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By

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APPLICATIONS OF HYPHENATED ANALYTICAL TECHNIQUES IN PHARMACEUTICAL INDUSTRY

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

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ABSTRACT

Analytical chemistry plays a critical role in pharmaceutical research and development. A typical analysis includes separating and identifying active pharmaceutical ingredients and impurities, determining the quantity of counterions, residual solvents, moisture, heavy metals in drug substances and drug products, and analyzing drugs and their metabolites in biological fluids. Analytical method development and validation are arguably the most important procedures in analytical research and development, because the qualities of drug substance and drug product are controlled by using analytical methods. This dissertation focuses on the application of various analytical techniques towards pharmaceutical industry.

An application of headspace gas chromatography/flame ionization detector (HSGC/FID) for determination of residual solvents in drug product has been studied in order to increase productivity of drug analysis in the pharmaceutical industry. The conditions of HS sampler and GC were optimized to make the HSGC method more sensitive, efficient and reproducible. The examples of real drug substance analyses demonstrate the broad application potential of this HSGC method in the determination of residual solvents in drug substances.

A simple, sensitive and robust liquid chromatography-tandem mass spectrometer (LC-MS/MS) method was developed and validated for highly polar aminoglycoside compounds gentamicin, kanamycin and apramycin. The effect of trichloroacetic acid (TCA) concentration on plasma protein precipitation and sample recovery was studied and an optimized concentration of 25-30% TCA were determined that gives the best sample recovery for aminoglycosides from rat plasma.

Simple, sensitive and robust LC-MS/MS methods were developed and validated for the determination of lipopeptide polymyxins and glycopeptide vancomycin in rat plasma. The effect of TCA concentration on sample recoveries was studied.

An efficient LC-MS/MS method was successfully developed and validated for determination of fifteen estrogens and metabolites in human serum. The sample derivatization procedures were optimized, and sample stability was assessed. The method was specific, accurate, precise, sensitive and linear within the calibration range. It had a comparable sensitivity to those from the typical published LC-MS/MS methods, while it had a much better LC separation

efficiency for separating all of the fifteen dansylated estrogens and metabolites with a significantly reduced elution time.

Both CIEF and MALDI-MS are frequently used in protein analysis, but hyphenation of the two is not investigated proportionally. One of the major reasons is that the additives (such as carrier ampholytes and detergent) in CIEF severely suppress the MALDI-MS signal, which hamper the hyphenation of the two. A simple CIEF-MALDI MS interface is used and to explore its application in proteomics research. This study is to develop a simple means to alleviate the signal suppressing from CIEF additives, such as carrier ampholytes and detergent, on MALDI- MS signals.

Chapter 1: INTRODUCTION

1.1 Background

Analytical chemistry plays a critical role in pharmaceutical research and development. A typical analysis includes separating and identifying active pharmaceutical ingredients and impurities, determining the quantity of counterions, residual solvents, moisture, heavy metals in drug substances and drug products, and analyzing drugs and their metabolites in biological fluids, e.g. plasma, urine and tissues [1-5]. Analytical method development and validation are arguably the most important procedures in analytical research and development, because the qualities of drug substance and drug product are controlled by using analytical methods. In order to develop successful analytical methods, three essential procedures should be established and verified, including sample preparation, analyte separation and detection [6].

The purposes of sample preparation are: 1) isolating the analyte(s) from the sample matrix, e.g. excipients of formulations, proteins from biological fluids (blood, tissues, etc.), which may interfere or damage the analytical instruments or systems; 2) dissolving and diluting the samples or analyte(s) with proper solvents to proper concentrations in solutions [7-9]. The precision and

accuracy of an analytical method depends largely on the reproducibility of the sample handling process, such as weighing, dissolution, and transferring steps [10,11]. Commonly used sample preparation procedures include: dissolution, sonication, solid phase extraction, liquid liquid extraction, and headspace (HS) sample extraction, *etc* [6].

A successful separation may significantly reduce or eliminate the interferences from the sample matrix, other analytes and impurities, and enhance the accuracy, precision and sensitivity of the analytical method(s). A particular separation technique is usually selected based on the chemical and physical properties of the sample or analyte, such as melting point, boiling point, functional groups, molecular weight, pKa, polarity, volatility, solubility and stability [3,6,9]. Chromatography techniques are the most commonly used separation systems, instruments such as gas chromatography (GC), high performance liquid chromatography (HPLC), super critical fluid chromatography (SFC), capillary electrophoresis (CE), and thin layer chromatography (TLC).

An appropriate detector(s) must be utilized to monitor or to detect the analyte with high accuracy, precision and sensitivity. Most of the commonly used chromatography instruments are connected with different types of

detectors. For example, GC used to be connected with a flame ionization detector (FID), thermal conductivity detector (TCD), sulfur chemiluminescence detector (SCD), and electron-capture detector (ECD). HPLC and CE usually use ultraviolet absorbance detectors or fluorescence detectors.

However, the pharmaceutical industry has increased its desire to adopt more stringent sample information requirements, higher sensitivity for impurities, and higher throughput for drug discovery and development [6]. The application of existing separation techniques to hyphenate with different types of detectors is the most popular way to meet these increasing industry standards [1,5,12]. One of the most exciting hyphenated technique was introduced by Hirschfeld in 1960's by the combination of GC and mass spectrometry (MS) [13,14]. By combining different techniques, analytical chemists can maximize the advantages of all instruments to provide rapid, reliable and valuable data for pharmaceutical analysis. Due to its high selectivity and sensitivity, MS is the most used of hyphenated techniques connecting with different separation systems, such as GC-MS, LC-MS, and CE-MS [12,15-19]. These hyphenated techniques are broadly adopted by the biopharmaceutical industry today.

In addition, analytical method validation is a critical procedure to evaluate and verify the method reliability, ruggedness and robustness with a series of

parameters, i.e. specificity, accuracy, precision, linearity and range, sensitivity, sample stability, etc.

The objectives of this dissertation were to develop and validate a number of analytical methods using a series of modern analytical technologies, e.g. HSGC-FID, LC-MS/MS, CE-MS, etc, while following scientific and practical pharmaceutical industry guidelines.

1.2 Headspace Gas Chromatography/Flame Ionization Detection

Headspace, as a sample of pretreatment methods, is commonly used to collect the releasing volatile compounds from solid or liquid carrier and couple with GC separation to detect the organic impurities and contaminations in active pharmaceutical ingredients or environment samples.

1.2.1 Description of Headspace (HS)

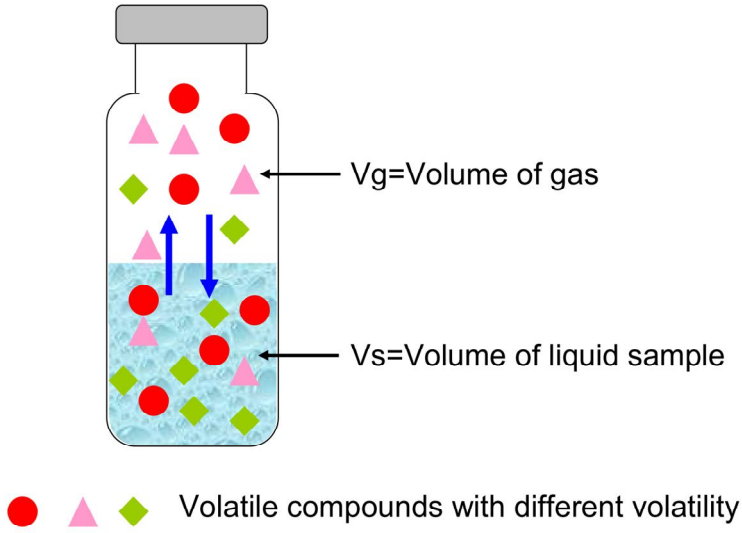


Figure1.1. Description of Headspace Sampling.

The headspace system is represented by the sealed vial (see in Figure 1.1). The distribution of the analyte between a gas and a liquid upon equilibrium is called equilibrium constant. When giving enough time and at a certain temperature, the analyte can reach its equilibrium and the relative amount of analyte in two phases is kept at a constant ratio.

After the analyte has equilibrated in the HS vial, the GC injector uses a sample probe to puncture the HS vial septum. A carrier gas (Helium) flushes through the vial and pressurizes the gas phase into the GC injector loop. Once it enters into the loop, the gas phase is transferred to the GC separation system by

passing a heated transfer line to avoid any condensation during the transferring process [20].

There are two types of the HS sampling techniques, static HS and dynamic HS sampling [21]. The static HS sampling is more easily automated than the dynamic HS sampling with purge and trap, but the dynamic HSGC has a higher sensitivity. This is because the static HS sampling requires longer equilibration time at the same equilibration temperature [22,23]. In order to increase the sensitivity of static HS sampling, the sample should be equilibrated at a higher temperature with a longer equilibration time.

1.2.2 Description of Gas Chromatography/Flame Ionization Detection

GC is based on the partitioning of the analyte between a stationary phase (liquid coating on the separation column) and a mobile phase (carrier gas) [24]. The carrier gas is usually helium. After the sample is injected into the GC injection port, the sample is vaporized and pushed by the carrier gas to flush through a stationary phase, at which the analyte interacts by volatility and solubility properties. The analytes with a higher affinity interaction for the stationary phase come out later than those having less interaction. The eluted analytes finally reach the detector.

FID is the most commonly used GC detector. It is a typical destructive, mass related detector. According to the strength of the signal, the amount of organic compounds can be quantified by peak area. Its prominent advantage is almost as a universal detector for all organic compounds, especially for hydrocarbons. However, FID is not sensitive to water, carbon dioxide and other inorganic compounds [4,24].

