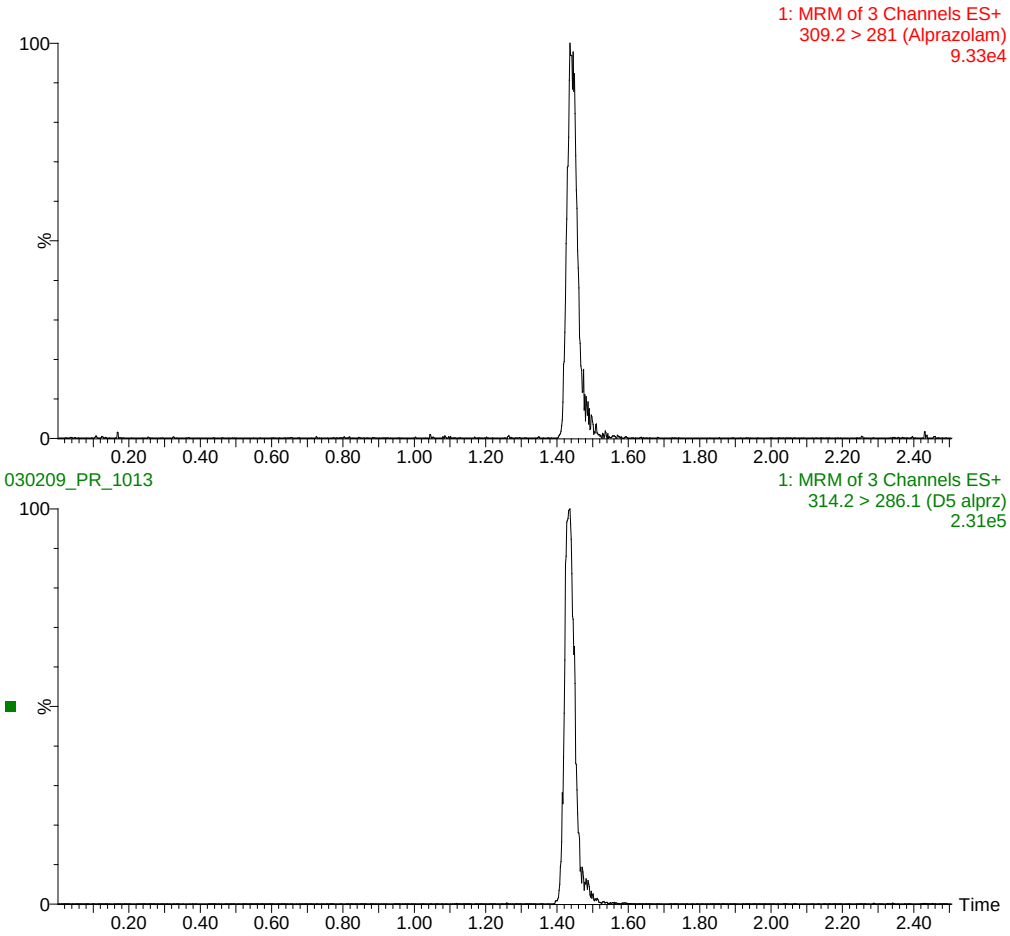


Figure 6.3.2 Separation of alprazolam (5 ng/mL) and D5 alprazolam derived from protein precipitated rat plasma on CMFD/MS.

Here we observe good peak shape for both of these analytes with a peak width of 4.2 seconds measured at the peak base for each of the analytes in the separation. This resulting peak width generated by the CMFD is slightly larger than the average peak widths of between 2.5 and 3 seconds, measured at peak base, that are normally generated by standard 2.1 mm i.d., 1.7 µm columns. However this result is still acceptable for the scale of chromatography that was employed with the CMFD.

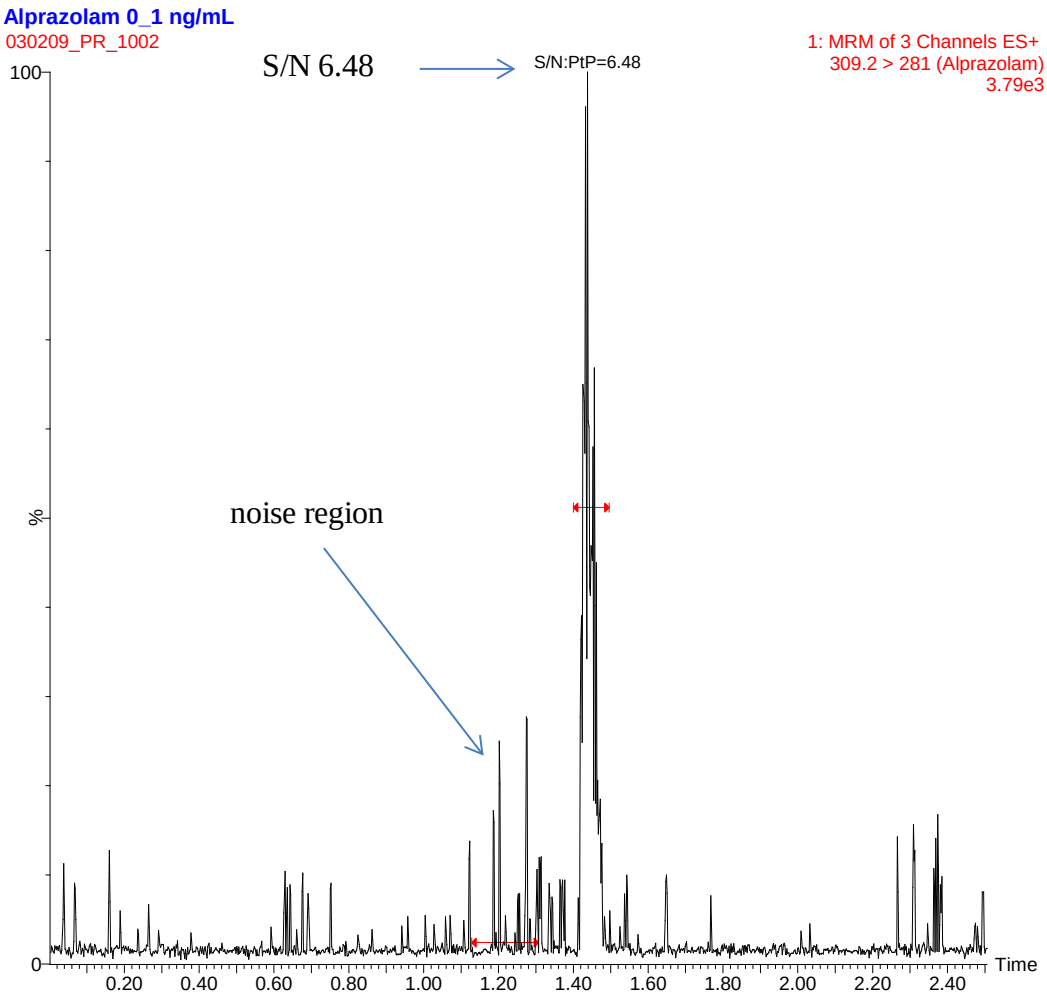


Figure 6.3.3 Limit of detection for alprazolam assay at 0.1 ng/mL level.

The limit of detection (LOD) was determined to be at the 0.1 ng/mL where a signal to noise value of 6.48 was obtained (**Figure 6.3.3**). While the lower limit of quantification (LLOQ) was determined to be 1 ng/mL at this level, the calibration line was created with all deviation (residual) values below 15 percent. **Figure 6.3.4** shows the calibration line for alprazolam over the range of 1 ng/mL to 500 ng/mL. The correlation coefficient was determined to be greater than 0.99 for the multi replicate calibration curve.

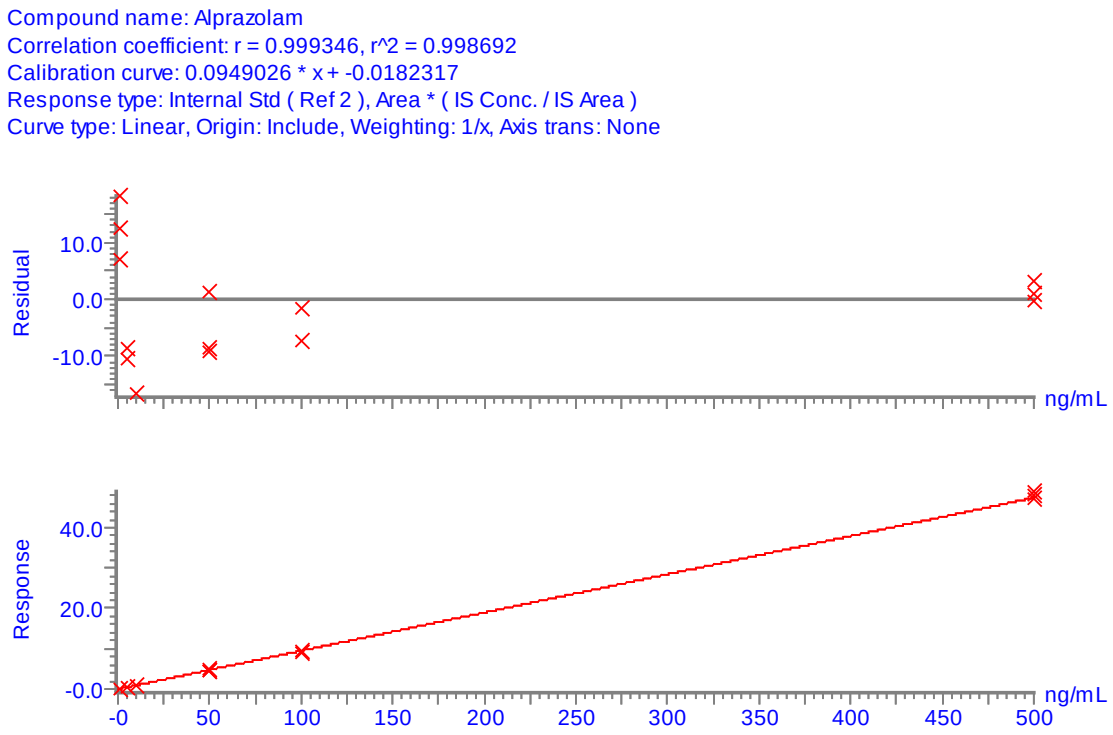


Figure 6.3.4 Calibration line and residuals for alprazolam assay.

This data shows that the system is capable of generating data of sufficient quality and sensitivity with appropriate throughput (<5 minute cycle time), for discovery bioanalysis.

A further assay was developed on the CMFD/MS system utilizing the DBS format. DBS has gained much attention and ongoing research in the bioanalytical community and in the literature for the last few years [5, 7, 8]. The purported advantages of this technique include: First, the need for remarkably lower blood volumes and second, easier shipping and storage, often at ambient temperatures. This leads to a simplification of the blood collection process and a reduction in the costs involved. Moreover, in preclinical studies the number of animals can be reduced due to the need for lower blood volumes which is in accordance with the 3R requirement of animal studies (replacement, reduction, refinement). Sitamaquine and D10 deuterated

sitamaquine were obtained from Chet Bowen (GSK Upper Marion PA, USA). Samples were prepared onto a Whatman FTA© DMPK card as outlined in section 6.2.8.

Sitamaquine (**Figure 6.3.5**) 8-aminoquinoline analog and is GlaxoSmithKline's (GSK) potential new once-a-day oral treatment for visceral leishmaniasis [9].

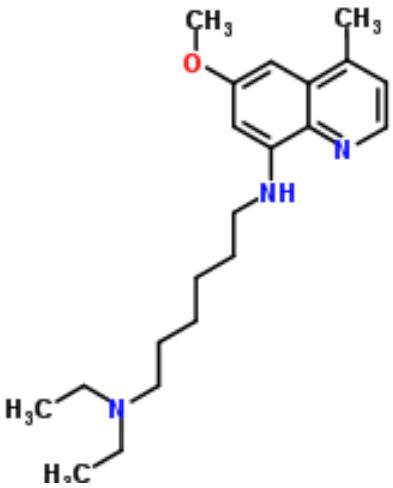


Figure 6.3.5 Structure of sitamaquine.

An example chromatogram produced by the CMFD/MS system for the sitamaquine assay is shown in **Figure 6.3.6**. The peaks for both the sitamaquine and IS both show an increase in peak tailing compared to the previous alprazolam example. However, the peak width is still rather impressive as the peak width at base generated for both analytes is in the order of 3 seconds at peak base.

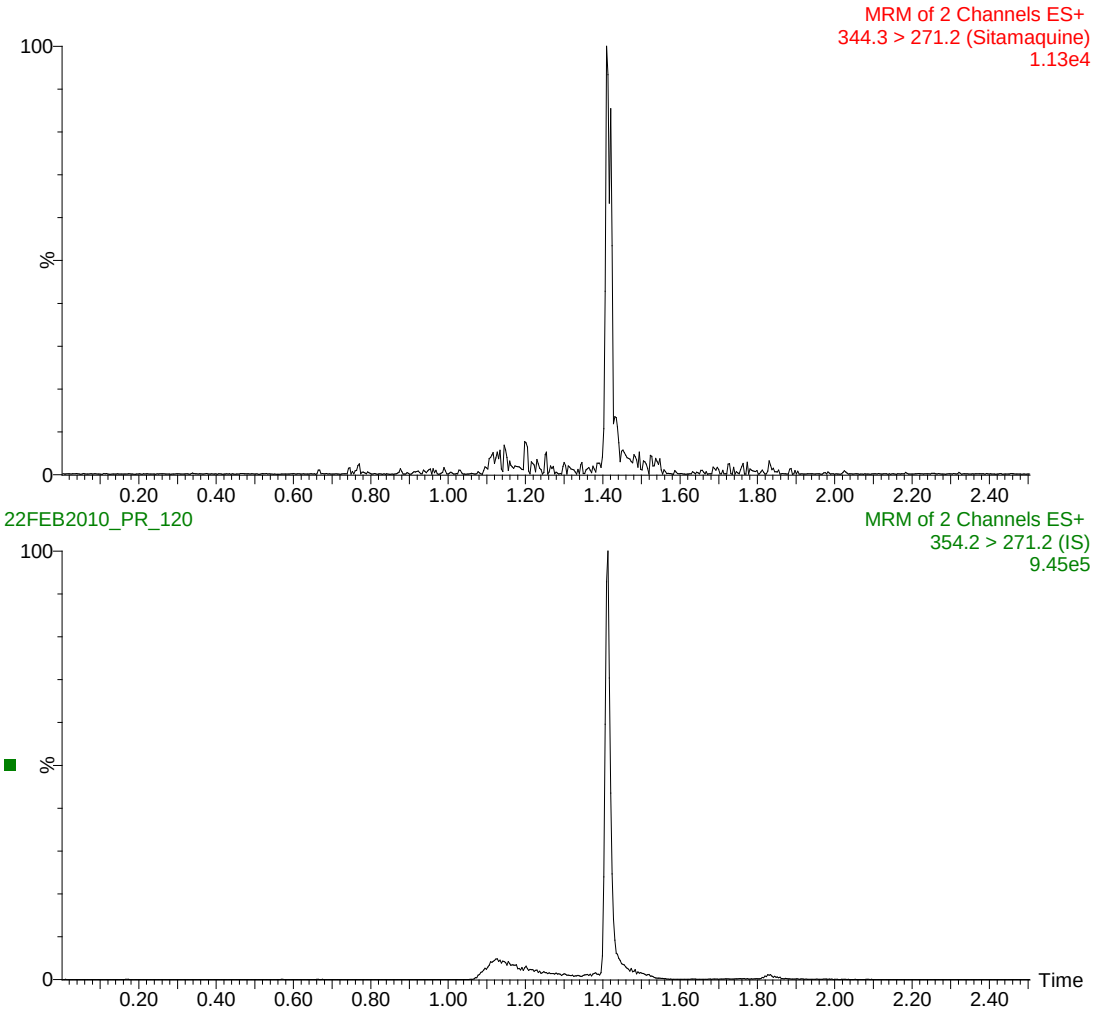


Figure 6.3.6 Separation of sitamaquine (50 ng/mL) and D10 sitamaquine derived from DBS of whole rat blood on CMFD/MS system.

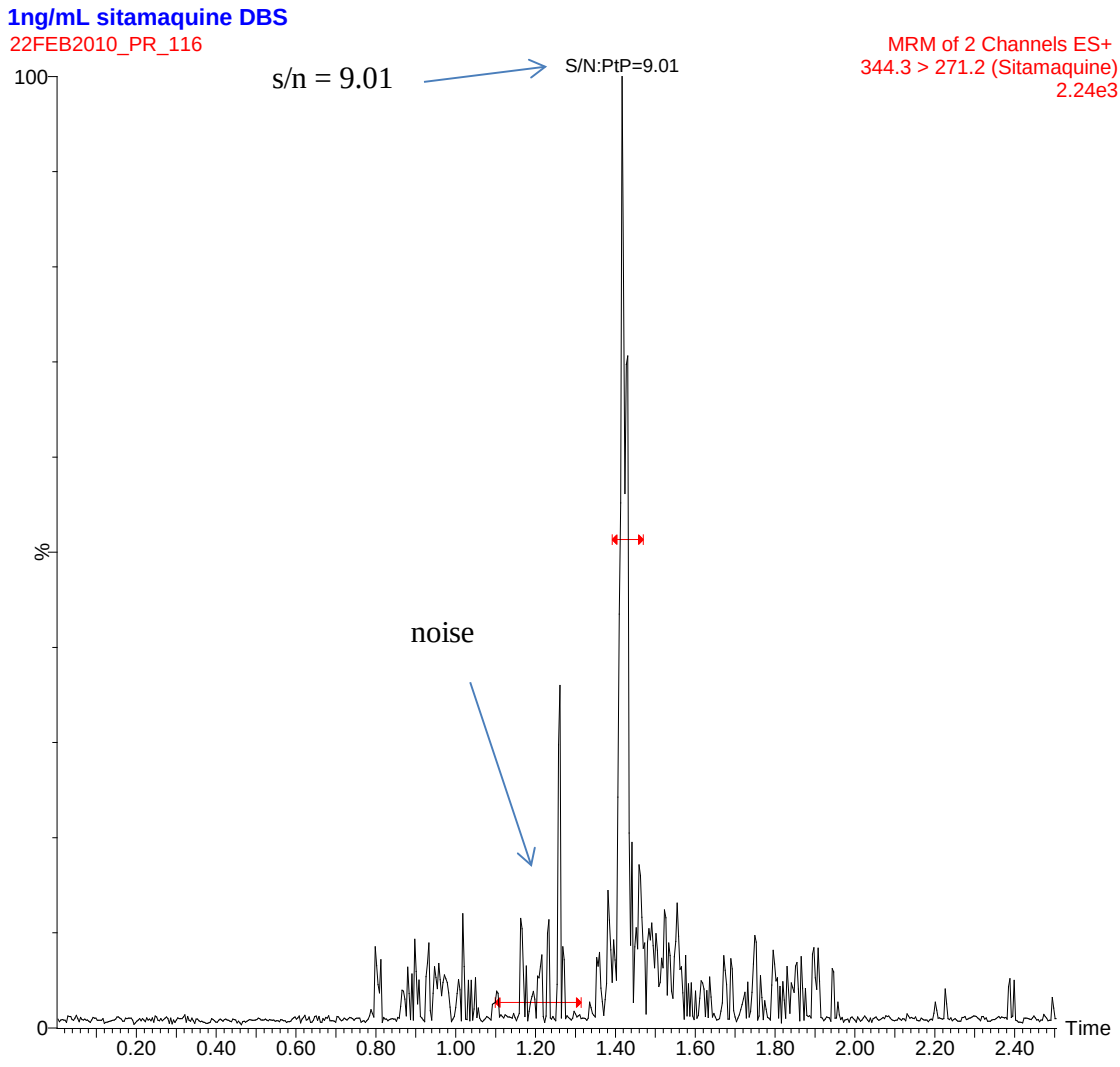


Figure 6.3.7 Limit of detection for sitamaquine DBS assay at 10 ng/mL level.

The limit of detection (LOD) was determined to be at the 10 ng/mL where a signal to noise value of 6.48 was obtained (**Figure 6.3.7**). While the lower limit of quantification (LLOQ) was determined to be 50 ng/mL as at this level, the calibration line was created with all deviations (residual) within 15 percent of the theoretical value. **Figure 6.3.8** shows the calibration line for sitamaquine over a range of 50 ng/mL to 10,000 ng/mL. The correlation coefficient was determined to be greater than 0.99 for the multi replicate calibration curve.

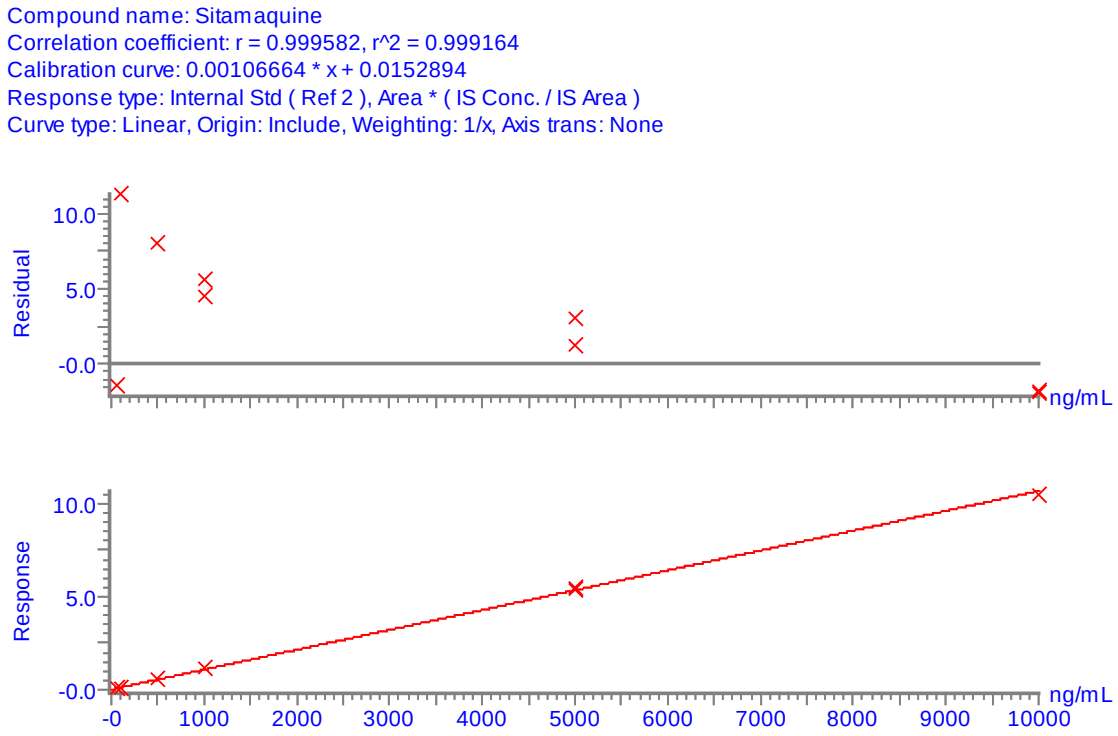


Figure 6.3.8.Calibration line and residuals for sitamaquine assay.

As with the previous alprazolam assay the CMFD/MS system is capable of generating data of sufficient quality and sensitivity with appropriate throughput i.e. < 5 minute cycle time. This means that the methods are truly fit for purpose from an economic point of view.

The chromatographic performance of the CMFD/MS device is more than acceptable, especially if one considers that the injection volumes utilized in this study, (1.2 µL) is equivalent to loading 58.8 µL onto a standard 2.1 column. Also a LLOQ of 1 ng/mL is an acceptable value for many LC/MS bioanalytical assays of pharmaceuticals in biofluids during discovery DMPK studies. This injection volume represents 17 percent of the volume of the unpacked, open cylinder volume in µLs. As shown by Figure 5.3.11 in the previous chapter, when using a simple protein precipitation approach, the volume that can be loaded onto the system is limited. Thus the overall detection limit may not be improved compared to that of a 2.1mm i.d. column packed with the same

material and operated at the same mobile phase linear velocity, as a greater volume of sample can be therefore loaded onto this i.d. column that may not be feasible with the 0.3 µm i.d. device utilized in this study. The CMFD/MS devices thus may have two main areas of application; i) when sample volume is limited and ii) when samples containing the analyte of interest can be concentrated into a sufficiently small volume. However in this case, depending on analyte concentration, a situation of creating a mass overload situation could prove detrimental as severe band broadening may occur. This situation will be illustrated in section 6.3.3.

6.3.2 Metabolic profiling of propranolol

Drug metabolism plays a key role in the drug discovery and development phases, as critical sites of metabolism or soft spots are identified [10], and differences in species metabolism need to be investigated [11]. The study of the drug metabolism of new chemical entities can reveal information not only on the fate of a molecule but also changes in the endogenous metabolic profile resulting from drug administration, yielding useful potential biomarker information [12, 13]. This information can be used to detect the presence of potentially toxic metabolites and monitor the efficacy of a treatment [14, 15]. In this section of Chapter 6, the application of the CFMD/MS for the analysis of metabolites of the model beta blocker propranolol following incubation with rat liver microsomes, is presented.

The applicability of the CFMD approach for *in vitro* drug metabolism analysis was evaluated using an incubation of the betablocker propranolol with rat liver microsomes. As indicated in the experimental section, MS data were acquired using a combination of full scan MS mode, MS/MS mode, precursor ion scan mode, survey scan mode, and

MRM-Dual Scan mode. In this last mode the tandem quadrupole mass spectrometer is able to rapidly switch between MS and MS/MS mode during a single run [16].

The positive ESI LC/MS TIC full scan trace for the gradient LC analysis of 1µL injection of the 1µMol incubation of propranolol is shown in **Figure 6.3.9**.

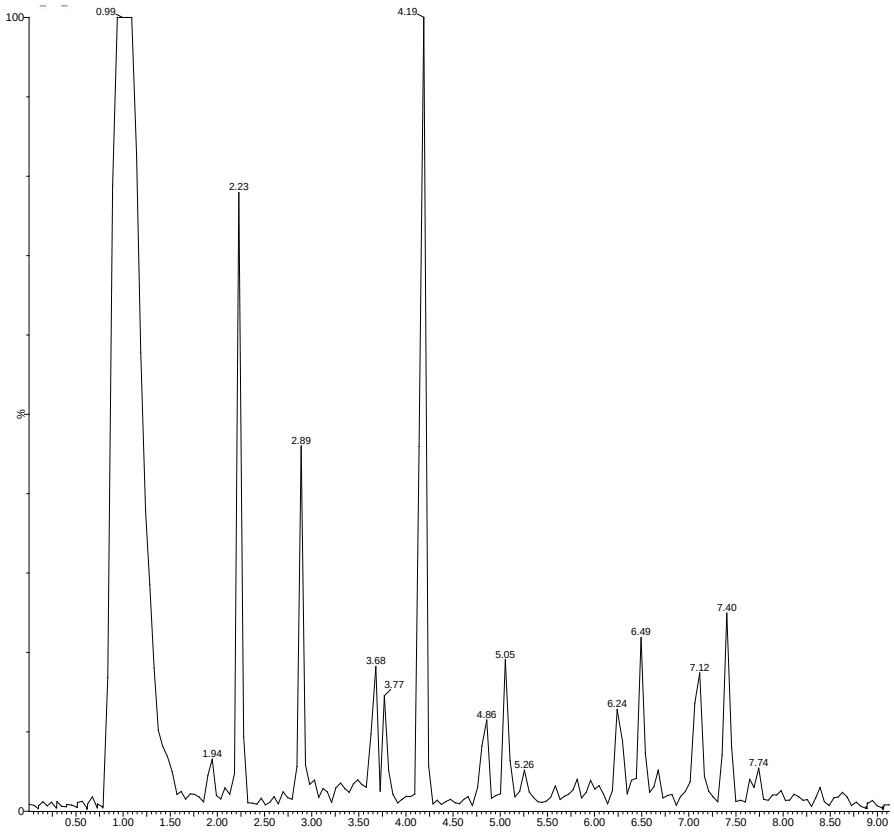


Figure 6.3.9 Full MS scan of CMFD/MS separation of propranolol microsomal incubation.

Herein some thirteen major peaks were detected via this gradient profile. The peak widths are somewhat wider than that normally obtained by traditional geometry columns packed with sub 2 µm particle materials, where the peak width is typically 2-3 seconds. Although the peak capacity is less than that of standard analytical scale sub

2µm particle LC it is still sufficient to provide a high quality separation. The reduction in performance may be due to i) high sample load, ii) lower density of the packed column bed or iii) the percentage of organic in the sample that was loaded onto the column. Thus, considering that with a column length of 10 cm and an internal diameter of 300 µm, a 1 µL injection of sample represents 14 % of the column bed volume the resulting peak shapes are quite impressive.