1.2.3 Coupling Headspace with Gas Chromatography/Flame Ionization Detection

In the past, direct injection of volatile compounds into the GC column has been the most commonly used method [22]. It is widely used in pharmaceutical industry as a residual solvent analysis. However, in direct injection mode, all analytes in a sample solution are directly injected into the GC injector, but only less than 4 μL of sample may be injected into a capillary column under regular conditions, and those non-volatile analytes may not be able to be eluted out from a GC column, due to their high polarity, high boiling point or low volatility [25-28]. When the non-volatiles accumulate on a GC column, they may decrease the column efficiency, shorten the column lifetime, decompose themselves, generate artificial peaks and affect the following analysis [26,28].

In contrast, the major advantages of HS sampling over direct injection are that it may avoid the contamination from the non-volatile analytes to the GC column, and a much larger sample may be loaded to a HS sampler [20,25,27]. These may result in a low background from contamination and a method high sensitivity. Coupling HS with GC/FID, the sample throughput is highly increased and less maintenance is required compared with the direct injection.

1.3 High Performance Liquid Chromatography/Mass Spectrometry

During the development of chromatograohy techniques, HPLC has become the leading technique for the application of pharmaceutical separation analysis due to its high reliability and high throughput abilities. Meanwhile, MS can help to solve difficult pharmaceutical analysis by utilizing the various modes and ionization techniques. The combination of HPLC and MS is the most frequently used analytical technique in pharmaceutical industry for drug discovery and development.

1.3.1 Description of High Performance Liquid Chromatography

Nowadays, HPLC is the most popular separation method to analyze the vast majority of organic compounds, including polar or ionic molecules, which

are not suitable for gas chromatography [6]. Another advantage of HPLC is that its stationary and mobile phase can be distinctly different combinations to meet the maximum needs of sample separation.

HPLC, based on the different physical and chemical separation principles for the analyte between the stationary phase and mobile phase, is cataloged as: adsorption chromatography, partition chromatography, ion chromatography, size exclusion chromatography, and affinity chromatography [6,24]. The most commonly used partition chromatography, according to the relative polarity of the stationary phase and mobile phase, is classified as normal-phase partition chromatography and reversed-phase partition chromatography.

When the polarity of the stationary phase is larger than that of the mobile phase, it is called the normal phase chromatography (NPC). Silica or alumina based particles are highly polar stationary phases. The less polar or non-polar solvents, such as hexane or ether, are used as the mobile phases. In NPC, the less polar component comes out earlier than the polar component because it is more soluble in the non-polar mobile phase. The hydrophilic interaction chromatographic (HILIC) column is the ideal choice for polar and hydrophilic compounds since its stationary phase is based on bare silica. The highly polar compound is retained on the HILIC column to achieve a good separation.

Analytes, such as very polar aminoglycoside compounds, amino acids, and peptide, usually have strong retention on HILIC column in NPC separation mode [29-31].

When the polarity of the stationary phase is less than that of the mobile phase, it is called reversed phase chromatography (RPC). In RPC, the stationary phase is non-polar, such as a long hydrocarbon chain on silica core, whereas the mobile phase is relatively polar, such as water, acetonitrile, and methanol. Most commonly, the hydrocarbon chain of the siloxane is a C_8 chain (*n*-octyl) or a C_{18} chain (*n*-octyldecyl) [24]. The most polar component elutes out from the column first since it has less interaction with the stationary phase and is more favorable to dissolve in the polar mobile phase than less polar components [32]. Increasing the polarity of the mobile phase will keep the polar component on the column longer and allow for an increased retention time. Nowadays, RP-HPLC is the most polar separation mode applied in pharmaceutical industry [33-35].

1.3.2 Description of Electrospray ionization Mass Spectrometry (ESI MS)

1.3.2.1 Fundamentals of Electrospray ionization

ESI was first introduced by Yamashita and Fenn in 1984, and has become the most popular ionization technique for MS [36,37]. ESI is a soft ionization process which makes organic or bio-molecules generate multiple charged ions.

A diagram (Figure 1.2) illustrates the basic set-up and process in ESI ionization mechanism [38].

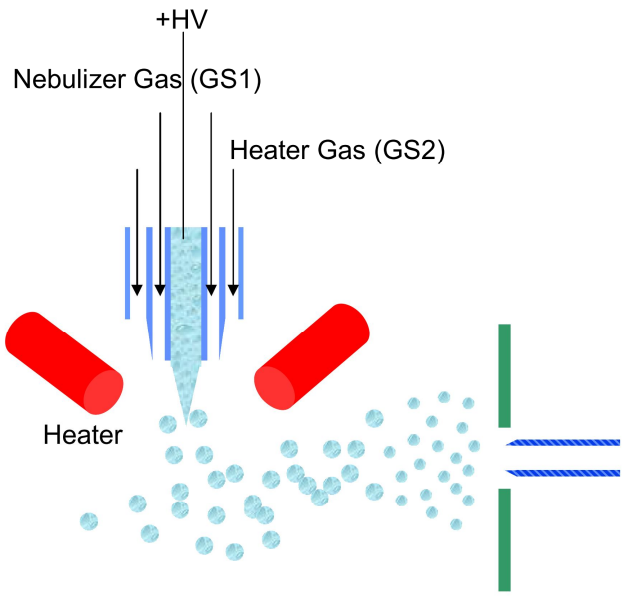


Figure 1.2. Scheme of Electrospray Ionization Process.

Currently, the most popular ESI source is the turbo ion source, which has a top down orthogonal spray (shown in Figure 1.2) to improve instrument robustness and to avoid neutral droplets from entering into the orifice hole. Otherwise, the MS instrument will be clogged and eventually lose the analyte signals.

1.3.2.2 Quadruploe and triple quadrupole mass analyzer

After fifty years of development, quadrupole and triple quadrupole MS are the most mature and most widely used small mass spectrometers. Quadrupole MS works as a mass filter and can achieve high resolution, high-mass range, fast analysis speed and low cost. Especially, triple quadrupole MS is the most powerful and popular mass analyzer to couple with HPLC to quantitate small molecules in pharmaceutical industry.

There are four types of MS scan modes for triple quadrupole MS: precursor ion scan, product ion scan, neutral loss scan and multiple reaction monitoring (MRM) scan. Compared with the other three scan modes, MRM scan mode is the most frequently used scan for quantitation purpose due to its ion selectivity [39].

MRM scan mode is illustrated as Figure 1.3 [39]. Sample mixtures containing P, M, and N are introduced into triple quadrupole MS. After ESI process, P, M, and N lose electrons and become charged ions. When these ion species pass through Q1 mass filter, only the selected M^+ precursor ion can enter into Q2 collision cell to collide with collision cell gas (CAD) gas and form fragmentations. In order to have a better selectivity for the monitored

compound, only the selected m/z fragmentations can pass through Q3 quadrupole mass filter, and the other product ions lose in Q3 quadrupole. In MRM scan mode, only the selected precursor-product ion pair can record by the detector, which maximizes the ion selectivity and minimizes the background noise from the sample matrix [39].

1.3.3 Coupling High Performance Liquid Chromatography with Mass Spectrometry

Nowadays, a lot of commercially available HPLC can couple with MS, which is contributed by the advanced interface design. For example, as shown in Figure 1.2, two symmetric turbo heaters and dried gas1 and gas2 can assist the solvent evaporation process. And the orthogonal spray mode also allows HPLC to carry up to 1 mL/min flow rate since most of the solvents go directly into the waste and only the margin area analyte is introduced into MS analyzer. Since the ionization process requires good solvent evaporation, the usage of non-volatile buffers or solvents will lead to damage the MS instrument and be prohibited to use. Only volatile buffers (containing formic acid, acetic acid, ammonia, formic acetate, and ammonia acetate, *et al.*) and mobile phase (water, methanol, and acetonitrile, *et al.*) are allowed to be introduced into HPLC followed by MS analyzer.

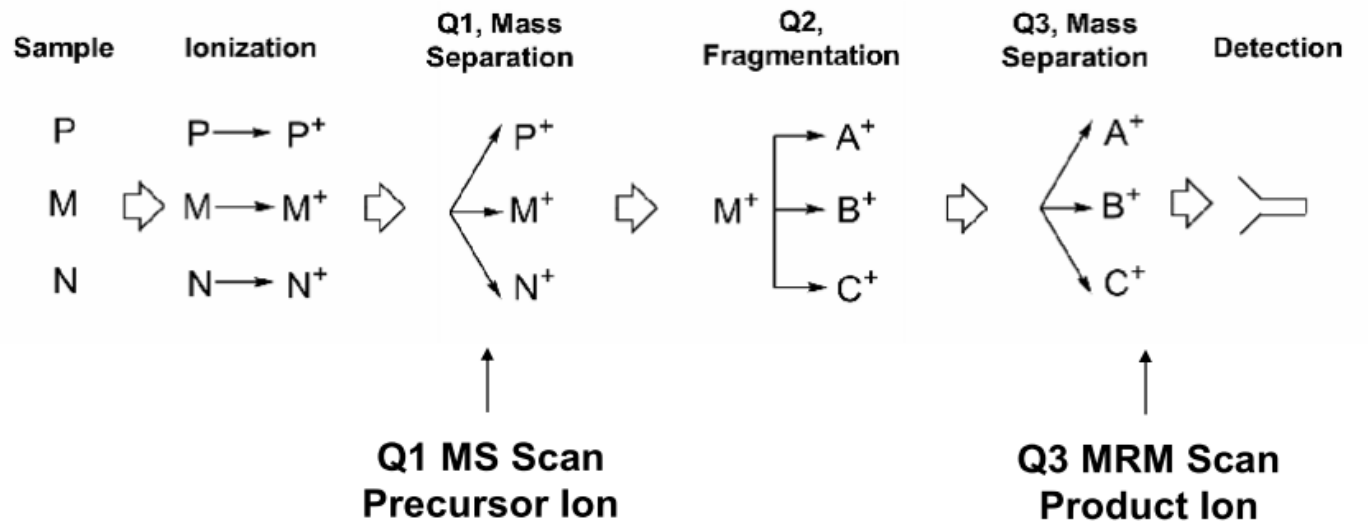


Figure 1.3 Schematic diagram of Triple Quadrupole Mass Analyaer as MS/MS Procedure.