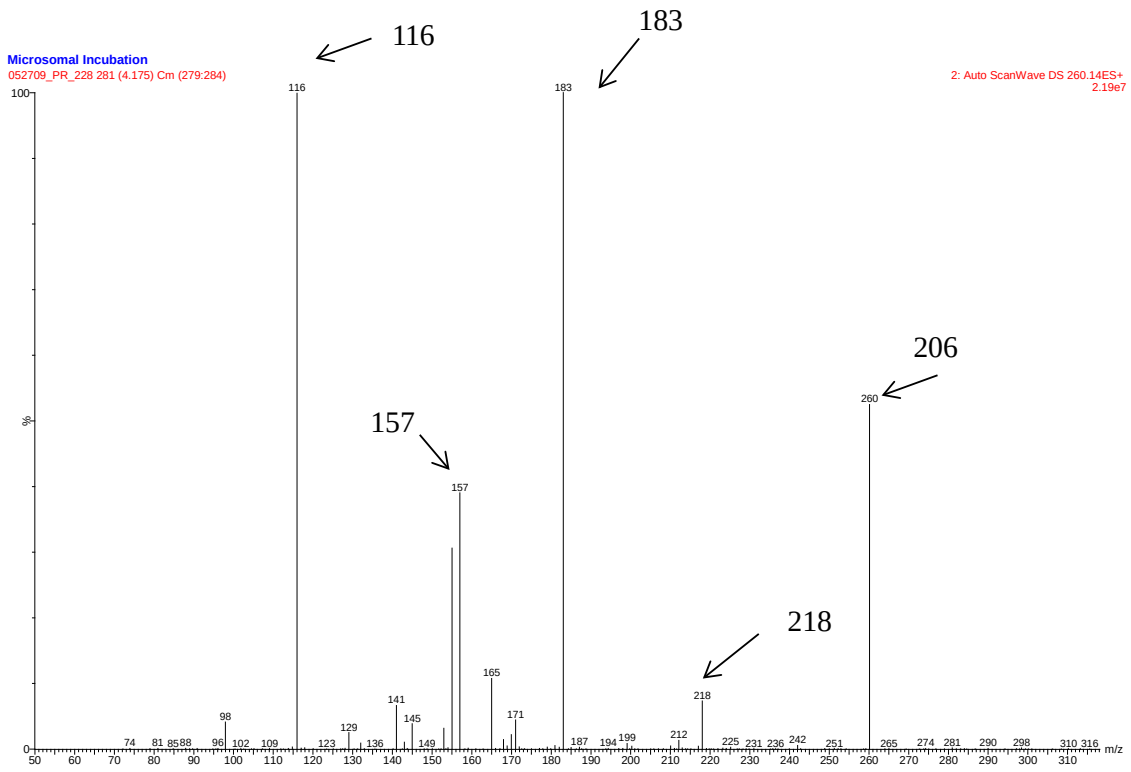


Figure 6.3.10 MS/MS spectra of propranolol.

Propranolol (m/z = 260) eluted with a retention time of 4.2 minutes and gave the MS/MS spectrum displayed in **Figure 6.3.10.** The fragmentation of propranolol gave rise to three major MS spectral peaks at m/z = 183, 157 and 116 and a minor peak at m/z 218. These MS/MS fragmentation results generated here in this study further

agreed with data reported from previous research[17]. Therefore these ions were utilized as diagnostic fragment ions to search for drug related metabolites in the sample.

A 1 µL sample of the 1 µMol incubation of propranolol was injected on to the CMFD/MS system operated in survey scan mode thereby collecting both full scan MS and MS/MS data dependent upon the intensity of the eluting peaks. The extracted ion chromatogram for the three fragment ions m/z = 183, 157 and 116 are shown in **Figure 6.3.11**. This data shows that the m/z = 116 ion detected a greater number of potentially drug related metabolites than the others (eight in total). Hence this was selected as the most suitable fragment for detecting possible drug related metabolites for further work. As these data were not derived from accurate mass determination the exact origin of the peaks cannot be confirmed based on it, nonetheless it remains a useful initial approach to metabolite detection.

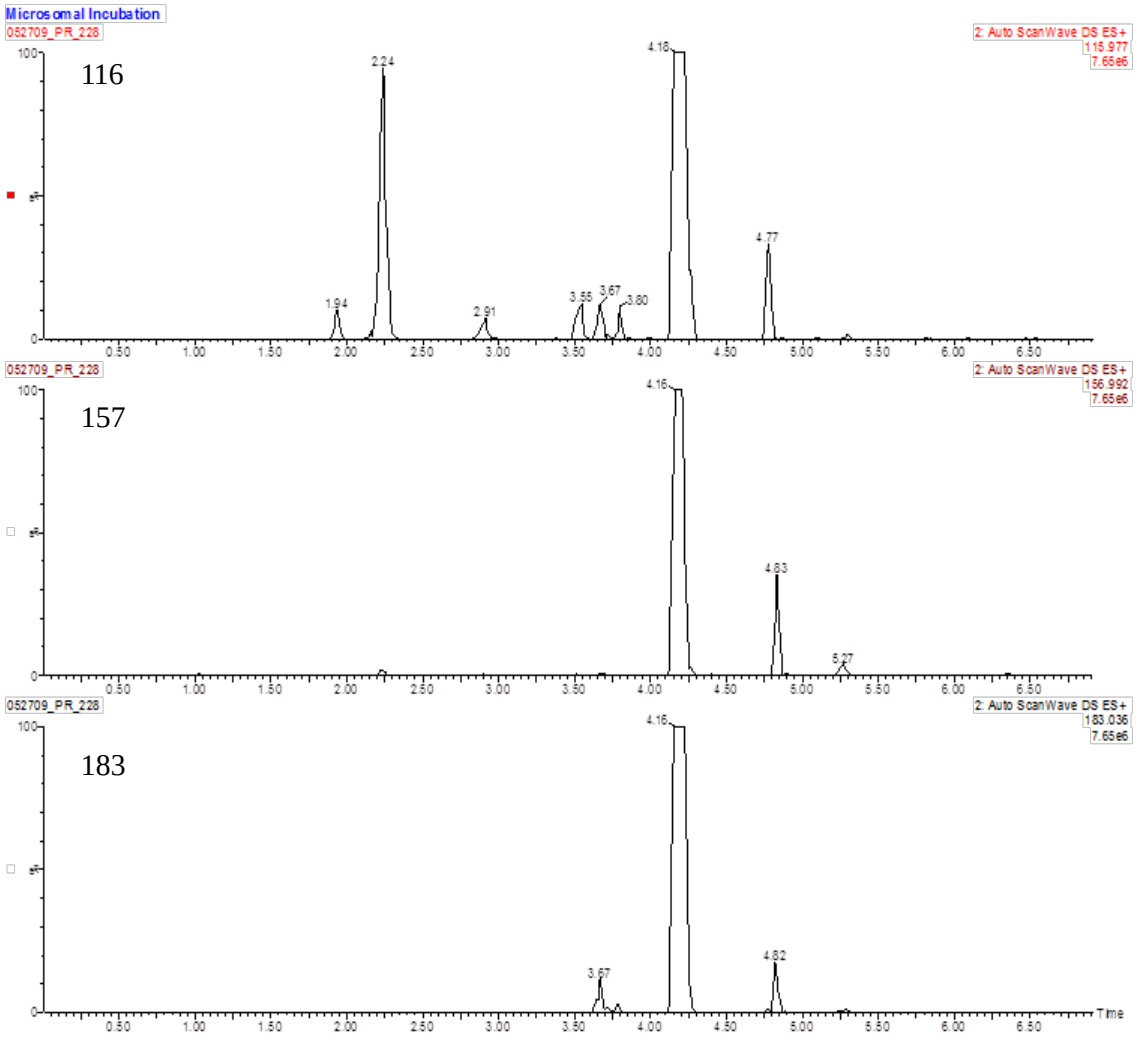


Figure 6.3.11 Survey scan results from microsomal incubation of propranolol using diagnostic fragment ions of (top) m/z 116, (middle) m/z 157, and (bottom) m/z 183.

The identification of the drug metabolites was confirmed by a review of the MS/MS spectra obtained from the survey scan mode analysis. In this mode of operation the mass spectrometer continuously monitors the LC chromatogram in full scan mode (in this case a range of 100 to 650 m/z in positive ESI). When an eluting peak's intensity exceeded a preset, user defined, threshold the instrument selected the base peak in the MS spectrum and switched to acquire an MS/MS spectrum of the base ion. After a predetermined number of scans the instrument automatically switched back to full scan

MS mode. The peaks eluting at retention times of 1.92, 2.24 and 2.90 mins gave rise to a base peak mass of m/z =452 and fragment ions of m/z = 276, 199 and 116, **Figures 6.3.12, 6.3.13 and 6.3.14.**

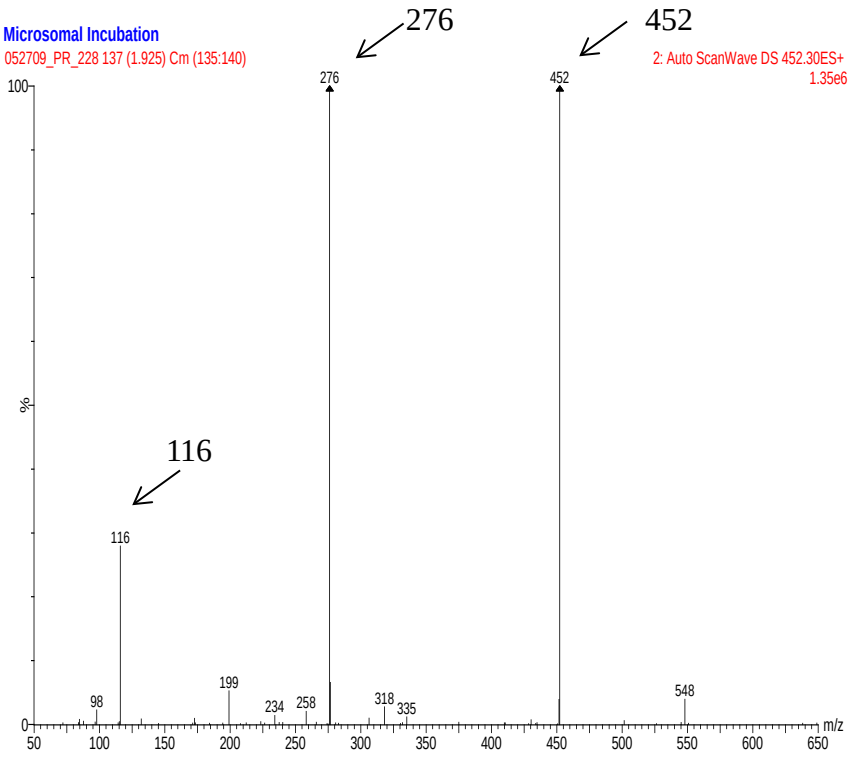


Figure 6.3.12 MS/MS spectra for hydroxyl glucuronide propranolol peak eluting at retention time 1.92 minutes.

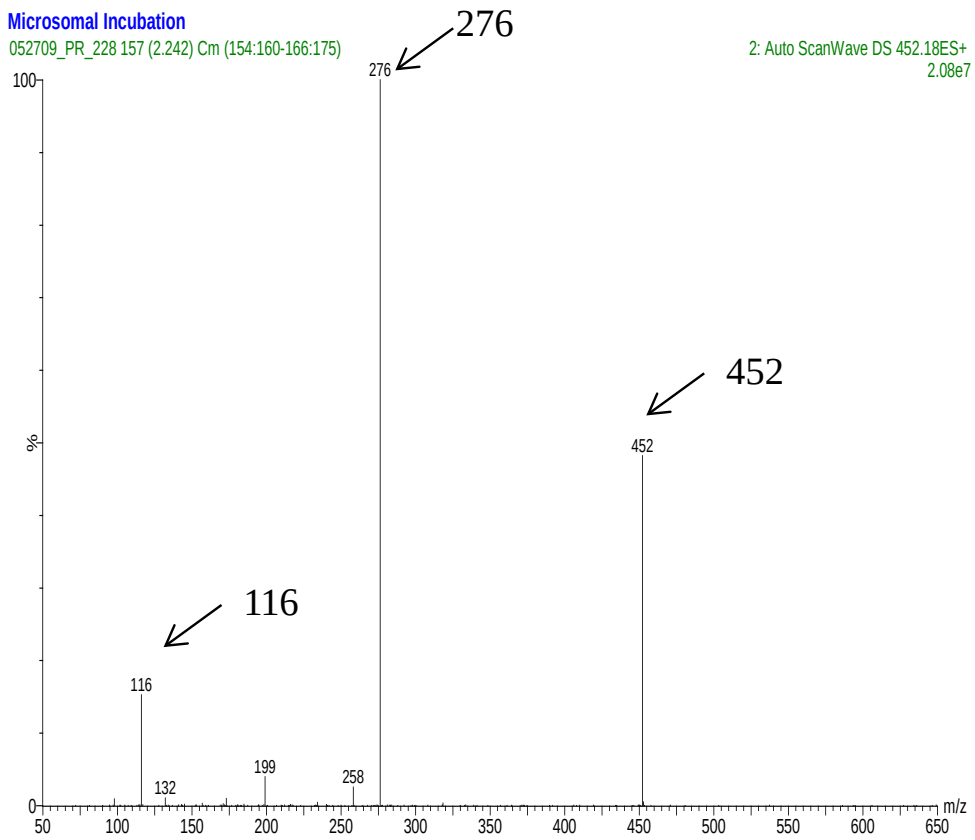


Figure 6.3.13 MS/MS spectra for hydroxyl glucuronide propranolol peak eluting at retention time 2.24 minutes.