1.4 Capillary Electrophoresis/Mass Spectrometry

At the present time, proteomics is the most promising area in academics and industry because it can offer a rich amount of information for cancer related biomarker studies. CE gives high separation efficiency for proteins and makes it possible for isolating low abundance biomarker proteins. MS utilizes the extremely sensitive detector and plenty of information to identify and quantify biomarker proteins. The coupling of CE and MS opens a new field for drug discovery and development based on biomarker studies.

1.4.1 Description of Capillary Electrophoresis

Capillary electrophoresis, including electrophoresis, chromatography and others, utilizes the capillary as the separation channel, the high-voltage electric field as the driving force, and a variety of sample physical properties as the separation mechanism (such as charge, size, isoelectric point, polarity) [24,40]. It is a micro-liquid separation technique, which only needs a few microliters of the total sample and buffer consumption. CE is another significant progress for analytical chemistry after HPLC separation

technique. It makes up the analytical subject from the microliter level to the nanoliter or sub-nanoliter level, and offers a great opportunity to proteomics [41].

There are several separation modes for capillary electrophoresis, such as capillary zone electrophresis (CZE), capillary gel electrophoresis (CGE), and capillary isoelectric focusing (CIEF) [40]. In this dissertation, CIEF is the main focus area for protein separation and will be discussed in detail as follows.

1.4.2 Fundamentals of Capillary Isoelectric Focusing

1.4.2.1 Description of Carrier Ampholytes

Among different CE techniques, CIEF is the most attractive separation technique for proteomics research, due to its high resolution and auto-biological sample focusing ability, especially when used for ampholytes, such as proteins and peptides [40,42]. In CIEF separation, carrier ampholytes are the key componenets for building up the pH gradient in capillary. In 1961, Svensson first introduced the term of carrier ampholyte, which claims that in an electric field a continuous and stable pH gradient is established by the mixtures of ampholyte compounds [43]. Those

compounds are the synthetic heterogeneous mixtures of isomers of polyamino polycarboxlic acids [42,44]. They have some unique physical and chemical properties, such as low molecular weight (1,000 – 15,000 Da), high buffering capability, good solubility, good electric conductivity, and absence of biological effects, to ensure them as the carriers to separate different proteins or peptides [42].

1.4.2.2 Capillary Isoelectric Focusing Process

In CIEF (see Figure 1.4), a mixture of carrier ampholytes and proteins is introduced into a capillary by pressure, and a DC voltage is applied at the same time. A pH gradient is generated and the analyte is separated based on the different pI, spontaneously concentrating themselves in their pI ranges, which are specific for each ampholyte analyte with net zero charge. When the proteins are focused in the capillary, the protein zones are mobilized toward a detector by hydrodynamic mobilization.

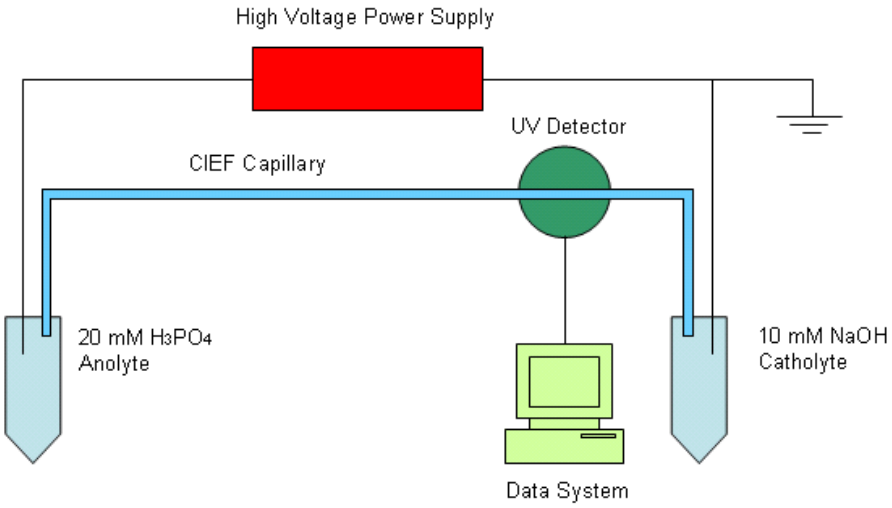


Figure 1.4 Schematic diagram of apparatus for CIEF.

1.4.3 Description of Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS)

1.4.3.1 Fundamentals of Matrix-assisted Laser Desorption/Ionization

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) is a recently developed type of soft ionization mass spectrometry. The instrument consists of two parts: matrix-assisted laser desorption ionization ion source (MALDI) and time of flight mass analyzer (TOF) [24]. The principle of MALDI is to use the sample and matrix mixture to form a thin film of crystals on the MALDI target plate. The plate is loaded into the high vacuum ion source. When the laser beam shots onto the sample spot, the crystal absorbs the laser energy

and transfers this energy to the biological molecules, and the ionization is induced, which is the process of proton transfer to or from the biological molecules. Finally, the biomolecules like proteins or peptides are charged and form the precursor ions [45,46]. High voltage is applied to the sample plate, accelerating ions into the flight tube and detected by mass analyzer.

1.4.4 Coupling Capillary Isoelectric Focusing with Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

The development of interfaces between CE and MS has been reviewed and proven as much more difficult than the interface of LC and MS [47-50]. Here, only interfaces between CIEF and MS are briefly summarized [51,52]. After the process of protein focusing in CIEF separation, the key thing is the sample individual collection and transportation to MS detection instruments. The biggest problem is the transferring step can distort the pH gradient in the capillary, which leads to a broadened focus zone, loss of resolution between two bands, expended elution time, and poor reproducibility [42,51]. In order to decrease the influence of band broadening and coupled with MS, researchers have developed several different methods to individually collect or on-line transfer fractions [53-57]. The mobilization can utilize either by

electrophoretic flow with sheath liquid assistant or by hydrodynamic flow with the elevation of the inlet capillary end.

The on-line CE-electrospray ionization (ESI) MS hyphenated method is more suitable for automation. Tang *et al.* first accomplished the coupling of ESI-MS with CE [19,58]. Different interfaces have been applied to improve the CE-MS coupling, such as coaxial liquid-sheath flow, microdialysis membrane device, and sheathless nanoflow interfaces, to minimize the influence from the additives in CE separations [49,50,54]. Foret *et al.* coupled CIEF with MALDI-TOF MS because MALDI-TOF MS can tolerate relatively high amounts of these additives [59]. Furthermore, off-line coupling of CIEF to MALDI MS can provide the high resolution from CIEF separation and the high mass accuracy and structural information from MALDI.

CIEF can be coupled with MALDI-TOF MS in several different ways: fraction collection, directly depositing the effluent onto MALDI plate, or separation in microchip channels and then using microchip as a target plate [60-63]. Among different methods available, direct spotting is the simplest approach. Wang *et al.* successfully used a porous joint made of a cellulose

acetate membrane for off-line CE-MALDI experiment for neuropeptide analysis of complex tissue samples [64].

1.5 Dissertation Synopsis

This dissertation focuses on the application of various analytical techniques towards pharmaceutical and bioanalytical problems. Chapter 2 will presents an application of HS-GC/FID towards a problem of interest to the pharmaceutical industry. The goal of this study is to develop and validate an efficient and sensitive generic HS-GC method for determination of residual solvents in drug substance in order to increase productivity of drug analysis in the pharmaceutical industry [65].

In Chapter 3, a study of highly polar aminoglycoside compounds, such gentamicin, kanamycin, and apramycin, will be discussed in biological matrix by using LC-MS/MS. The goals of this study are not only introduce a method for analysis of aminoglycoside compounds, but also compare the behaviors of amimoglycoside compounds on a HILIC column and a hydrophobic column. The effect of trichloroacetic acid concentration on plasma protein precipitation and sample recovery efficiency will be discussed in this chapter [66].

Chapter 4 will present simple, sensitive and robust LC-MS/MS methods for the determination of peptide drugs, such as lipopeptide polymyxins and glycopeptide vancomycin in biological matrix. The purpose of this study is to develop and validate a general bioanalytical method based on the same principle for the antibacterial peptide compounds [67].

In Chapter 5, a study of female hormones and their metabolites in human serum will be presented by the application of LC-MS/MS. Many concerns of the existing methods will also be studied in Chapter 5, such as sample derivatization, method specificity, sensitiviy, separation efficiency and long running time. Compared to the vast existing analytical methods in the literature, the LC-MS/MS used in this dissertation can be precessed quickly and having the similar or better sensitivity. This procedure will largely accelerate the sample throughput efficiency in the diagnosis of hormone related cancers.

Chapter 6 will describe a simple CIEF-MALDI MS interface in order to explore its application in proteomics research. The main goal of this study is to develop a simple means to alleviate the signal suppressing from CIEF

additives, such as carrier ampholytes and detergent, on MALDI MS signals [68].

Chapter 7 will give an overall conclusion and discuss the future directions of the research presented in this dissertation.

Chapter 2: Headspace GC/FID for the analysis of residual solvent in drug substance.

2.1 Introduction

Residual solvents are critical impurities in drug substances, drug products and excipients, because they may cause toxicity and safety issues, and affect physicochemical properties of drug substances and drug products. In order to control residual solvent contents in drug substances, products and excipients, ICH Q3C guideline provides specific criteria for class 1 solvents (5)--known or suspected human carcinogens or environmental hazards, class 2 solvents (26)--suspected of other significant but reversible toxicities, and class 3 (28) solvents--low toxic potential to man [69]. Therefore, determination of residual solvents becomes a necessary procedure for quality control of drug substances and drug products to meet regulatory expectations and ensure patient safety.