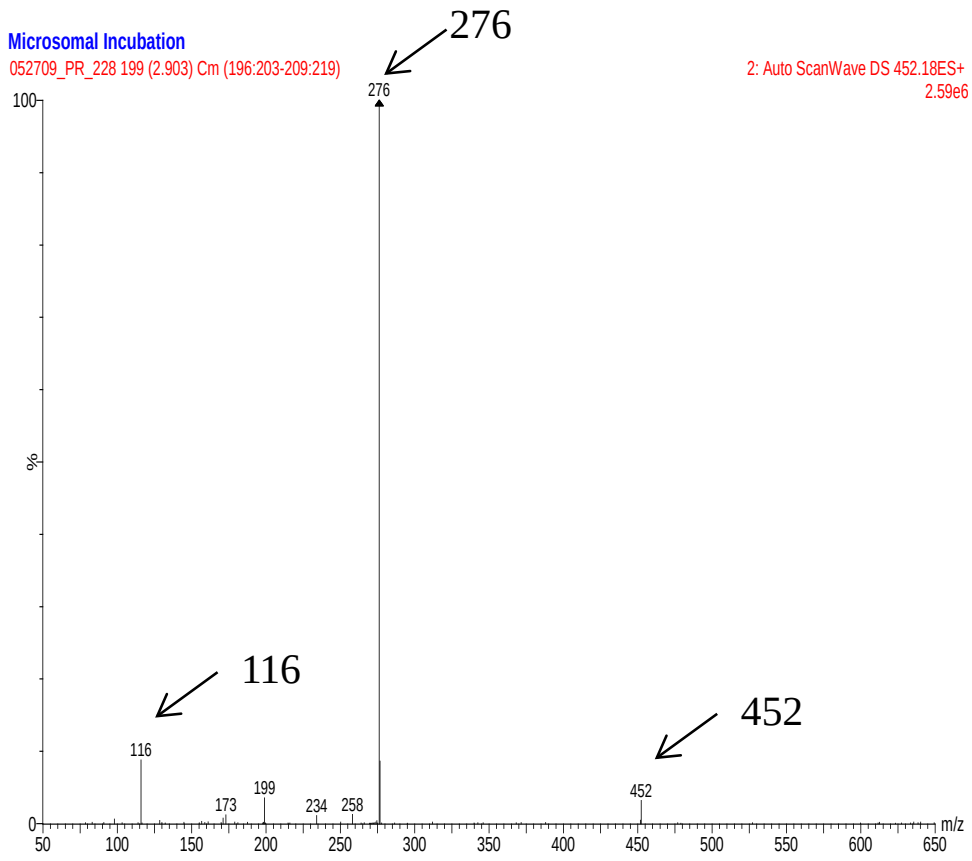


Figure 6.3.14 MS/MS spectra for hydroxyl glucuronide propranolol peak eluting at retention time 2.90 minutes.

These peaks can be rationalized as the phase II hydroxyl glucuronide metabolites of propranolol. There is a characteristic loss of 176 Da from 452 ⇒276 relating to the loss of the glucuronic acid moiety. The peak eluting at 2.90 minutes also had the characteristic base peak of m/z =452, however, this peak was much weaker than those peaks eluting at 1.92 and 2.24 minutes with the most intense peak being the m/z = 276 ms/ms fragment ion. This data suggests that this metabolite peak maybe thermally labile with the glucuronic acid group dissociating in the MS source as there has been past evidence of this phenomenon or has undergone in source fragmentation [18].

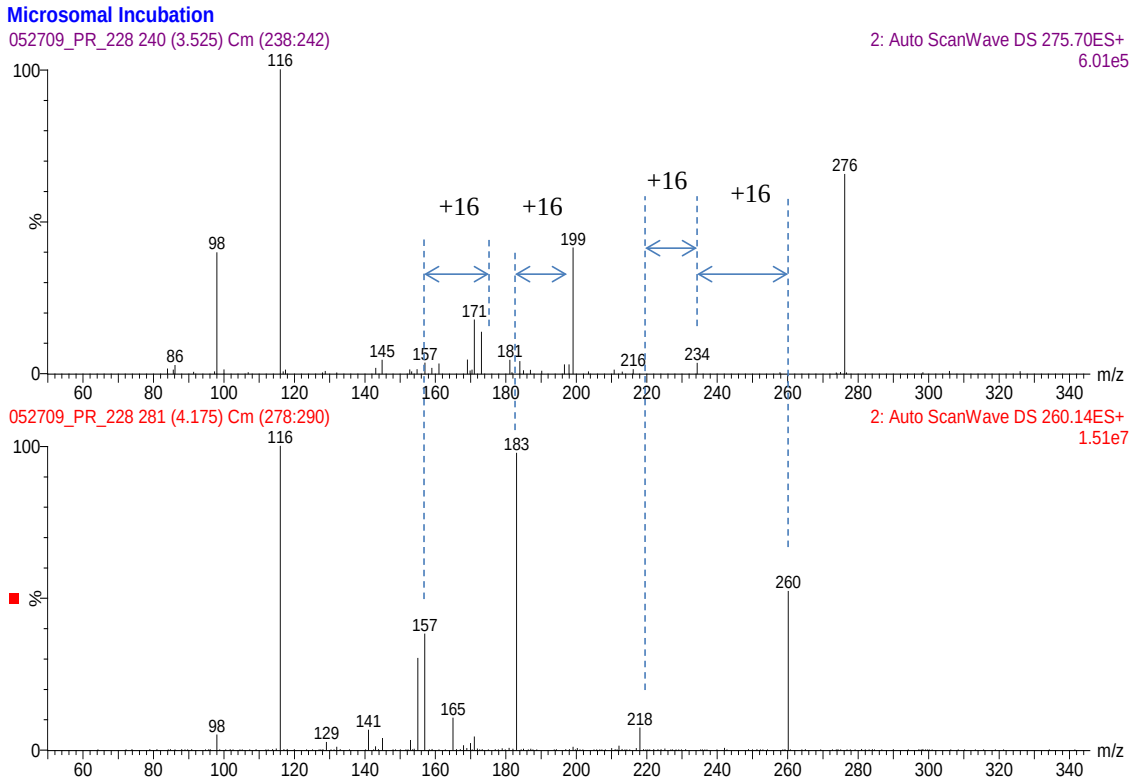


Figure 6.3.15 Comparison of the MS/MS spectrum of a hydroxyl propranolol metabolite (top) and propranolol standard (bottom).

The 276 ion is can be rationalized as resulting from the hydroxylation of the propranolol molecule. The peak eluting with a retention time of 3.53 mins gave rise to a peak with an m/z value of 276. A comparison of the MS/MS spectrum obtained for this peak (top) with that of the propranolol peak (bottom) is given in **Figure 6.3.15**.

From this it is evident that the ion 276 is +16 to the 260 ion in the propranolol spectrum, the 234 ion is +16 to the 218 ion in the propranolol spectrum, the 199 ion is +16 to the 183 ion from propranolol and the 173 on is +16 to the 157 ion in the propranolol spectrum. The 116 ion is present in both spectra suggest that the site of hydroxylation metabolism may not be on the aliphatic part of the propranolol molecule, **Figure 6.3.16**,

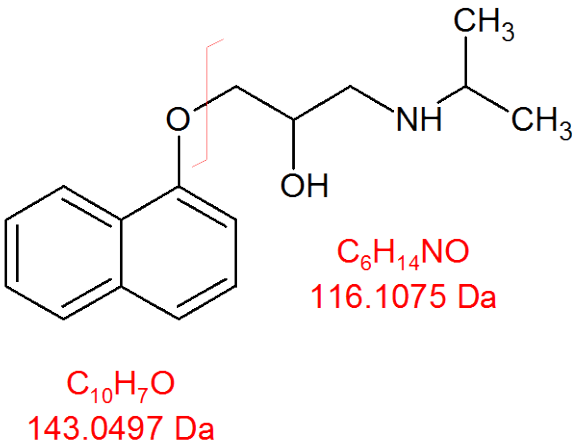


Figure 6.3.16 Structure of propranolol and MS/MS fragment of m/z 116.

The MS/MS spectra derived from the peaks eluting with retention times of 3.67 and 3.80 minutes are displayed in **Figures 6.3.17 and 6.3.18.** The MS/MS analysis of both LC peaks (3.67 and 3.80 mins) yielded the product ions m/z =436, 260 and 116. The ions m/z = 260 and 116 were derived from the propranolol molecule whereas the peak m/z = 436 can be rationalised as metabolism to a glucuronide, thus these two peaks are most probably glucuronide metabolites of the propranolol molecule itself. **Table 6.3.1** summarizes the potential metabolites of propranolol indentified by diagnostic fragment ions and their respective retention times.

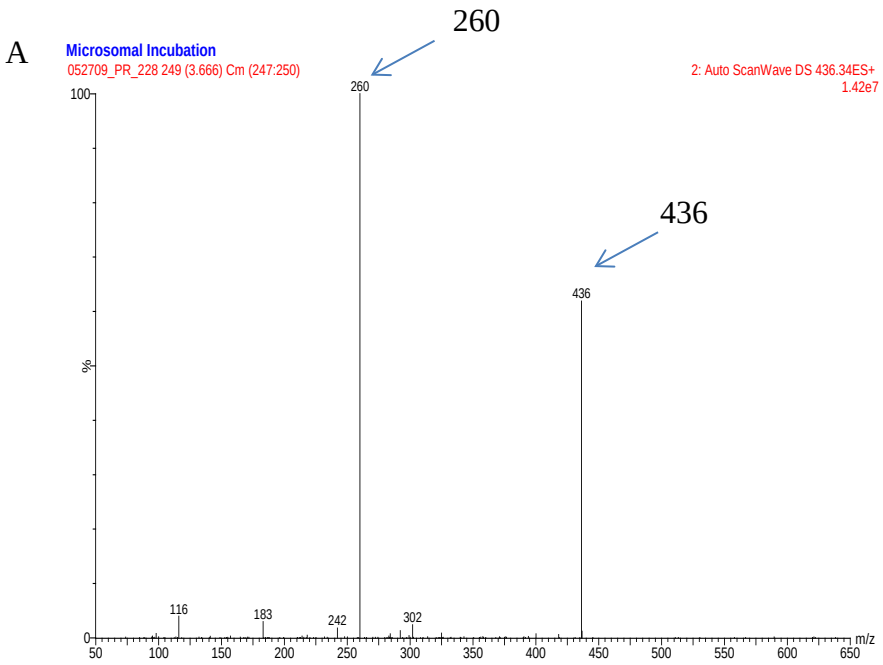


Figure 6.3.17 MS/MS spectra of glucuronide metabolites of propranolol eluting at retention time 3.67 minutes.

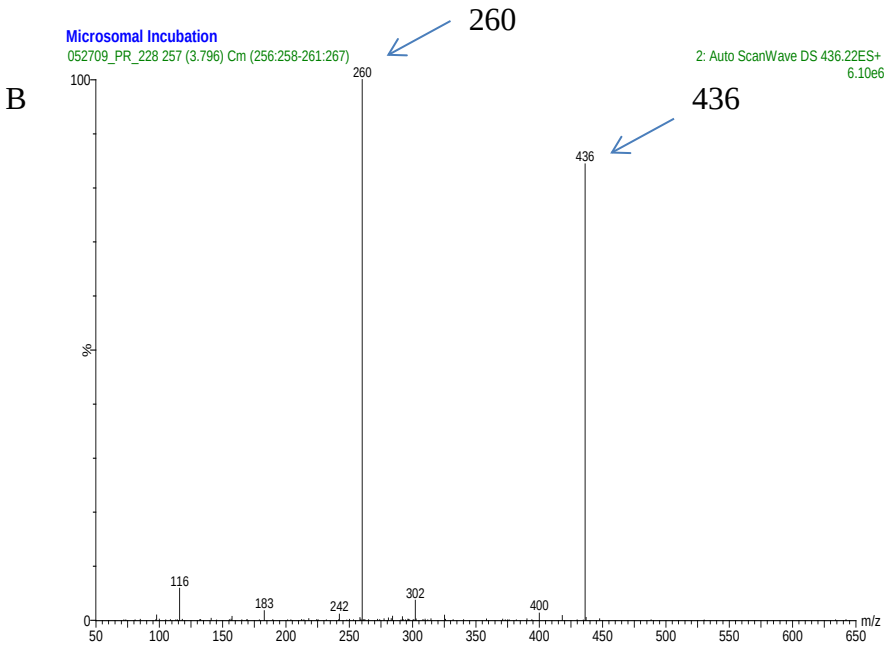


Figure 6.3.18 MS/MS spectra of glucuronide metabolites of propranolol eluting at retention time 3.80 minutes.

Peak Identification	Retention Time (mins)	Precursor ion (M+H)	Fragment Ions (M+H)
Propranolol	4.19	260	183, 157, 116
Hydroxyl Metabolite	3.53	276	199, 116
Glucuronide metabolite	3.67	436	436, 260, 116
Glucuronide metabolite	3.8	436	436, 260, 116
Hydroxyl Glucuronide metabolite	1.92	452	452, 276, 116
Hydroxyl Glucuronide metabolite	2.24	452	452, 276, 116
Hydroxyl Glucuronide metabolite	2.9	452	452, 276, 116

Table 6.3.1 Summary of retention times of propranolol and potential propranolol metabolites.

6.3.3 Application to metabonomic or biomarker discovery studies

As stated in Chapter 4, metabonomics has been defined as "Quantitative measurement of multivariate metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification" and has been primarily utilized to provide information for the wide-spread understanding of global metabolic regulation and biological system failure [19]. Initial work in the area of metabonomics was focused upon understanding the mechanisms of drug toxicity in mammalian systems [20-23].

Much of the initial metabonomics research employed either NMR or GC-MS as the primary platforms to acquire analytical data [24-26]. Although NMR and LC/NMR are used in drug metabolism studies, LC/MS is by far the dominant analytical platform for the acquisition of qualitative and quantitative data in drug metabolism and pharmacokinetics (DMPK) studies because of the inherent sensitivity, speed and throughput of the technique. The application of LC/MS to metabonomics studies has provided complementary data to that obtained by NMR, often producing assays of greater sensitivity [27-29].

The vast majority of the LC/MS metabonomics methodologies employed the use of formic acid based organo-aqueous gradient approaches [30-32], on 2.1 mm i.d. columns. Previously an investigation utilizing basic mobile phases with ESI positive mode MS was presented by Rainville *et al*. [33]. In this section of Chapter 6 the use of the CMFD/MS system with both acidic and basic mobile phases was utilized as a strategy to increase the potential information that could be obtained during a metabonomics experiment.