Developing and validating an efficient and sensitive generic analytical method for the determination of residual solvents may significantly increase productivity of an analytical laboratory in the pharmaceutical industry. Determination of residual solvents using GC with a

flame ionization detector (FID) is the most common technique in the pharmaceutical industry, because of its high separation efficiency and sensitivity for volatile organic compounds. GC analysis may be performed by either direct injection or HS sampling [22]. The advantage of the direct injection mode is that all analytes in a sample solution are directly injected into the GC, leading to a lower sample load or sample requirement and a simpler analytical procedure. But, the high boiling/melting point or polar components of the sample may not be eluted through a GC column, and they may contaminate the GC injection port and/or column. In contrast, HS sampling can prevent this from occurring, but it limits the analysis to those solvents being evaporated from the HS only, and it requires a larger sample load. In addition, the analysis time can be longer due to sampler equilibration prior to injection on column.

There are two types of HS sampling techniques, static HS and dynamic HS sampling. The static HS sampling is more easily automated. Dynamic HS sampling with purge and trap is less suitable for automation but has a higher sensitivity [22,70]. Currently, static HSGC with FID is more popular for analyzing residual solvents in drug substances [25,27,71-73] and drug products [21,26,28] in the industry. Static HS sampling is based on

thermostatic partitioning of volatile compounds in a sealed vial between the sample diluent and the gas phase. Sample diluent is a critical factor affecting HSGC method sample load, sensitivity, HS equilibration temperature and time. A good sample diluent for analyzing residual solvents in drug substances or drug products should have a high capability for dissolving a large amount of samples, a high boiling point and a good stability. There are a number of commonly used sample diluents for HSGC analyses, such as water, DMSO, N,N-dimethyformamide (DMF), N,N-dimethylacetamide (DMA), benzyl alcohol (BA), 1,3-dimethyl-2-imidazolidinone (DMI), and mixtures of water-DMF or water-DMSO [74].

Water is a good diluent for water soluble samples and analytes, because it is clean, stable and inexpensive. However, many organic synthetic drug substances and drug products have low water solubilities, which would limit the sample load. Meanwhile, using water as a diluent may also lead to a lower method precision than organic solvent, like DMF [25]. When a mixture of water-DMF or water-DMSO is used as a sample diluent, it may increase the solubility of many drug substances or drug products, and decrease the partition coefficient of the analytes, resulting in better transfer of analytes from the diluents to the gas phase, and improved method

sensitivity [21,27,28]. If the sample diluent uses these aqueous mixtures, two other important factors, HS equilibration temperature and time, must be taken into consideration for obtaining HS equilibration efficiency. It is required that the HS equilibration temperature should be lower than the boiling point of the sample diluent. Otherwise, if the sample was equilibrated at or above the boiling point of the sample diluent, e.g. water at 100 °C, a large amount of sample diluent may be vaporized (at 100 °C), resulting in a dangerously high sample vial pressure, and a flood of the sample diluent and analytes to the GC system. This means that if water or water-organic mixture is chosen as the sample diluent, the HS equilibration temperature must be lower than 100 °C, i.e. 75-80 °C [25,27,28,74]. However, more than half of the organic solvents listed in ICH guideline Q3C may not be fully vaporized below 100 °C, because their boiling points are higher than 80 °C. In order to increase method sensitivity, equilibration at a low HS oven temperature requires a longer equilibration time, e.g. 30 to 90 minutes [25,27,28,74], to obtain a good phase distribution of the volatile compounds between the gas phase and the sample diluent.

In contrast, those organic solvents, e.g. DMSO (b.p. 189 °C), DMF (b.p. 153 °C), DMA (b.p. 166 °C), BA (b.p. 204 °C), and DMI (b.p. 105 °C),

may provide better solubilization of sample, and they also have higher boiling points than water. When they are used as the sample diluents for HSGC, higher method sensitivity due to better solvent recoveries and improved method precision were observed [70,74,75]. However, DMF, DMA and BA are not very stable at high temperature and are susceptible to degradation when exposed to ultrasonic wave energy during sample preparation. The degradants from high HS equilibration temperature or sonication process during sample preparation may interfere with the analyses of the residual solvents [74]. Since DMSO is more stable at high temperature than the other solvents, e.g. DMF and BA, and has a higher capacity of dissolving drug substances and drug products, as well as a higher boiling point than water, it is a better sample diluent for HSGC analyses.

A number of parameters may affect GC method sensitivity and separation efficiency, such as sample injection split ratio, GC carrier gas linear velocity or flow rate and oven temperature program (isocratic or gradient). The typical GC parameters for a generic separation of residual solvents in previous publications are: split ratio 1:5-20; carrier gas linear velocity 20-36 cm/s; oven temperature at 40 °C isocratic, or with gradient programming from 40 °C to 90-160 °C at 5-10 °C/min [25,27,70-72,74,75].

These parameters may be optimized for separation efficiency and detection sensitivity for determining specific ICH Q3C solvents.

The objective of this study was to develop and validate a generic HPGC method which has a shorter sample equilibration time, a better separation for most of the interested solvents, a higher sensitivity and a broader concentration range. We selected 4 mL of DMSO as the sample diluent for 200 mg of drug substance in order to develop a generic HSGC method with efficient HS equilibration, GC separation and high detection sensitivity. We assessed a number of HSGC parameters, as listed in Table 2.1. Since class 1 solvents (5) are highly carcinogenic or toxic, they are generally avoided in pharmaceutical manufacturing. Only ICH class 2 (26) and class 3 (28) solvents were evaluated during this method development. The method validation was performed to demonstrate the method specificity, accuracy, precision, linearity and sensitivity. There are a number of calibration methods for the determination of residual solvents in drug substances by HSGC, such as calibrations using external standard, internal standard and standard addition, but there are no significant differences among these approaches with respect to accuracy and precision [27,76]. Therefore, we used an external standard approach in this method, and

evaluated the drug substance matrix impacts on residual solvents recoveries using four synthetic small molecule drug substances during method validation.

2.2. Experimental

2.2.1 Reagents and chemicals

The drug substances were synthesized by Astrazeneca Pharmaceuticals LP (Wilmington, USA). Solvents used were of ≥ 98% purity, and purchased from the following sources: acetone, acetonitrile, n-heptane and toluene from Fisher Scientific (Fairlawn, NJ, USA); formic acid and acetic acid from Acros Organics (Geel, Germany); ethyl ether from J. T. Baker (Phillipsburg, NJ, USA). The remaining organic solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 2.1 HSGC parameters for the method development and validation. Reprinted from [65] with permission.

Parameter	Evaluated settings for development	Optimized settings for validation
HS		
Equilbration temperature	125, 140, 150 °C	140 °C
Transfer line temperature	135, 140, 150 °C	140 °C
Loop temperature	125, 140, 150 °C	140 °C
Vial pressure	9 psi	9 psi
Vial equilibration time	8, 10, 15 mim	10 mim
Vial pressurization time	0.4 min	0.4 min
Loop size	1 mL	1 mL
Loop equilibration time	0.5 min	0.5 min
Loop fill time	1 min	1 min
GC		
Inlet temperature	200 °C	200 °C
Carrier (He) flow rate	1.5, 1.8, 2.0 mL/min (28-40 cm/s)	1.8 mL/min (constant flow, approximately 30-33 cm/s)
Inlet split ratio	1:0, 1:1, 1:2, 1:5	1:1 at the split flow of 1.9 mL/min
Oven temperature gradient	hold 0, 1, 3, 5 min at 35 °C	0-3 min at 35 °C
	ramping at 2, 3, 4, 5, 8, 10 °C/min	3-21.75 min, ramping to 110 °C at 4 °C/min,
	ramping to 240-280 °C at 10, 20, 30, 40 °C/min	21.75-25 min, ramping to 240 °C at 40 °C/min
	hold at 240-280 °C for 2-10	25-30 min at 240 °C
FID temperature	260, 280, 300 °C	260 °C
Detector gas flows	hydrogen 30-40 mL/min, air 300-400 mL/min	hydrogen, air, make up at 35, 350, 23.2 mL/min, respectively

2.2.2 Instrumentation

An Agilent 6890A GC equipped with an FID and a 7694 HS sampler was used for the experiments. The HSGC system was controlled using Agilent Chem32 software, and data acquisition and processing were accomplished using Thermo Atlas software. The GC column was an Agilent DB-624 (6% cyanopropylphenyl/94% dimtheyl polysiloxane) fused silica capillary column, 30 m long, 0.32 mm I.D., 1.8 μm film thickness (Part No. 123-1334, serial No. US1613334H). The HSGC parameters assessed during the method development and validation are listed in Table 2.1.

2.2.3 Standard solutions

The ICH Q3C class 2 and 3 solvents (54) were prepared at about 1000 ppm individually in DMSO, and injected to the HSGC system. Since ten of these 54 solvents were not suitable for the current method due to their high boiling points or high polarities, only 44 of these solvents were used for the method validation experiments. In order to obtain good separations and proper signal strength, these 44 solvents were separated as three groups, I (26), II (9) and III (9), respectively. The concentrations of these solvents were prepared in ranges of 0.2-15,000 ppm (from the quantitation limit to

full GC chromatogram scale) by sequential diluting high concentration stock mixture solutions with DMSO to 10 concentration levels, because the sensitivity of these solvents to FID varies significantly. Two identical samples were prepared for the accuracy test for each group mixture at 20-900 ppm levels. All the solvent concentrations were calculated based on 200 mg drug substance being dissolved in 4 mL of DMSO. For the HSGC analysis, 4 mL (sample load) of the standard solutions was pipetted into an Agilent 20 mL headspace sample vial and immediately sealed with a Teflon-lined septum and an aluminum crimp cap (Agilent, Wilmington, DE, USA).