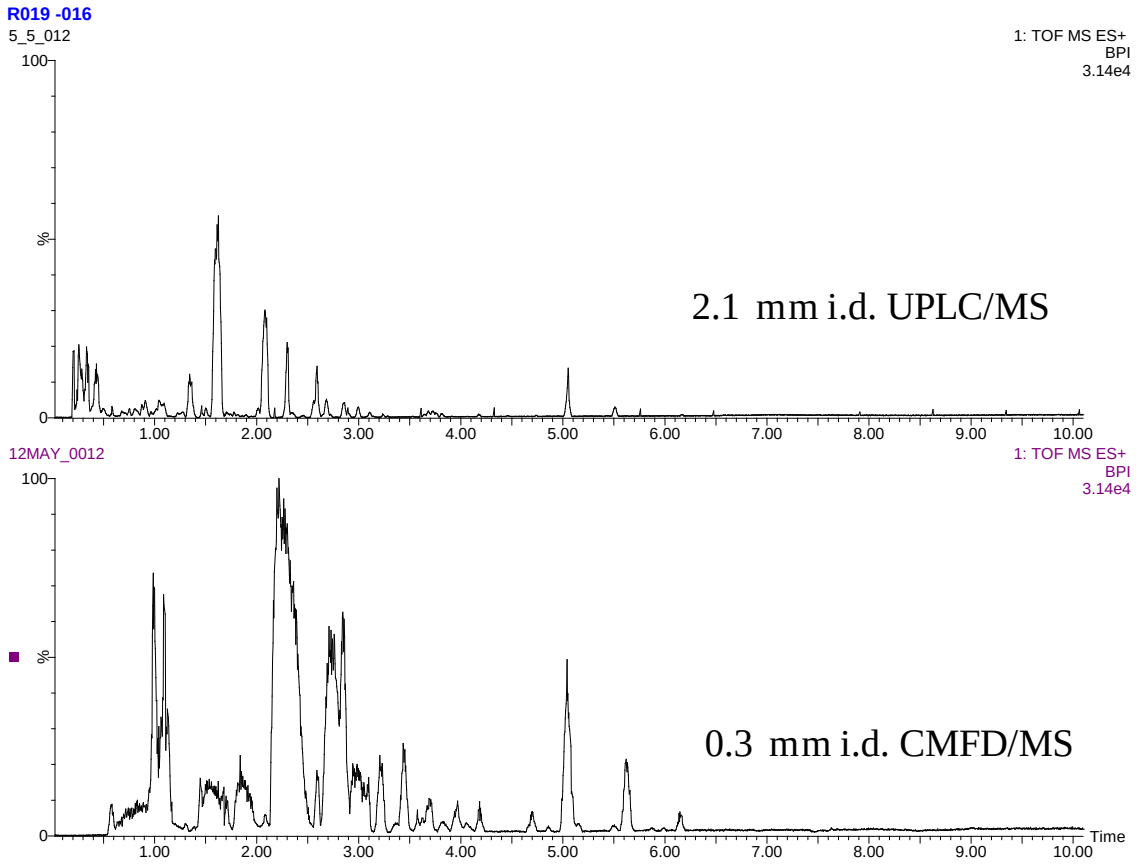


Figure 6.3.19 Separation of axenic rat urine by conventional 2.1 mm i.d. UPLC and on the prototype CMFD/MS system.

Figure 6.3.19 illustrates a separation of urine from axenic rat number nineteen on both a standard 2.1 mm i.d. UPLC column and the CMFD. In these chromatograms we observe an overall increase in the MS response for the analytes when they were separated under the CMFD/MS conditions compared with the conventional LC/MS approach. Many of the analytes in the chromatogram run under the CMFD/MS conditions show acceptable peak shape with minimal tailing, however there is evidence of overload of some of the analytes in particular those that elute between 2.3 and 2.8 minutes.

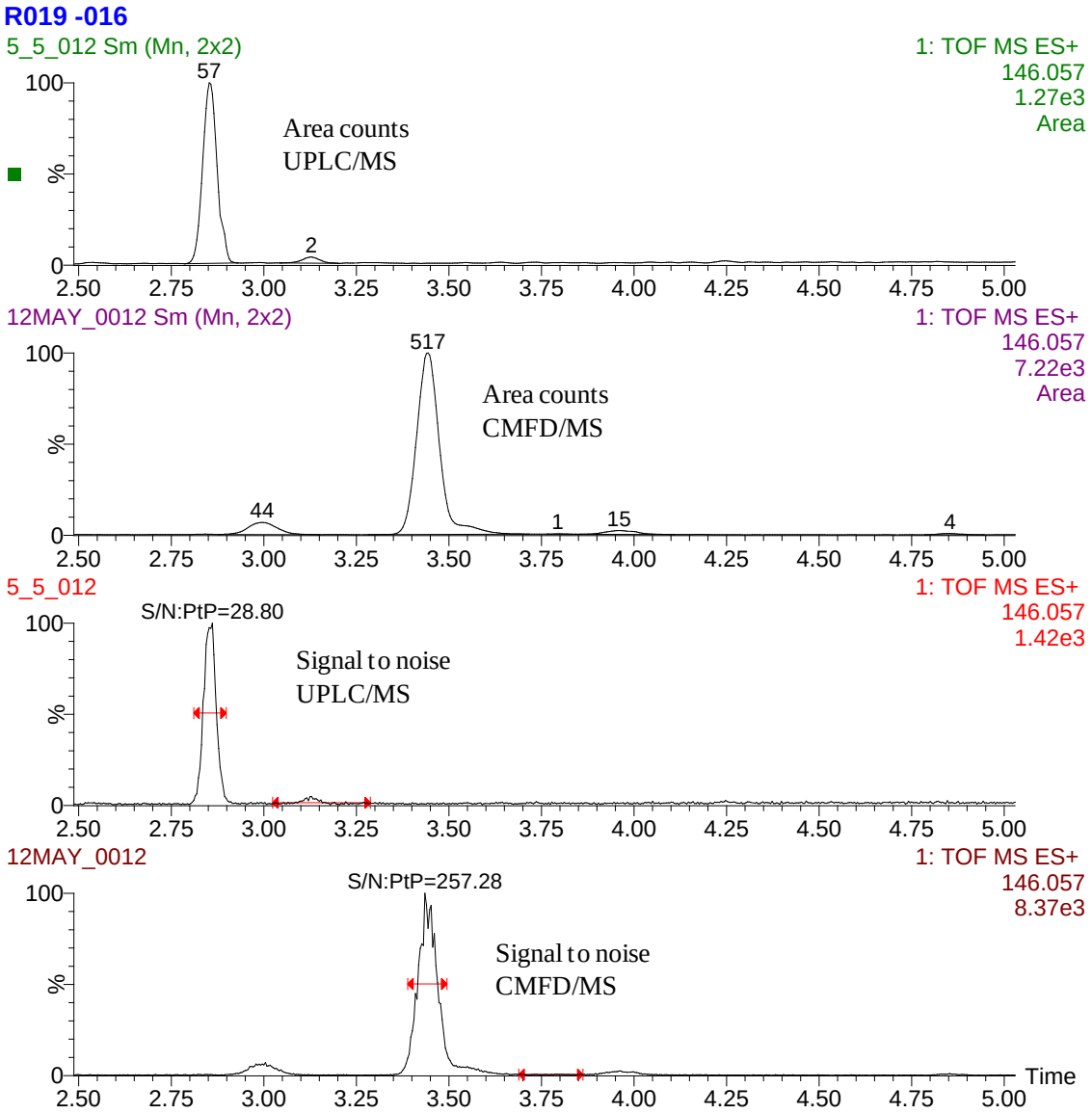


Figure 6.3.20 XIC of analtye with m/z 146.057 derived from axenic rat urine on conventional UPLC/MS and prototype CMFD/MS systems.

In the example shown in **Figure 6.3.20** the area counts, signal and signal to noise values are shown for the analyte with m/z of 146.057. Here we observe that the area counts have increased from 57 to 517, which is an increase of 9.1 fold. The overall signal utilizing the unsmoothed integrated data showed an increase from 1420 to 8370 *i.e.* a 5.9 fold increase, and a signal to noise increase from 28.8 to 257.3 or 8.9 fold. This

data is in agreement with the acquired results illustrated for the small molecule probe pharmaceuticals in Chapter 5. The peak width at base for this particular analyte however increased from 4.2 seconds on the UPLC/MS system to 7.8 seconds on the CMFD/MS system. While the peak width for this particular analyte is greater than those generated by previous analysis, one explanation for this could be related to the chemical structure of the analyte, since wider peak widths than the typical 3 second peak widths was also generated for this analyte by the UPLC/MS system.

The data in **Figure 6.3.21** shows the XIC for another analyte found in the sample (m/z 182.079). In this example, multiple peaks are present with the same m/z. The boxed in area highlights the separation of closely eluting peaks with the same m/z separated both by UPLC/MS and the CMFD/MS system. In this example the UPLC/MS separation showed increased resolution of the highlighted peaks compared to the CMFS/MS separation. This example illustrates the balance that can exist between the desired or required resolution of analytes from each other as well as from other matrix components, and the amount that can and or should be loaded for the desired sensitivity increase.

The utilization of high pH mobile phases as outlined in the previous chapters was again demonstrated with the analysis of the axenic rat urine in conjunction with the CMFD/MS system. However, theses separations carried out utilized ammonium acetate buffer systems adjusted to a pH value of 10. This modification was a result of the inability of the nano ACQUITY LC module to operate at the previously utilized basic pH of 10.5 as was described in Chapter 5, due to the dissolution of internal silica tubings within the LC system. The data shown in Figure 6.3.22 illustrates the vast differences in the profile of the sample that can be observed when complex biofluid

samples such as urine are separated under both the traditional acidic modified mobile phase and the basic modified mobile phase.

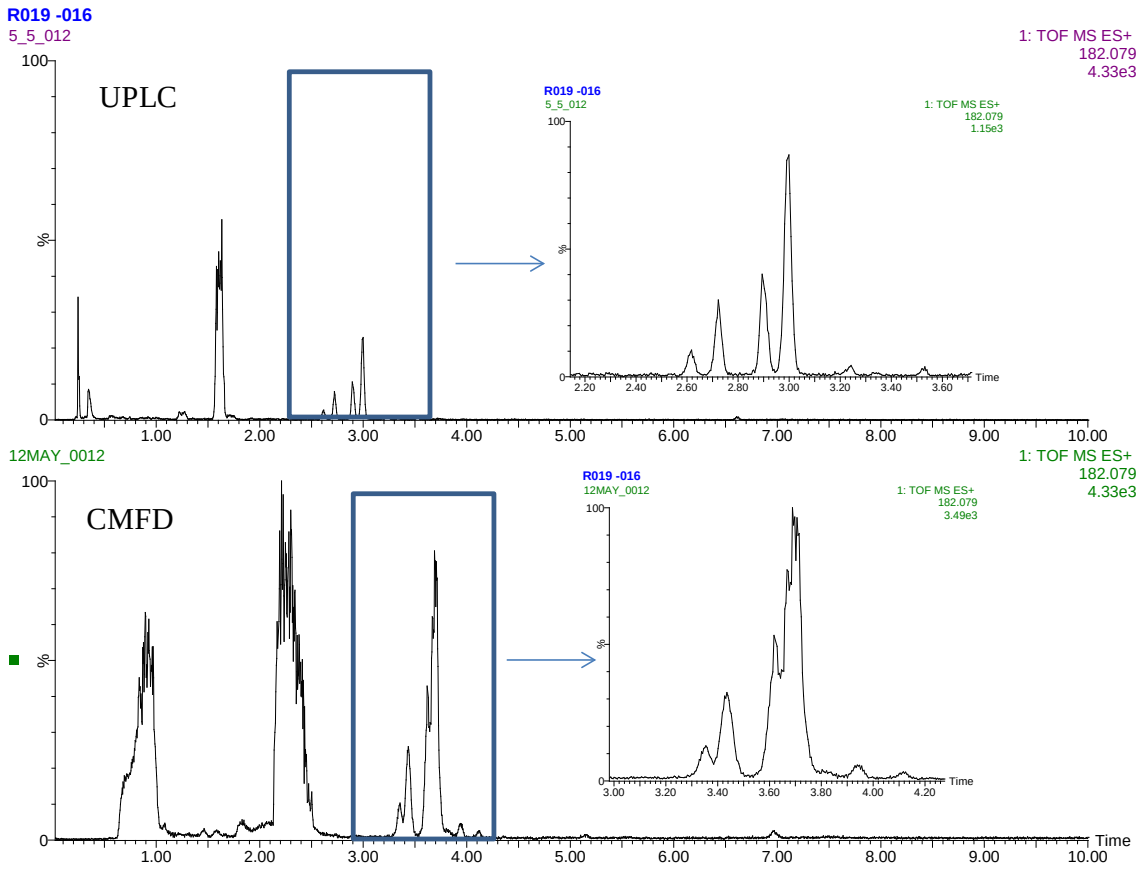


Figure 6.3.21 XIC of analtye with m/z 182.079 derived from axenic rat urine on conventional UPLC/MS (top chromatogram) and prototype CMFD/MS systems (bottom chromatogram).

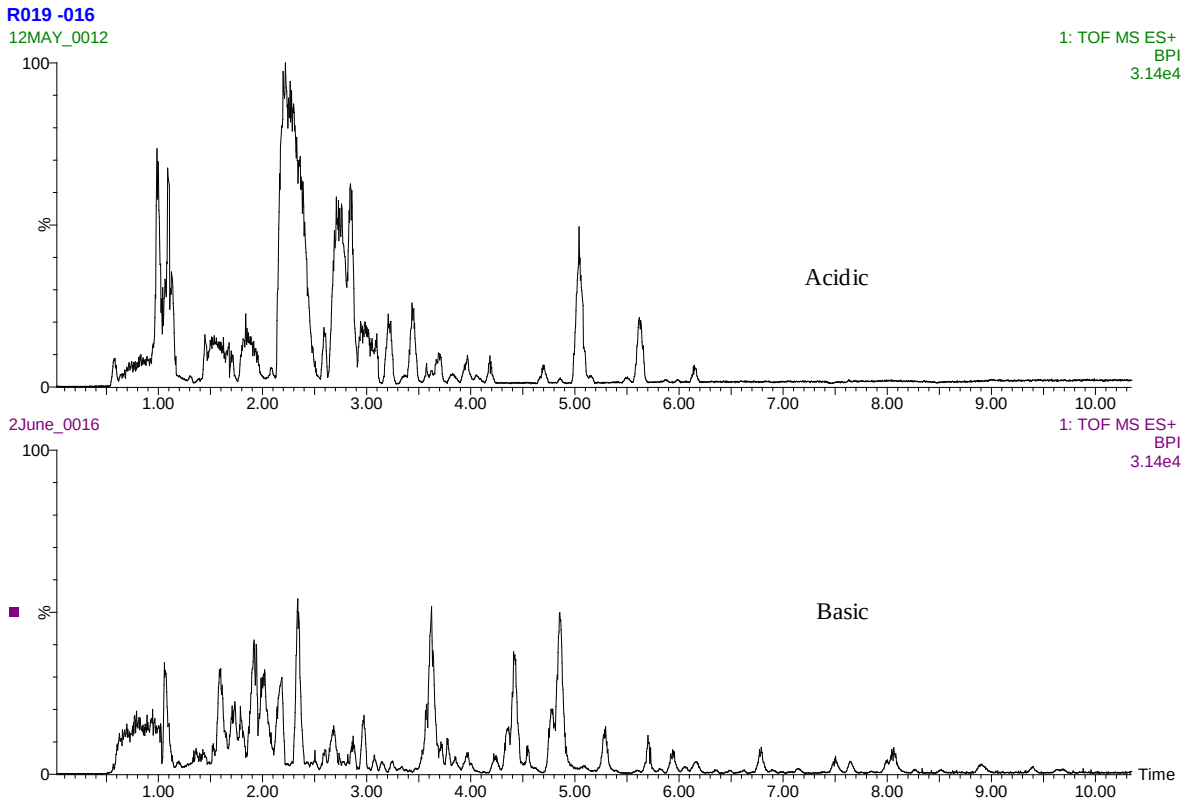


Figure 6.3.22 Linked axis display of full MS scan of axenic rat urine separated with CMFD/MS system under acidic (top chromatogram) and basic (bottom chromatogram) mobile phase conditions.

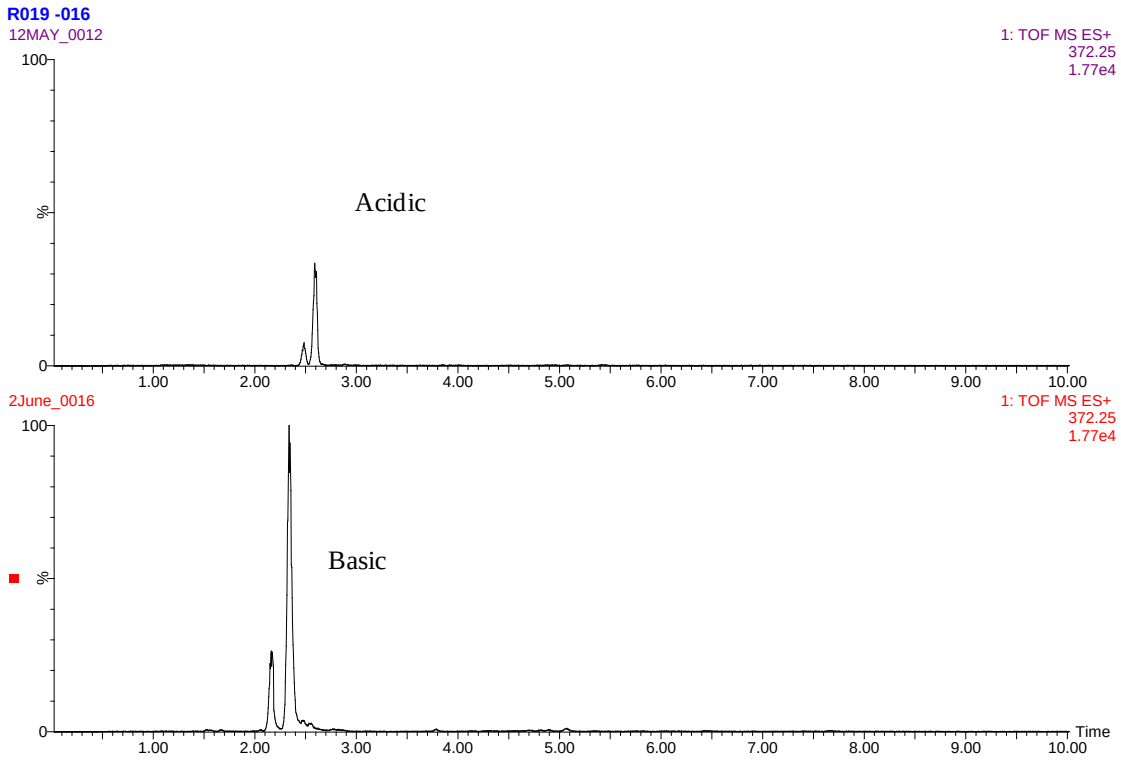


Figure 6.3.23 XIC of analyte with m/z of 372.250 analyzed with CMFD/MS system under acidic (top chromatogram) and basic (bottom) mobile phase conditions.

Again as was shown in Chapters 2 - 4, increases in analyte response can be increased when the MS is operating in ESI positive ionization mode with a basic modified mobile phase. Here we also observe that the resolution of the two analytes with m/z of 372.250 maintained baseline resolution. Also observed in **Figure 6.3.23** is the retention time shift of the analytes towards the void volume of the separation. This example again indicates that increases in MS sensitivity while operating under these basic conditions is not related to increased analyte retention and subsequent elution in a higher organic composition as the contributing variable responsible for the increase in MS response. Therefore these increases in sensitivity obtained with the basic mobile phases could result in significant sensitivity increases compare to traditional 2.1 mm i.d. scale chromatography analyzed under acidic mobile phase conditions and could therefore be

fully capable of obtaining similar information for small sample volumes of biofluids in metabonomic or other types of studies.

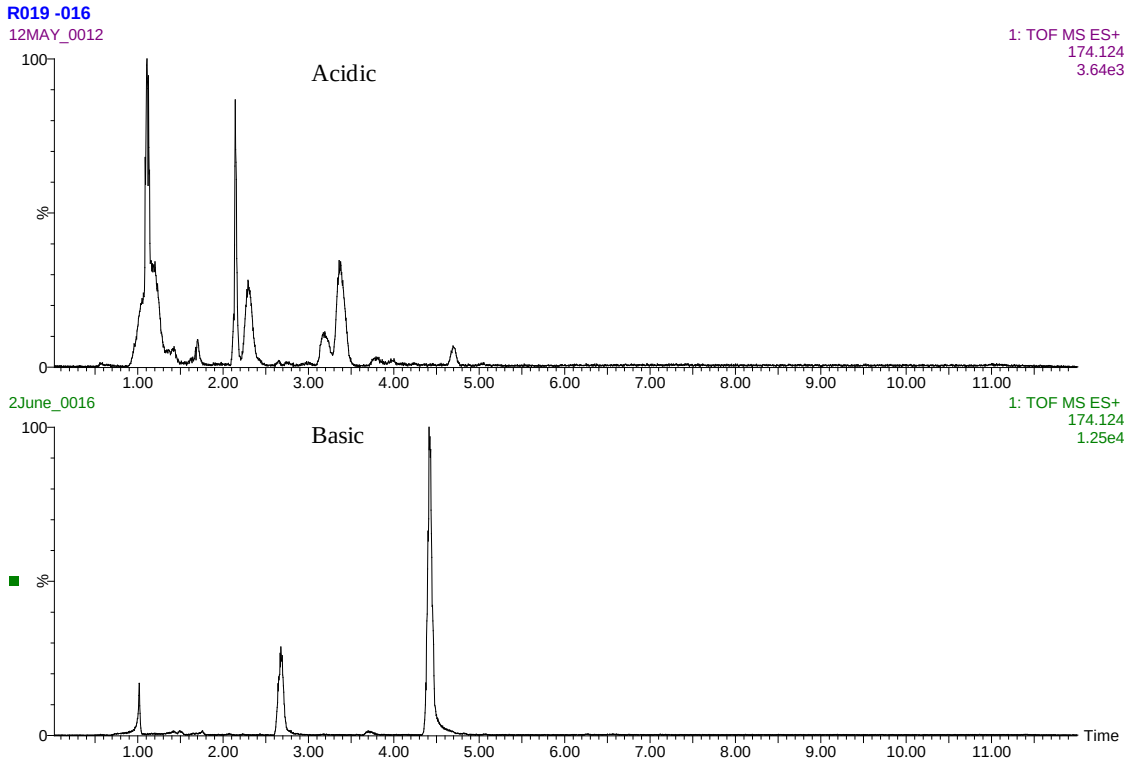


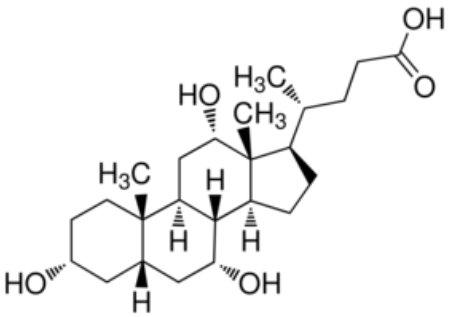
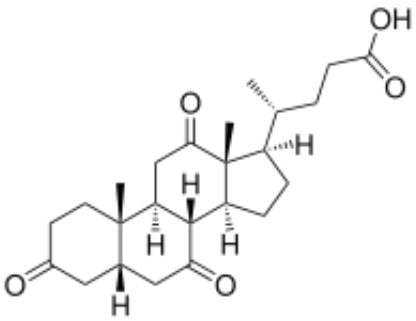
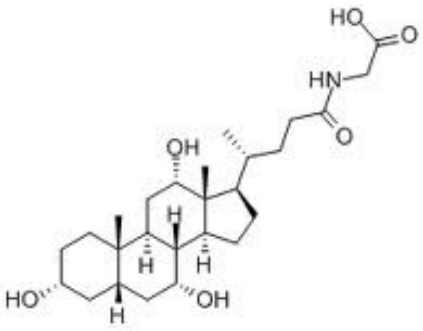
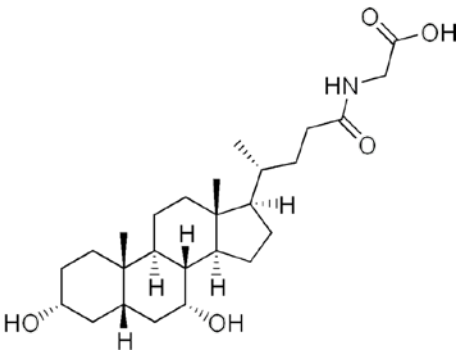
Figure 6.3.24 XIC of analyte with m/z of 174.124 analyzed with CMFD/MS system under acidic (top chromatogram) and basic (bottom) mobile phase conditions.

Figure 6.3.24 illustrates the differences in response of analytes with the same m/z when exposed to traditional acidic and basic mobile phase conditions. In this example, vast differences are observed in the separation profile for these analytes with a m/z of 172.124 as was also observed on the full scan data from **Figure 6.3.22**. These examples indicate the potential of these separation conditions to be implemented as a strategy for increased analyte detection for studies focused on the profiling of complex samples such as biofluid samples.

6.3.4 Bile acid profiling in dog

Bile is a complex biofluid and the source of important cholesterol derivatives such as the bile acids which are involved in the emulsification of lipids via the formation of micelles to aid in lipid digestion. The dispersion of lipid molecules fat into these micelles provides for an increased surface area for the action of lipases, which actually digests the triglycerides. As triglycerides are broken down into fatty acids and monoglycerides, they are absorbed by the villi on the intestine walls. After being transferred across the intestinal membrane, the fatty acids reform into triglycerides, before being absorbed into the lymphatic system through lacteals. Without bile salts, most of the lipids in food would be excreted in feces, undigested. Therefore without the action of bile acids this essential biomolecule would not be to be utilized by humans and other mammals.

Under normal health circumstances bile acid levels exist in relatively low concentrations in both plasma and urine, however in a diseased state the concentration and composition of bile acids can become disturbed. Therefore the profiling of bile acids provides for a tool into the determination of the liver and intestinal activity[34] . In earlier published work with rats, the utilization of UPLC coupled with ToF mass spectrometry was presented for the purpose of profiling bile acids from both dogs and rat samples [35]. In this section of Chapter 6, the use of the CMFD/MS system for the purposes of profiling bile acids from dog bile is presented. **Table 6.3.2** shows common bile acids that were detected in this study.

Bile acid	m/z ESI-	Structure
Cholic acid	407.5635	
Deoxycholic acid	401.5158	
Glycocholic acid	464.6148	
Glycochenodeoxycholic acid	448.6154	

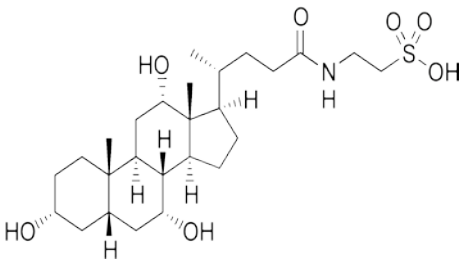
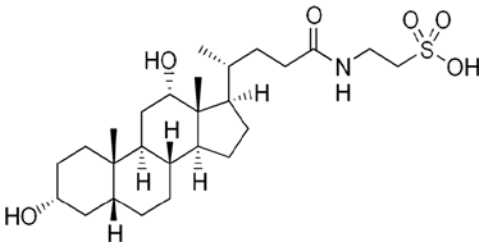
Taurocholic acid	514.6951	
Taurodeoxycholic acid	497.6877	
Taurochenodeoxycholic acid	498.6957	

Table 6.3.2 Description, m/z ESI negative mode and structures of bile acids profiled.

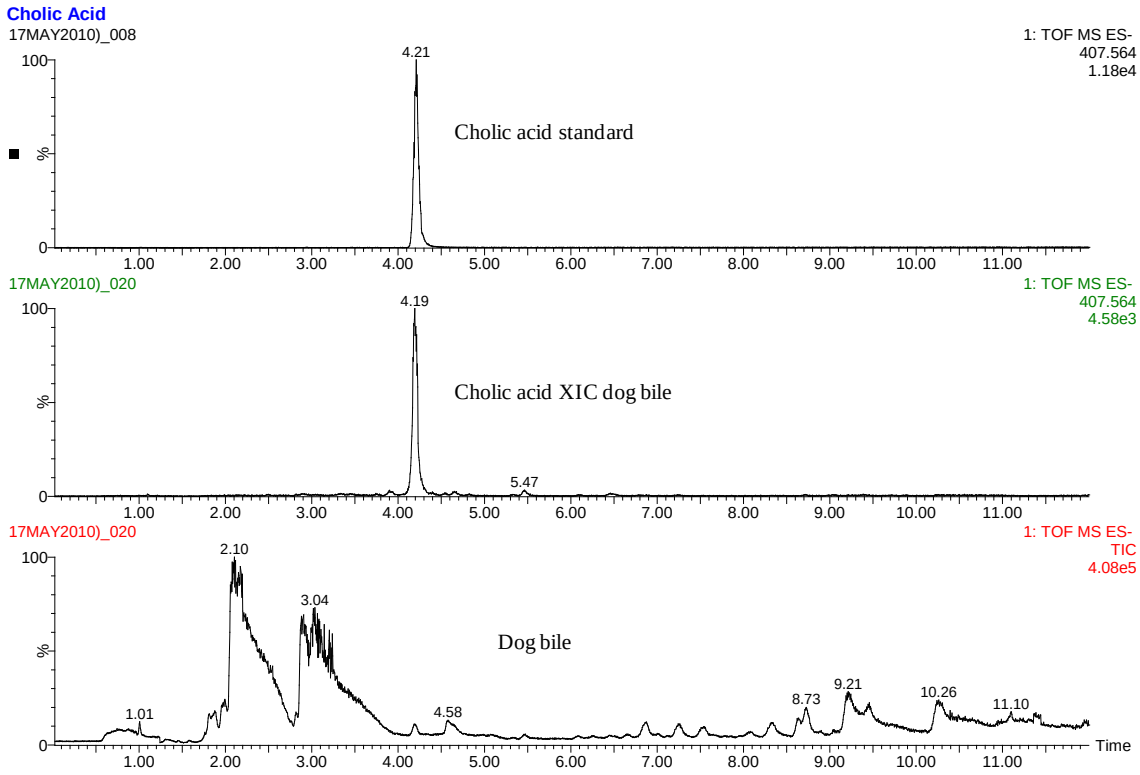


Figure 6.3.25 Cholic acid standard, XIC of cholic acid from dog bile, and dog bile separation.