2.2.4 Drug Substance Sample Solution

The four drug substances were dissolved individually (200 mg each) in 4 mL of blank DMSO or in the three group mixtures at both working concentration (20-900 ppm) and low concentration (2-90 ppm) levels. For example, 200 mg for one drug substance was weighed in seven HS sample vials, 4 mL of blank DMSO was added into the first vial; then either 4 mL of groups I, II or III solvent mixtures at working concentration levels (20-900 ppm) was added; or 4 mL of groups I, II or III solvent mixtures at low concentration levels (2-90 ppm) was added. All the solvent concentrations

were calculated based on 200 mg drug substance being dissolved in 4 mL of DMSO. The sample solutions were vortexed using a Thermolyne mixer (Dubuque, IA, USA), and sonicated for 5 minutes using a Bransonic 3200 (Danbuty, CT, USA) to dissolve all samples completely. The samples for each drug substance were prepared in duplicate.

2.2.5 Procedure

During the HSGC method development, in order to select the most appropriate system parameters to obtain the best separation, sensitivity and time efficiency, 54 class 2 and class 3 solvents, and typical samples of the 3 groups of solvent mixtures were injected under a variety of conditions, e.g. at different HS oven temperatures (125-150 °C), equilibration time (8-15 minutes), GC gradients (35-280 °C, ramping speed 3-10 and 10-40 °C/min), carrier flow rate (28-40 cm/sec), sample split ratio (1:1-5:1), etc. The final HSGC conditions used for method validation were obtained based on optimized HS and GC parameters.

The HSGC system was equilibrated under the experimental conditions by injecting 3 blank DMSO samples every day before sample sequence injections. Each of the ICH Q3C class 2 and 3 solvents (54) was

injected once separately to determine method specificity and signal response sensitivity. Since 10 of these 54 solvents are unsuitable for the current method due to their high boiling points or polarities, only 44 of these solvents were used for the method validation experiments, and they are separated as three groups according to their retention behavior and detectability by FID.

The method validation experiments of these 44 solvents were performed by injecting the 3 groups of solvents sequentially from low to high concentrations to determine the method specificity, sensitivity, linearity, accuracy and precision. Each of the 3 groups of solvent mixtures at working concentrations (20-900 ppm for accuracy and precision testing) and at low concentrations (2-90 ppm for sensitivity testing) were injected six times on the first day of experiment, and one time in each of the following five days. The samples of four drug substances in blank DMSO and in three spiked groups of solvent mixtures at 2-90 ppm and 20-900 ppm levels were injected once to evaluate the method feasibility to drug substance and the impact of drug substance on the recoveries of those solvents.

2.3 Results and discussion

2.3.1 Optimization of HS conditions

The HS sampler has a number of parameters affecting the method sensitivity, precision, and efficiency, including: temperature (oven, transfer line, and loop), time (vial equilibration and pressurization, loop fill, and injection), pressure (vial and carrier gas) and phase ratio (vial size and sample volume). Selecting a proper sample diluent for HSGC analysis is very critical for method sensitivity, precision and sample equilibration temperature and time, and it will affect the final optimized HS conditions. When we evaluated HS equilibration temperature at 125 °C, 140 °C and 150 °C with equilibration times of 8, 10 and 15 minutes, many solvents with boiling point higher than 125 °C could not evaporate efficiently at 125 °C within 15 minutes, while a significant amount of DMSO evaporated at 150 °C even in 8 minutes, overloading the GC column, and interfering with the method separation efficiency. When the equilibration time at 140 °C was extended from 10 to 15 minutes, the recoveries of the 44 class 2 and class 3 solvents remained constant. Therefore, we determined that equilibrating at an oven temperature of 140 °C for 10 minutes was optimal. It was observed that when the temperatures of the injection loop and the transfer line were 10 °C

higher than the HS oven temperature of 140 °C or kept the same as that of the oven temperature, there was no significant change in solvent recoveries. However, when the HS sampler was equilibrated at 140 °C, those ICH Q3C solvents with a boiling point higher than 150 °C, as listed in Table 2.2, could not be analyzed by this HSGC method.

Table 2.2 ICH class 2 and class 3 solvents unsuitable for this HSGC method. Reprinted from [65] with permission.

Number	Solvent	FW (g/mol)	Boiling Point (^{o}C)
1	Anisole	108	154
2	Cumene	120	152
3	Tetralin	132	206-208
4	Ethylene glycol	62	197
5	N,N-Dimethylacetamide	87	164-166
6	Formamide	45	210
7	Sulfolane	120	285
8	N-methyl pyrrolidone	99	202-204
9	Formic acid	46	101
10	Acetic acid	60	118

2.3.2 Optimization of GC separation

The choice of GC column is crucial for establishing an efficient and robust HSGC method. The Agilent DB-624 column (30 m X 0.32 mm I.D., 1.8 μm) is a commonly used column for residual solvents determination, because of its medium polarity. Most of the ICH Q3C class 2 and 3 solvents can be resolved by the Agilent DB-624 column except formic acid and acetic acid, due to their high polarities. To obtain efficient separation and sample

sensitivity, a number of GC parameters were evaluated when developing this method, such as the GC oven temperature gradient, carrier gas flow rate and sample split ratio: initial temperature 35 °C at different holding time (0, 1, 3, and 5 minutes), temperature ramping rate (2, 3, 4, 5, 8, 10 °C/min), carrier flow rate (1.5, 1.8, and 2.0 mL/min) and split ratio (splitless or 1 to1-5 ratio). Our data indicated that the GC parameters listed in section 2.2.2 were the most efficient combination for separation and sensitivity of this method. Under these optimized conditions, 44 of class 2 and class 3 solvents were analyzed by this method. The separation efficiency of this method is better than previously reported methods, because more class 2 and class 3 solvents can be resolved by this method. Another advantage of our generic HSGC method is its capability to separate most of the frequently used solvents in a considerably shorter time (total running time is 40 minutes, including 10 minutes for HS vial equilibration and 30 minutes for GC separation) compared to previously reported methods [25,27].

2.3.3 Method validation

2.3.3.1 Specificity

The typical HSGC chromatograms of 44 ICH Q3C class 2 and 3 solvent standards are shown in Figure 2.1. As indicated in the retentions of

these solvents in Table 2.3, most of these solvents (33) are well separated from each other and DMSO, but some of the solvents in Group II and Group III are incompletely resolved with those in Group I, such as Ethyl formate (III) and 2-Propanol (I). However, there are rare cases when a drug substance contains more than five residual solvents at or around meaningful detection limits. For example, each of the four drug substances evaluated in this study contain a mixture of two to four residual solvents at or above the quantitation limits of this HSGC method, as shown in Figure 2.2. That means this HSGC method is a suitable approach in many pharmaceutical applications for screening and determining the 44 ICH Q3C solvents.

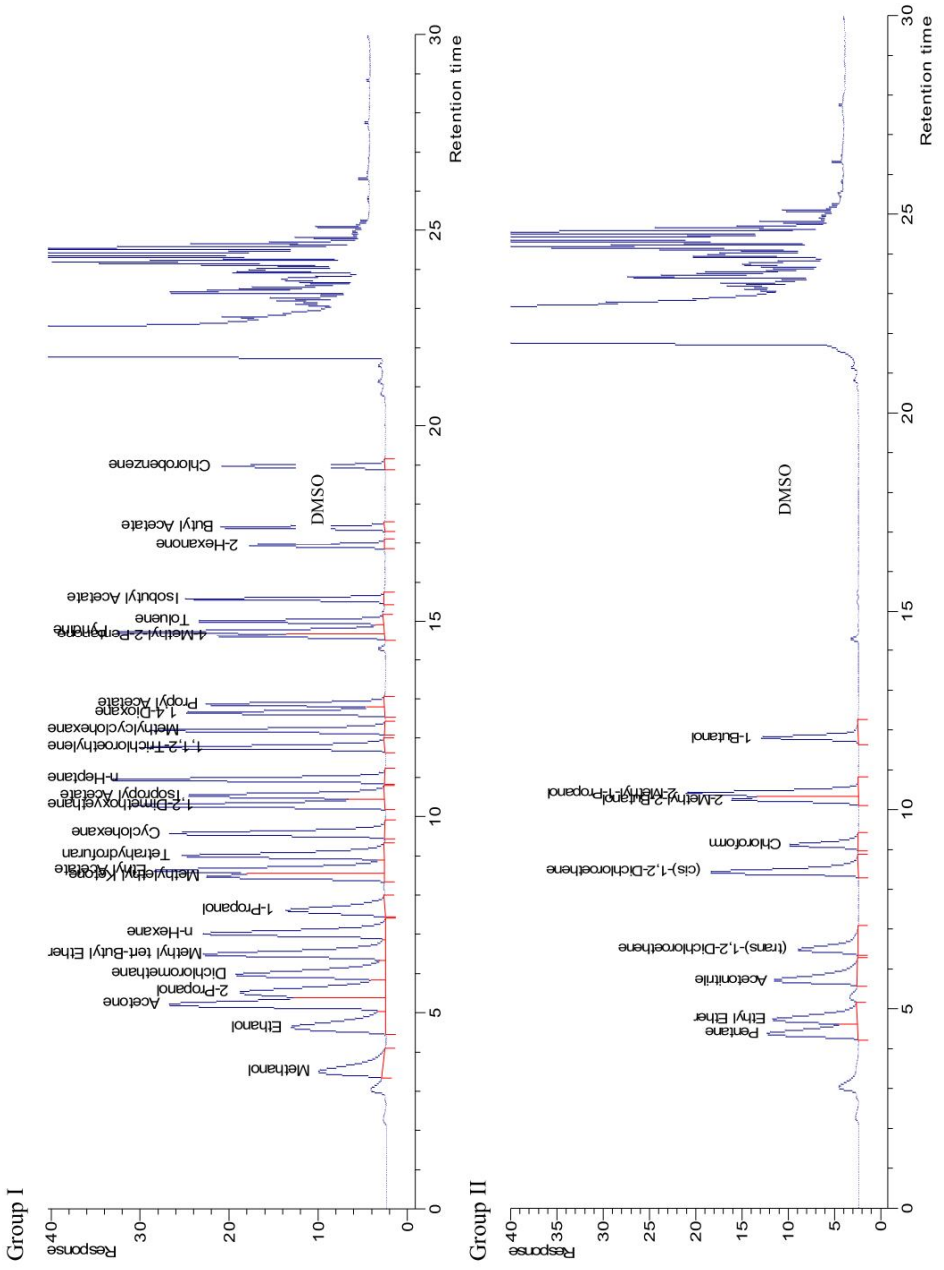
2.3.3.2 Linearity

The method linearity was investigated using ten concentration levels ranging from 0.2 to 15 000 ppm, and the linearity of each solvent was assessed using linear regression. Since the sensitivity of each of the 44 solvents to FID varied significantly, the concentration ranges of each organic solvent were adjusted during the sample preparation procedures to obtain a relatively reasonable peak height for each organic solvent and to cover appropriate linear ranges. As shown in Table 2.3, the regression coefficients (r^2) of these 44 solvents are within the range of 0.9990-1.0000. The

intercepts of these regression lines are less than 2% of the high calibration concentrations (20-900 ppm) for all 44 solvents. This means that the 44 solvents have linear responses within the calibration ranges studied, which are broader than ICH guideline detection range of 50-5 000 ppm.

2.3.3.3 Accuracy

Accuracy of this method was determined by analyzing duplicate sample preparations of the three groups of the ICH Q3C solvents at working concentration levels (20-900 ppm level). As shown in Table 2.4, the bias values (the difference between the measured value and the theoretical value) of these 44 solvents are equal or less than ± 2.7% of the theoretical values. The results indicate that the HSGC method has sufficient accuracy for screening and determining the 44 solvents studied at the working concentration level.


Group I
Response
Retention time
Methanol
Ethanol
Acetone
2-Propanol
Dichloromethane
Methyl tert-Butyl Ether
n-Hexane
1-Propanol
Methylethyl Ketone
Ethyl Acetate
Tetrahydrofuran
Cyclohexane
1,2-Dimethoxyethane
Isopropyl Acetate
n-Heptane
1,1,2-Trichloroethylene
Methylcyclohexane
1,4-Dioxane
Propyl Acetate
4-Methyl-2-Pentanone
Pyridine
Toluene
Isobutyl Acetate
DMSO
2-Hexanone
Butyl Acetate
Chlorobenzene
Group II
Response
Retention time
Pentane
Ethyl Ether
Acetonitrile
(trans)-1,2-Dichloroethene
(cis)-1,2-Dichloroethene
Chloroform
2-Methyl-2-Butanol
2-Methyl-1-Propanol
1-Butanol
DMSO

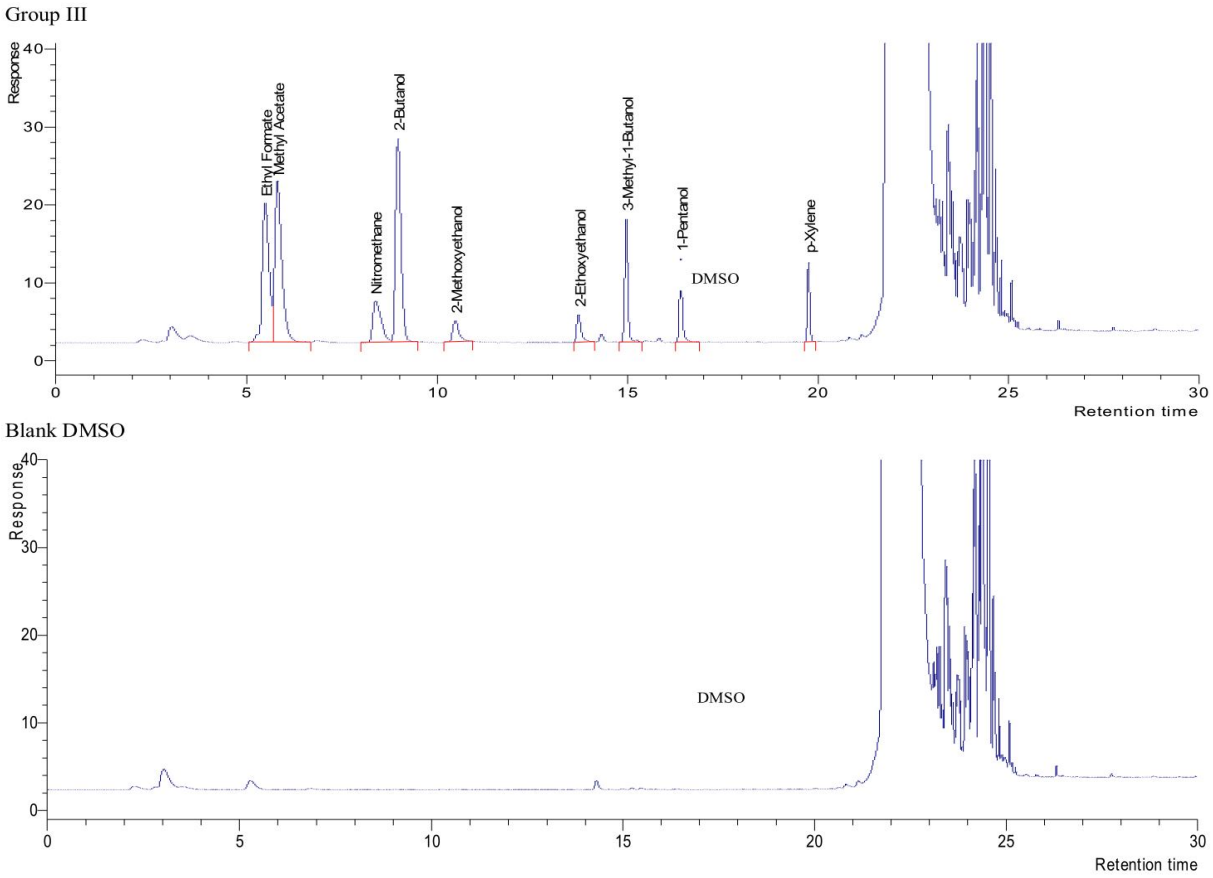


Figure 2.1 Typical chromatograms of 44 ICH solvents at 20-900 ppm. Reprinted from [65] with permission.

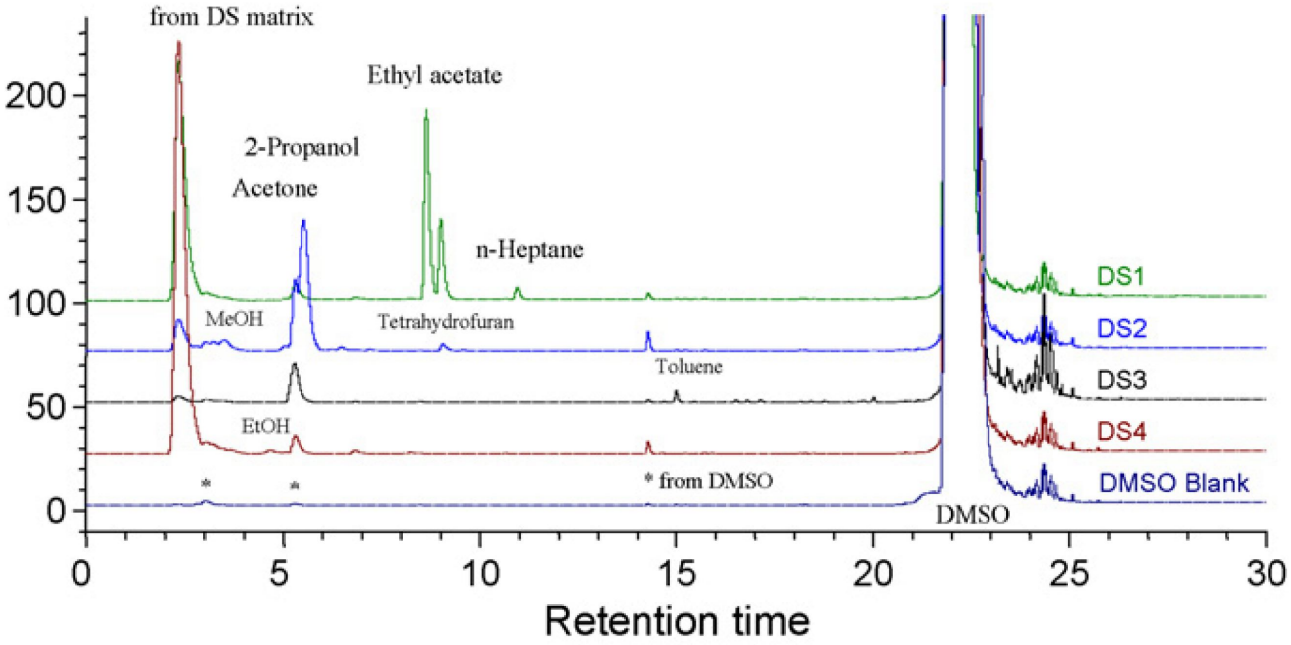


Figure 2.2 Typical chromatograms of residual solvents in four drug substances. Reprinted from [65] with permission.

Table 2.3 Retention times and linearity of 44 ICH class 2 and class 3 solvents. Reprinted from [65] with permission.