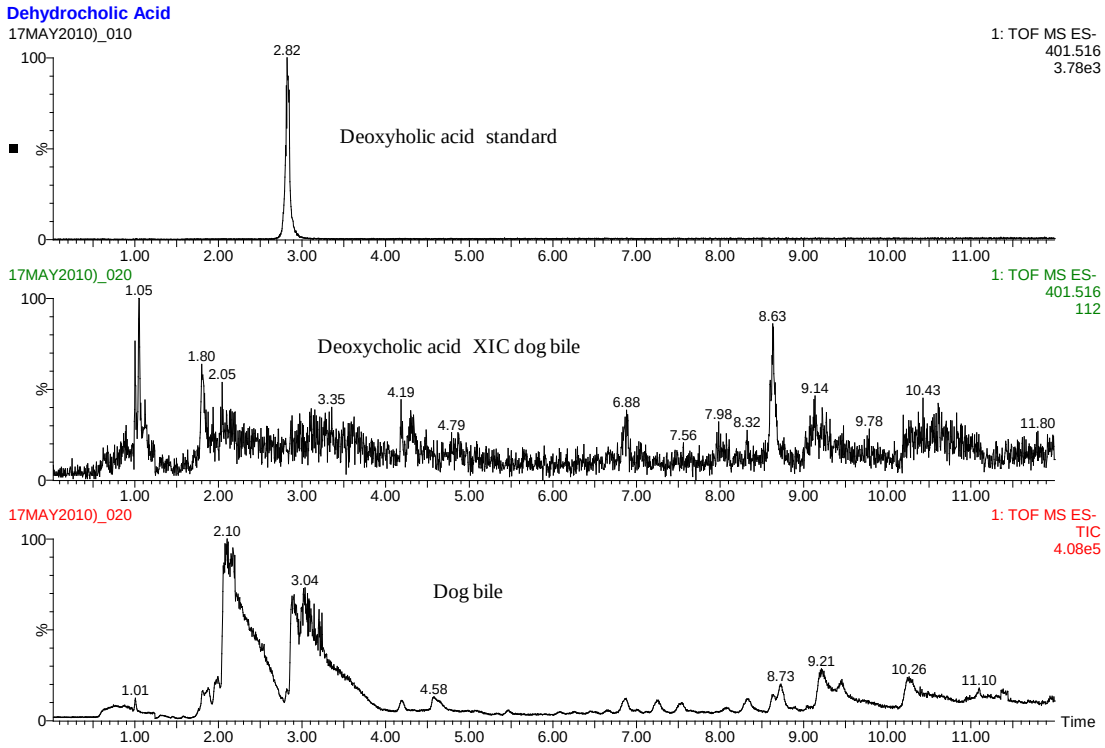


Figure 6.3.26 Deoxyholic acid standard, XIC of deoxycholic acid from dog bile, and dog bile separation.

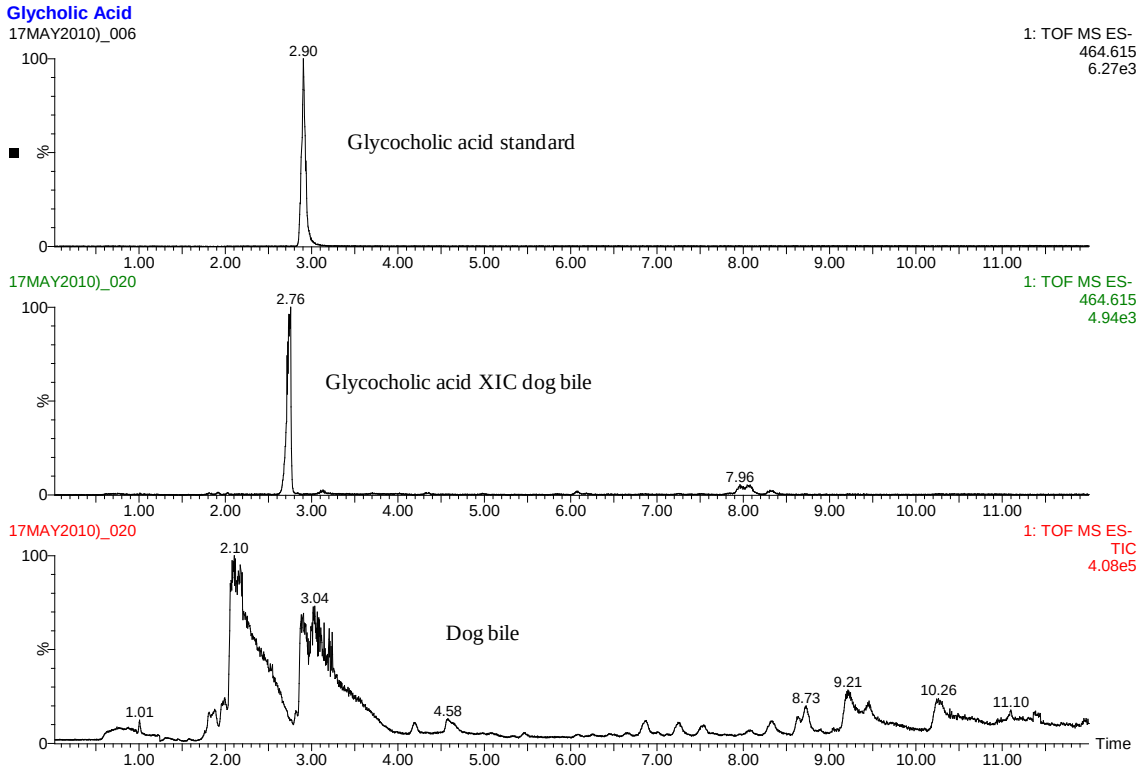


Figure 6.3.27 Glycocholic acid standard, XIC of glycocholic acid from dog bile, and dog bile separation.

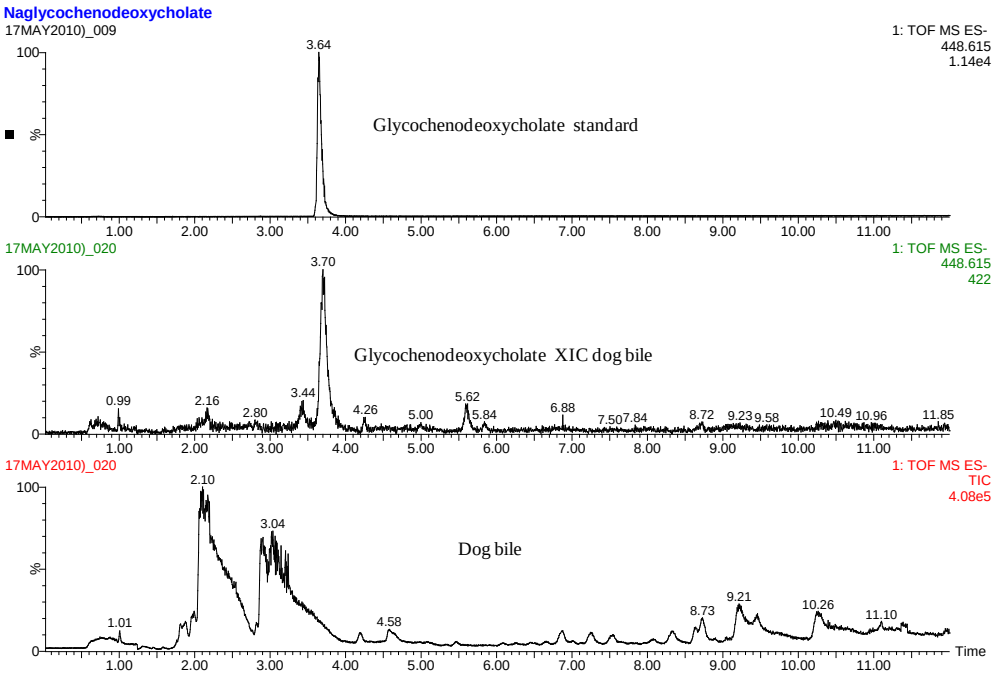


Figure 6.3.28 Glycochenodeoxycholic acid standard, XIC of glycochenodeoxycholic acid from dog bile, and dog bile separation.

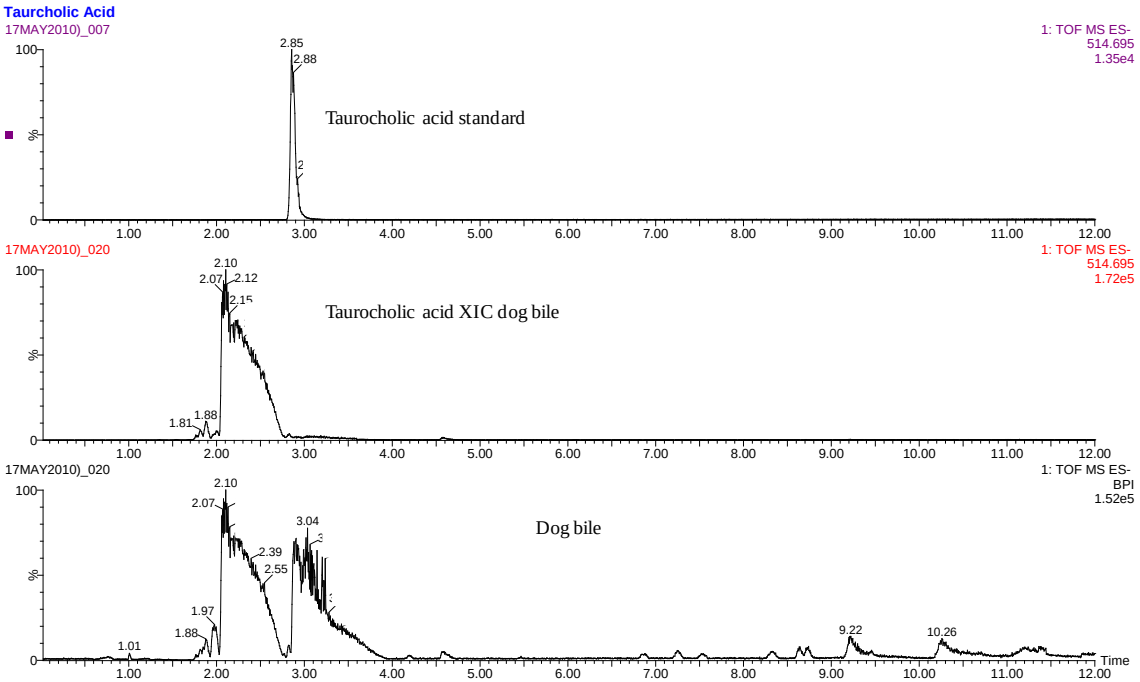


Figure 6.3.29 Taurocholic acid standard, XIC of taurocholic acid from dog bile, and dog bile separation.

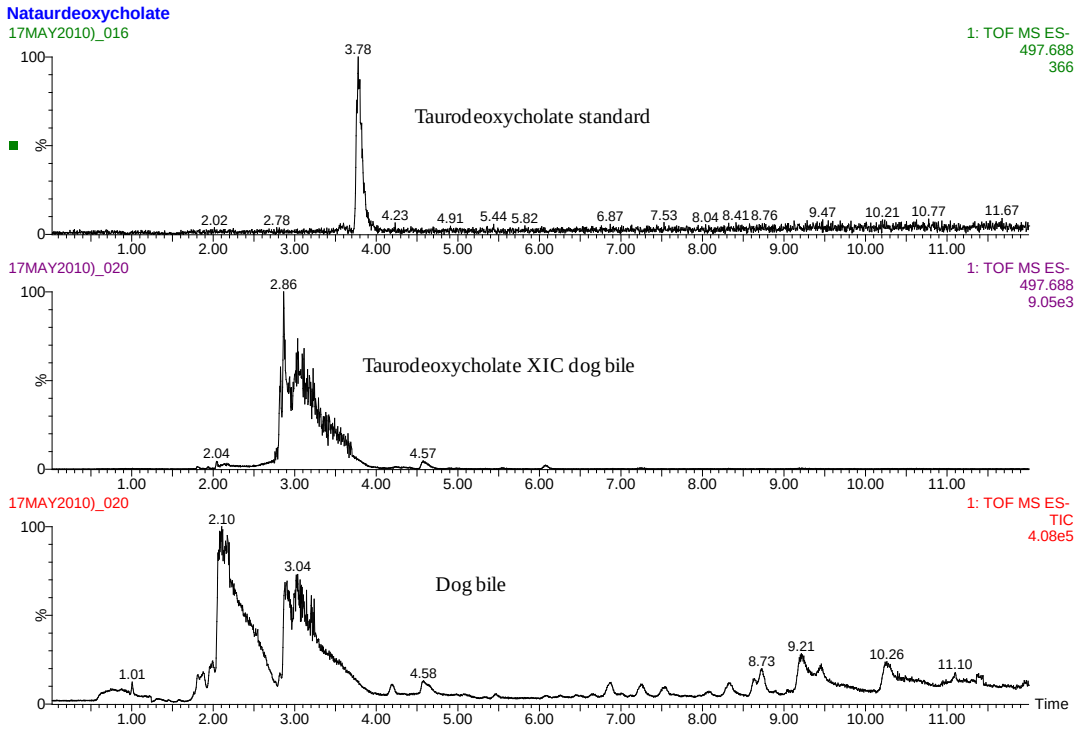


Figure 6.2.30 Taurodeoxycholic acid standard, XIC of taurodeoxycholic acid from dog bile, and dog bile separation.

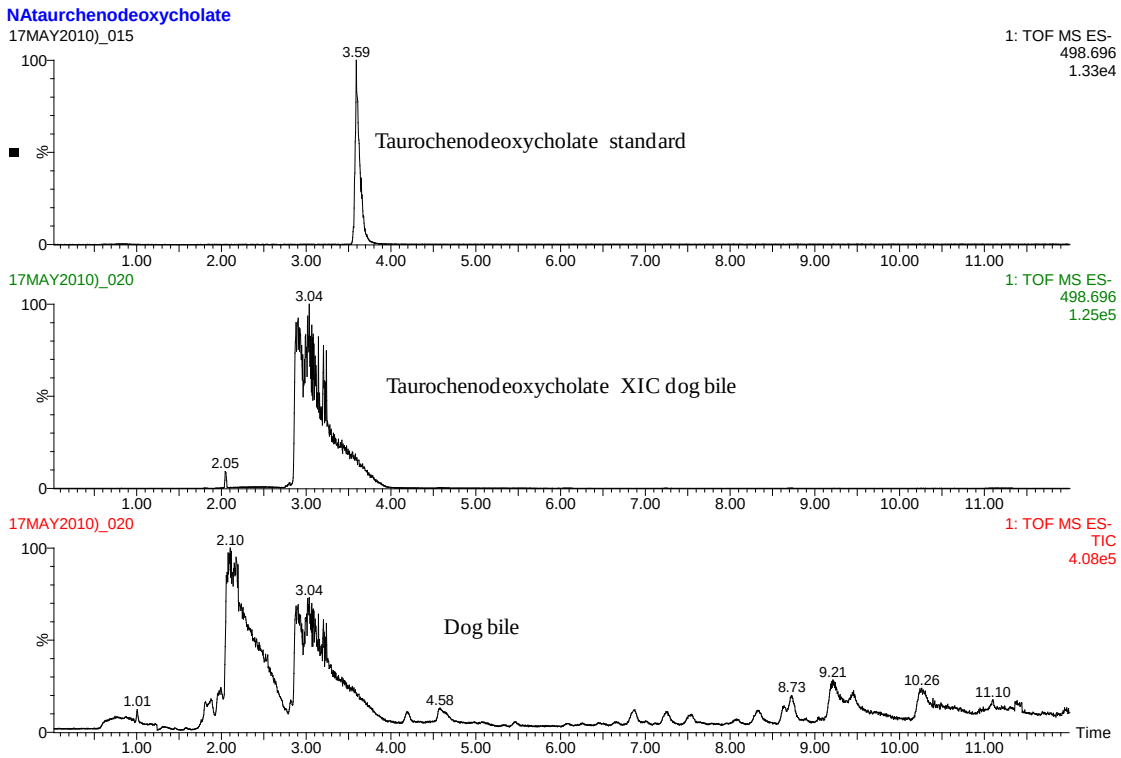


Figure 6.2.31 Taurochenodeoxycholic acid standard, XIC of taurochenodeoxycholic acid from dog bile, and dog bile separation.

The data shown in **Figures 6.3.25** through **6.3.31** illustrates the ability of the CMFD/MS system to successfully operate in ESI negative ionization mode. The data further shows the ability of the system for the separation of bile acids and in the profiling of bile acids from diluted dog bile. The peak shifts for the bile acids taurocholic, taurodeoxycholic and taurochenodeoxycholic acids are likely due to the high concentration of these bile acids in the dog bile sample. Each of these bile acids exhibit a shark fin like appearance whereby the tail end of the peaks are at the same retention time as the standard, however the overall retention time of the peak has shifted towards the void volume [36]. The lack of MS signal produced by the bile acids deoxycholic acid and glychochenodeoxycholic acid could be due to two possibilities. The first is that the relative levels of these two bile acids in the diluted dog bile are below the detection limits of the MS. The second possibility for the lack of detection could be related to ion suppression as both of the analytes co-elute with taurodeoxycholic and taurochenodeoxycholic acid, both of which are present at large concentrations [37, 38].

6.4 Conclusion

The utilization of the CMFD/MS system for the analysis of biofluid samples was shown in both ESI positive and ESI negative ionization modes. The use of the CMFD/MS system was shown to allow for the analysis of small sample injection volumes from plasma prepared by protein precipitation and from dried blood spots. Peak widths generated for these two analytes were in the order of between three to five seconds at base. Limits of detection for alprazolam and sitamaquine prepared as previously stated respectively were 0.1 ng/mL and 10 ng/mL. Both assays proved linear for three orders of magnitude and residual values for a multipoint calibration line proved to within the criteria as set forward by the US FDA for bioanalytical validation criteria. Cycle times produced for both of the assays were within five minutes therefore illustrating the high-throughput potential of the device.

This device was successfully utilized for the profiling of metabolites from the beta blocker drug, propranolol. The CMFD/MS system was shown capable of separating some thirteen related metabolites from the parent drug. The device was coupled with tandem quadrupole MS operating in survey scan lead to the detection of hydroxyl and glucuronide metabolites utilizing diagnostic fragment m/z of 116, 157 and 183. This data indicated the potential for high sensitivity, high resolution drug metabolism studies where sample volumes may be limited.

Further separation of complex biofluid samples derived from axenic rats and bile from dogs again indicated the separation and sensitivity capabilities offered by the CMFD/MS system. Comparisons of analyte response derived from axenic rat urine showed an increase in signal of almost six fold and an increase in signal to noise of nine fold with respect to conventional UPLC. The utilization of basic mobiles phases was

shown to be possible with the CMFD. This combination of basic mobile phase and CMFD/MS operating in ESI + mode showed the differences in separation profile that could be obtained by implementing these conditions. Increases in sensitivity where again shown for analytes under the basic conditions compared to traditional acidic conditions for analytes detected in the axenic rat urine. This data would indicate that the coupling of the CMFD/MS system with basic mobile phases would be a viable strategy for metabonomics studies. Furthermore, in the case where only small sample volumes are available, this strategy of pH profiling on the CMFD/MS system with its increased sensitivity capabilities over traditional 2.1 mm i.d. columns, may lead to a significant increase in the ions detected over the traditional utilized LC/MS methodology.

The CMFD/MS device was also shown to be capable of operating in ESI – mode. The successful separation of analytes from dog bile was carried out. Chromatographic peaks identified as bile acids were shown to be of gaussian peak shape unless in a state of mass overload. The most concentrated bile acids identified by accurate mass MS and by authentic standards were found to be taurocholic, taurodeoxycholic and taurochenodeoxycholic acids. The peak shape and decrease in retention of these analytes was indicative of a mass overload condition with the concentration and injection volume that was utilized for the bile separation.

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Chapter 7

Final Conclusions and Future Research Directions

But now my task is smoothly done,

I can fly, or I can run,-Milton

7.1 Final Conclusions

The direction of this research project was derived from two distinct approaches as a means to increase the sensitivity and information obtained from the analysis of biofluid samples under electrospray positive mode LC/MS. The first approach demonstrated the benefits of using basic solutions such as ammonium hydroxide and ammonium acetate to modify the aqueous mobile phase in LC/MS analysis operating in electrospray positive ionization mode. Both small molecule pharmaceuticals and endogenous compounds in biofluids showed increases in signal, signal-to-noise, and retention in comparison to traditional acidic modified mobile phase LC/MS.

An increase in the chromatographic retention of poorly retained small molecule pharmaceuticals such as ranitidine was observed compared to identical LC/MS separations carried out under traditional acidic modified aqueous mobile phases. This increase in retention time could therefore be incorporated as a strategy to reduce any potential matrix interferences that could occur near the void volume of a LC/MS separation. The study did not however show a significant increase in retention for the predominant analytes that were detected in the column void volume found in urine samples under basic mobile phase conditions compared to the same analysis acquired with the acidic mobile phase. Therefore although the strategy of utilizing basic mobile phases increased the retention of many of the pharmaceutical compounds in the study it was not successful with the highly polar compounds detected in the void volume of the urine samples tested.

The base modified mobile phase further made it possible to resolve endogenous compounds found in urine that had previously co-eluted under traditional acidic aqueous mobile phase LC/MS separations. Furthermore, the increase in the MS response was

not primarily related to the late elution of analytes in a higher organic composition under basic conditions which is often the interpretation for the increase in MS response under these conditions. This was shown with compounds such as alprazolam and fluticasone which maintained identical retention times under both pH conditions and still showed increased MS signal when chromatographed under the basic conditions. Moreover analytes found in rat urine that exhibited a shorter retention time under basic versus acidic mobile phase conditions also showed increases in MS response.

The effect of the mobile phase pH on the choline-containing phospholipid fraction present in protein precipitated rat plasma from four different sources, was grossly unaffected by the change in mobile phase pH. However, the basic modified aqueous mobile phase in combination with methanol yielded the highest response for the choline-containing lipid fraction of the prepared plasma sample. Therefore, although there was an increase in MS response for many of the pharmaceuticals tested in Chapter 2, as well as increased retention, careful assessment to ensure co-elution of pharmaceuticals with the choline containing phospholipids should be assessed if implementing the use of basic mobile phases as matrix interferences could exists.

These high pH conditions were then utilized in combination with a 1.7 µm C_{18} chromatographic column to produce a simple, sensitive, and specific LC/MS assay for alprazolam in rat plasma The limit of quantification using protein precipitation was determined to 0.01 ng/mL. The variability of the assay was determined to be less than 6% calculated via multiple QC sample levels. The assay was shown to be linear over a range of 0.01 to 10 ng/mL. The system and column was also shown to be robust as the system was operated under these conditions continuously over a 4 day period and 1000 injections of protein precipitated plasma samples without any deterioration of chromatographic peak shape or increase in system backpressure. This strategy has been

further utilized in the DMPK laboratory at Waters to produce high sensitive assays for other compounds such as fluticasone, and clopidogrel.

Moreover the application of basic mobile phases in LC/MS operating in electrospray positive ionization mode in metabonomic studies showed great promise. The technique provided complementary data to that obtained with traditional acidic based mobile phases and resulted in an increased in the overall number of ions detected during the study. The analysis of a real sample set of control rats and rats dosed with hydrazine revealed that both the acidic and basic system were capable of differentiating between the 0-hr and 48-hr time points.

The second approach was the development and implementation of a 0.3 x 100 mm i.d. ceramic microfluidic device packed with 1.7 µm C_{18} BEH chromatographic particles and coupled to a compatible ESI MS source for the analysis of biological samples for use in DMPK laboratories. The device showed average chromatographic efficiencies of 9038 plates compared to 10219 plates for standard silica capillary columns packed with the identical sorbent. Gradient performance utilizing a diverse mix of compounds yielded a peak capacity of 55 as the average peak widths for all analytes was 0.11 minutes for a 6 minute separation.

Resolution of the probe pharmaceutical alprazolam and associated hydroxyl metabolite was maintained between 1.2 and 1.5 for four different devices. Testing of the device with plasma samples prepared by protein precipitation resulted in over 1000 injections being carried out over approximately a one week period while maintaining resolution of alprazolam and its associated hydroxyl metabolite. Furthermore no dramatic increase in system back pressure was observed over this test period.

The nano-scale system utilized in this work was unable to withstand the high pH (10.5) utilized in Chapters 2 through 4. This was most likely due to the dissolution of the silica-tubing resulting in line plugging and subsequent system overpressure. However, the ceramic material appeared stable following incubation with the pH 10.5 solution as the morphology and appearance was observed to be similar before and after incubation at this elevated pH. Prolonged operation of the system at a pH of 10 was obtainable and these conditions were utilized to analyze the small molecule pharmaceutical alprazolam during a continuous six day period without incidence or failure of the system. These mobile phase conditions were further utilized in the separation of endogenous components found in rat urine.

MS source optimization studies indicated the need for operation of lower capillary voltages as many of the small molecules tested ionized best under condition of less than 3.0 kV applied to the capillary. Further a closed source resulted in less background noise observed and therefore was implemented during the course of the work carried out in Chapter 6. The resulting MS source built to operate under these conditions and with the flow rates required to operate the 0.3 x 100 mm ceramic microfluidic device yielded an increase in signal-to-noise for many small molecule pharmaceuticals when compared to the traditional high-flow source fitted with a narrow-bore (75 µm i.d.) capillary.

This LC/MS configuration was then successfully utilized in the analysis of small molecule pharmaceuticals in rat plasma prepared by protein precipitation. The configuration was further utilized in the separation of metabolites from microsomal incubation of propranolol. Operation of the system under negative electrospray ionization mode was demonstrated utilizing bile acids as the test molecules. Furthermore, the profiling of bile acids was successfully carried out with diluted dog bile. Finally, the utility of this configuration was shown operating under both high and

low pH mobile phase combinations for the analysis of urine derived from rats dosed with the model toxin hydrazine as an example of the utility of such a device for metabonomics or biomarker discovery studies.

7.2 Future Research

Future research should focus on several areas to further explore the utilization of both of the approaches investigated within this body of work. These could include:

1. The influence on the stability, and increase, or decrease in MS signal obtained from phase I and phase II small molecule drug metabolites when utilizing high pH mobile phases compared to tradition acidic mobile phases analyzed under ESI + LC/MS.

2. While the investigation of the use of the high pH mobile phase showed that there was no retention time shift for the choline-containing phospholipids found in protein-precipitated plasma, an investigation on the use of this technique as a means to either reduce or increase matrix effects could prove useful.

3. Basic mobile phase pH has been shown as a means to increase the loading capability of basic pharmaceuticals for preparative chromatography. A similar investigation carried out on capillary or nanoscale chromatography may prove useful as a means to further increase bioanalytical assay sensitivity by increasing the mass loading capability at this chromatographic scale.

4. Further investigation into the mechanism of small molecule ionisation in basic solutions under electrospray positive mode conditions.

5. Investigation into other ceramic microfluidic i.d. dimensions and channel shapes on chromatographic performance, robustness and LC/MS assay sensitivity.

6. As previous “chip” based devices utilize a trap and analytical column configuration in one device, a comparison on the advantages of this approach versus a separate trap and analytical configuration would be useful. The later approach would provide for a disposable guard column type format as well as the ability to have multiple chromatographic sorbent configurations that could prove useful during method development as this configuration would be more flexible for method development compared to the traditional approach of both trap and analytical media on the same chip.

7. Packing of the ceramic microfluidic device with “fused-core” or “poroshell” type particles and a comparison of the chromatographic separation capability, influence on system pressure and analyte loading capacity compared to the 1.7 µm porous particles utilized in this could shape the future of capillary and lower scale chromatography. Further packing the ceramic microfluidic device with other packing materials such those utilized in hydrophilic interaction chromatography could be carried out and may prove useful.