Organic Solvents	b.p. (°C)	Retention Time (min)	Range (ppm)	r^2
Methanol (I)	64.7	3.53	8.9-8868	0.9996
Pentane (II)	36.1	4.40	0.4-401	0.9996
Ethanol (I)	78.3	4.66	8.8-8837	0.9994
Ethyl ether (II)	34.6	4.75	0.6-571	0.9999
Acetone (I)	56.0	5.22	12.6-6320	0.9998
Ethyl formate (III)	54.0	5.50*	8.8-4402	0.9999
2-Propanol (I)	82.0	5.54	17.6-8803	0.9996
Acetanitrile (II)	81.0	5.74	4.4-4402	0.9997
Methyl acetate (III)	56.9	5.82*	1.8-4474	0.9999
Dichloromethane (I)	40.0	6.00	14.9-14857	0.9997
1,2-Dichloroethene (II)	47.5	6.51*	7.1-7112	0.9999
Methyl tert-butyl ether (I)	55.2	6.52	1.2-1185	1.0000
n-Hexane (I)	69.0	7.04	0.5-524	0.9998
1-Propanol (I)	97.1	7.64	18-8998	0.9994
Nitromethane (III)	100.0-103.0	8.39*	7.3-18194	0.9990
1,2-Dichloroethene (II)	60.0	8.45*	7.1-7112	0.9999
Methylethyl ketone (I)	79.6	8.50	3.9-3864	0.9996
Ethyl acetate (I)	76.5	8.65	4.3-5305	0.9999
2-Butanol (III)	99.0	8.98	4.1-10342	0.9998
Tetrahydrofuran (I)	65.0-67.0	9.03	2.9-2845	0.9999
Chloroform (II)	60.5	9.13	11.8-11840	0.9999
Cyclohexane (I)	80.7	9.60	0.6-623	1.0000
2-Methyl-2-butanol (II)	102.0	10.28*	4.6-4564	0.9995
1,2-Dimethoxyethane (I)	85.0	10.32	7.0-6946	0.9998
2-Methyl-1-propanol (II)	108.0	10.44*	6.4-6416	0.9999
2-Methoxyethanol (III)	124.0	10.48*	24.7-12352	0.9990
Isopropyl acetate (I)	85.0-91.0	10.56	2.8-2816	1.0000
n-Heptane (I)	98.0	10.97	0.6-547	1.0000
1,1,2-Trichloroethylene (I)	87.2	11.79	9.3-9344	0.9998
1-Butanol (II)	117.0	11.83*	6.5-6478	0.9996
Methylcyclohexane (I)	101.0	12.23	0.6-616	1.0000
1,4-Dioxane (I)	101.1	12.68	8.3-8264	0.9997
Propyl acetate (I)	102.0	12.89	2.8-2841	0.9999
2-Ethoxyethanol (III)	135.0	13.71	23.8-11904	0.9993
4-Methyl-2-pentanone (I)	115.0-117.0	14.64	2.6-2560	0.9996
Pyridine (I)	115.2	14.77	7.9-7855	0.9997
3-Methyl-1-butanol (III)	131.2	14.98*	4.1-10355	0.9994
Toluene (I)	110.0-111.0	15.04	1.4-2774	0.9994
Isobutyl acetate (I)	118.0	15.60	2.8-2800	0.9999
1-Pentanol (III)	138.0	16.41	4.2-10424	0.9997
2-Hexanone (I)	126.0-128.0	16.98	2.6-2596	0.9996
Butyl acetate (I)	126.0	17.43	2.8-2816	0.9997
Chlorobenzene (I)	131.0	19.00	3.6-3552	0.9994
p-Xylene (III)	138.4	19.77	0.3-689	0.9997

( ) indicates the corresponding groups.
*overlapped or partially overlapped peaks

Table 2.4 Accuracy and precision of 44 ICH solvents at working concentrations. Reprinted from [65] with permission.

Peak No.	Compound	Accuracy	Precision (RSD%, n=6)	
		Bias%	Intraday	Interday
Group I				
1	Methanol	1.54	1.76	1.53
2	Ethanol	-0.40	2.28	1.87
3	Acetone	-1.07	1.47	1.76
4	2-Propanol	0.94	1.82	1.90
5	Dichloromethane	-0.05	1.36	1.68
6	Methyl tert-butyl ether	-0.46	1.53	1.33
7	n-Hexane	-1.04	1.98	0.92
8	1-Propanol	0.23	1.79	1.58
9	Methylethyl ketone	-2.69	1.88	1.47
10	Ethyl acetate	1.65	1.56	1.91
11	Tetrahydrofuran	-0.74	1.64	1.42
12	Cyclohexane	-0.91	1.73	1.00
13	1,2-Dimethoxyethane	-0.10	1.63	1.19
14	Isopropyl acetate	-0.28	2.16	1.42
15	n-Heptane	-0.78	2.02	0.69
16	1,1,2-Trichloroethylene	-0.42	1.55	1.31
17	Methylcyclohexane	-0.61	1.70	1.11
18	1,4-Dioxane	0.23	2.09	1.45
19	Propyl acetate	-0.20	1.82	0.97
20	4-Methyl-2-pentanone	0.80	1.70	1.81
21	Pyridine	-0.40	1.62	1.27
22	Toluene	-0.43	2.21	1.52
23	Isobutyl acetate	-0.02	1.57	1.29
24	2-Hexanone	0.05	1.53	1.23
25	Butyl acetate	-0.73	1.62	1.29
26	Chlorobenzene	-0.60	1.53	1.23
Group II				
1	Pentane	2.49	1.50	1.86
2	Ethyl ether	-0.04	2.01	1.99
3	Acetonitrile	-0.95	1.68	1.55
4	(trans)-1,2-Dichloroethene	0.86	1.45	1.92
5	(cis)-1,2-Dichloroethene	0.49	1.37	1.67
6	Chloroform	-0.90	1.85	1.67
7	2-Methyl-2-butanol	-1.63	2.15	2.19
8	2-Methyl-1-propanol	2.34	1.96	1.87
9	1-Butanol	0.04	1.62	1.72
Group III				
1	Ethyl formate	-0.49	0.76	1.48
2	Methyl acetate	2.31	0.57	1.52
3	Nitromethane	1.42	1.59	1.69
4	2-Butanol	-1.28	1.30	2.02
5	2-Methoxyethanol	1.34	1.05	1.66
6	2-Ethoxyethanol	-2.50	1.14	1.50
7	3-Methyl-1-butanol	0.19	0.75	2.13
8	1-Pentanol	0.10	0.90	1.98
9	p-Xylene	0.42	1.28	1.14

Table 2.5 Limit of quantitation and limit of detection of 44 ICH solvents. Reprinted from [65] with permission.

Peak No.	Peak Name	QL ppm	Precision at QL (RSD%, n=6)		DL ppm
			Intraday	Interday	
Group I					
1	Methanol	2.11	8.53	5.35	0.63
2	Ethanol	3.68	6.70	8.94	1.10
3	Acetone	0.65	4.31	3.39	0.20
4	2-Propanol	1.87	7.57	6.81	0.56
5	Dichloromethane	3.71	4.50	4.83	1.11
6	Methyl tert-butyl ether	0.24	2.31	4.17	0.07
7	n-Hexane	0.10	2.72	6.27	0.03
8	1-Propanol	3.21	8.45	3.59	0.96
9	Methylethyl ketone	0.79	4.82	7.76	0.24
10	Ethyl acetate	0.68	2.19	2.95	0.21
11	Tetrahydrofuran	0.48	3.44	2.31	0.14
12	Cyclohexane	0.11	2.77	5.23	0.03
13	1,2-Dimethoxyethane	0.91	1.56	5.07	0.27
14	Isopropyl acetate	0.44	2.08	1.78	0.13
15	n-Heptane	**0.07**	1.95	2.57	**0.02**
16	1,1,2-Trichloroethylene	1.35	2.06	2.80	0.41
17	Methylcyclohexane	0.09	2.68	3.19	0.03
18	1,4-Dioxane	1.50	2.14	2.02	0.45
19	Propyl acetate	0.57	4.18	2.43	0.17
20	4-Methyl-2-pentanone	0.51	2.34	3.95	0.15
21	Pyridine	1.27	2.56	1.90	0.38
22	Toluene	0.28	1.94	3.35	0.08
23	Isobutyl acetate	0.47	3.26	4.93	0.14
24	2-Hexanone	0.74	2.58	2.96	0.22
25	Butyl acetate	0.64	3.23	4.40	0.19
26	Chlorobenzene	0.81	4.61	4.64	0.24
Group II					
1	Pentane	0.24	3.35	9.75	0.07
2	Ethyl ether	0.38	9.12	8.69	0.11
3	Acetonitrile	2.59	4.23	4.55	0.78
4	(trans)-1,2-	5.47	7.74	9.34	1.64
5	(cis)-1,2-	2.16	7.92	5.47	0.65
6	Chloroform	7.40	6.56	8.36	2.22
7	2-Methyl-2-butanol	1.47	4.72	8.17	0.44
8	2-Methyl-1-propanol	1.60	3.07	7.67	0.48
9	1-Butanol	3.08	8.13	3.99	0.93
Group III					
1	Ethyl formate	0.96	2.81	1.44	0.29
2	Methyl acetate	0.85	8.67	2.36	0.26
3	Nitromethane	3.64	9.43	10.15	1.09
4	2-Butanol	1.53	2.94	4.25	0.46
5	2-Methoxyethanol	**24.70**	7.07	2.60	**7.41**
6	2-Ethoxyethanol	18.31	7.26	2.43	5.49
7	3-Methyl-1-butanol	2.59	3.49	5.59	0.78
8	1-Pentanol	2.61	7.04	9.71	0.78
9	p-Xylene	0.23	6.91	5.59	0.07

2.3.3.4 Precision

The precision of the HSGC method was assessed by evaluating both method precision (intraday precision) and system repeatability (interday precision). The method precision is presented by the relative standard deviation of the response (RSD%, n=6) of six injections (six vials) of the same sample (groups I, II and III) at both a working concentration (20-900 ppm) and a lower concentration (2-90 ppm) on the same day. The relative standard deviations, RSD% of six injections of each solvent in the same day (intraday) were in the range of 0.57-2.28% at the 20-900 ppm level and of 1.56-9.43% at the 2-90 ppm level, respectively, as shown in Table 2.4 and Table 2.5. Similarly the relative standard deviations, RSD%, of six injections of each solvent in six consecutive days (interday) were in the range of 0.69-2.19% at the 20-900 ppm level and of 1.44-10.15% at the 2-90 ppm level, respectively, as shown in Table 2.4 and Table 2.5. These results indicated that this HSGC method has reasonable precision and system repeatability within the analytical range of determinations.

2.3.3.5 Method sensitivity

The sensitivity of this HSGC method is presented as the quantitation limit (QL) with a signal-noise ratio of 10 to 1, and detection limit (DL) with

a signal-noise ratio of 3 to 1. As shown in Table 2.5, the QL values of the 44 solvents evaluated range from 0.07-24.70 ppm, and DL range from 0.02-7.41 ppm. The broad ranges of QL and DL are due to the differences of hydrocarbon content in different solvents. Since some elements, e.g., chlorine, oxygen and nitrogen, are incombustible, solvents containing these elements have lower molar combustion capacities than pure hydrocarbons, leading to lower detection limits by FID. However, our results demonstrate that this HSGC method is sensitive enough for determination of the 44 solvents in drug substances, because the QL values (0.07-24.70 ppm) of these solvents are much lower than the requirements of ICH guideline for class 2 and 3 solvents (50 ppm or higher in most cases).

2.3.3.6 Sample analyses and matrix impacts of drug substances

In order to demonstrate the suitability of the HSGC method for determination of residual solvents in real drug substances, and for evaluating the impact of the drug substance matrix on solvent analyses, we analyzed four synthesized small molecule organic drug substances from Astrazeneca Pharmaceuticals, Wilmington, Delaware. We also spiked the 44 solvents in 3 groups into the four drug substances at both the 20-900 ppm and 2-90 ppm levels. As shown in Figure 2.2 and Table 2.6, the eight solvents in the four

drug substances are successfully determined by this HSGC method, and these results are consistent with those results from direct injection GC methods. When the 44 solvents were spiked into these four drug substances at both the 20-900 ppm and 2-90 ppm levels, most of the spiked solvents could be recovered from 70% to 115% during the HSGC analysis, as shown in Table 2.7, especially at the higher concentrations. These results suggest that interferences from the drug substance matrix or from the impurity peaks in DMSO, e.g. the peak at 3.1 min, 5.4 min and 14.3 min, should not have a significant impact on this HSGC method at regular working concentration. However, attention should be paid to those solvents, e.g. methanol (for DS1and DS4) and 2-methoxyethanol (for DS1 and DS3), where obvious interferences were observed for particular drug substance at the low solvent concentration levels. A more specific method validation may be required when some solvents recoveries are extremely out of range due to drug substances interferences. For example, drug substance samples containing hydrocarbon residual solvents (e.g. hexane, pentane, etc.) should be analyzed with a smaller sample load, i.e. 20-30 mg, to cover the ICH determination range of 5-5000 ppm interest, because these hydrocarbons have lower QLs when using FID detection.

Table 2.6 Residual solvents in four drug substances. Reprinted from [65] with permission.

Drug Substance (DS)	Residual Solvent	RT (min)	Concentration (ppm)
DS1	Acetone	5.22	91.2
	Ethyl acetate	8.65	589.4
	Tetrahydrofuran	9.03	201.9
	n-Heptane	10.97	4.6
DS2	Methanol	3.53	246.4
	Acetone	5.22	270.8
	2-Propanol	5.54	1451.6
	Tetrahydrofuran	9.03	19.4
DS3	Acetone	5.22	254.8
	Toluene	15.04	9.7
DS4	Methanol	3.53	90.8
	Ethanol	4.66	109.6
	Acetone	5.22	112.3

Table 2.7 Recoveries of 44 ICH solvent spiked in four drug substances.

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Solvent	Recovery %							
	DS1		DS2		DS3		DS4	
Group I	**WL***	**LL***	**WL**	**LL**	**WL**	**LL**	**WL**	**LL**
Methanol	97.0	17.9	102.7	108.1	100.2	110.1	68.2	ND
Ethanol	97.9	**118.0**	103.4	105.8	95.4	**122.8**	106.0	126.9
Acetone	94.2	57.0	92.2	94.3	**75.1**	**360.3**	95.4	89.0
2-Propanol	103.1	**129.7**	101.3	112.8	95.9	ND	103.0	121.6
Dichloromethane	94.3	**116.8**	105.1	ND	90.5	**119.2**	91.8	99.5
Methyl tert-butyl ether	83.8	99.6	95.1	94.8	70.2	86.4	79.6	90.0
n-Hexane	79.8	104.1	89.2	89.3	66.6	79.9	74.8	109.7
1-Propanol	98.0	99.7	100.2	98.9	96.7	102.6	100.1	96.0
Methylethyl ketone	81.7	**238.2**	98.0	90.9	92.6	94.4	99.7	106.6
Ethyl acetate	112.3	**140.2**	94.4	95.7	85.7	96.1	91.8	108.2
Tetrahydrofuran	96.0	85.4	94.5	98.4	84.3	95.7	90.3	109.4
Cyclohexane	82.9	95.0	91.2	93.6	69.4	73.9	89.1	95.7
1,2-Dimethoxyethane	95.7	95.7	97.0	93.5	89.3	89.4	100.2	99.3
Isopropyl acetate	92.6	106.1	95.8	100.8	85.2	92.5	98.8	102.6
n-Heptane	81.7	102.4	90.7	94.3	66.7	71.3	86.9	98.4
1,1,2-Trichloroethylene	92.7	97.9	96.2	96.2	88.2	92.5	99.0	103.8
Methylcyclohexane	84.4	91.6	91.6	93.9	71.2	78.2	90.2	97.4
1,4-Dioxane	96.6	99.1	97.5	101.2	91.7	95.0	100.1	99.7
Propyl acetate	94.8	99.0	97.7	100.9	88.5	96.3	100.7	101.1
4-Methyl-2-pentanone	96.6	102.5	97.4	96.1	91.8	94.6	104.1	110.5
Pyridine	97.8	98.7	96.7	98.1	93.9	113.8	100.1	100.3
Toluene	92.9	98.3	94.8	103.3	88.6	85.4	98.3	99.6
Isobutyl acetate	95.4	96.4	97.8	92.3	92.5	95.2	101.9	99.8
2-Hexanone	96.4	99.4	97.8	99.1	92.7	91.0	101.5	103.0
Butyl acetate	97.0	99.6	97.8	101.6	93.9	102.3	102.7	104.2
Chlorobenzene	97.0	101.2	98.1	99.4	92.5	98.5	101.5	104.0
Group II								
Pentane	85.6	87.2	94.7	89.1	79.2	73.6	93.4	77.5
Ethyl ether	88.6	95.3	96.1	89.8	77.1	74.6	98.9	**162.3**
Acetonitrile	96.5	**122.6**	91.6	**170.4**	103.3	**140.9**	97.4	**120.3**
(trans)-1,2-Dichloroethene	87.1	100.4	93.6	79.1	87.6	95.2	92.9	90.1
(cis)-1,2-Dichloroethene	75.8	64.7	97.1	89.7	92.3	84.8	97.3	89.0
Chloroform	115.0	76.3	100.9	99.3	96.5	94.1	100.6	105.7
2-Methyl-2-butanol	97.8	99.4	94.7	98.1	96.5	96.1	100.3	96.5
2-Methyl-1-propanol	103.6	99.7	104.7	95.6	101.4	98.9	105.0	104.7
1-Butanol	101.2	103.0	101.1	91.2	100.0	99.3	101.2	103.1
Group III								
Ethyl formate	95.0	ND	**271.2**	**1751.6**	97.1	88.0	110.7	ND
Methyl acetate	85.2	88.0	95.1	ND	84.0	69.4	92.5	103.0
Nitromethane	**278.2**	ND	109.8	**120.7**	98.4	110.8	91.4	93.2
2-Butanol	**124.0**	ND	108.3	**197.8**	88.6	95.5	97.2	105.4
2-Methoxyethanol	94.7	ND	105.6	110.0	92.3	48.4	101.8	94.9
2-Ethoxyethanol	94.7	98.0	103.8	88.6	81.2	96.5	105.0	107.0
3-Methyl-1-butanol	97.0	100.3	103.8	109.8	105.6	ND	108.1	111.2
1-Pentanol	98.7	99.5	106.8	103.1	91.3	99.9	107.8	108.4
p-Xylene	89.8	92.8	93.0	97.3	91.9	88.4	99.0	103.9

* WL stands at working concentration level (20-900 ppm); LL stands at low concentration level (2-90 ppm)

2.4 Conclusions

In this study, a generic HSGC method is successfully developed and validated for the determination of 44 ICH Q3C class 2 and 3 residual solvents in drug substances. The method is specific, accurate, precise, linear, sensitive and efficient. DMSO was selected as the sample diluent due to its high capacity for dissolving organic drug substances, stability and high boiling point. The conditions of HS sampler and GC were optimized to make the HSGC method more sensitive, efficient and reproducible. This method has a much shorter sample equilibration time, a better separation for many solvents, a higher sensitivity and a broader concentration range comparing with the previously published methods. The examples of real drug substance analyses demonstrate the broad application potential of this HSGC method in the determination of residual solvents in drug substances. This method meets ICH guideline requirements, and may be suitable for residual solvent determinations in a variety of pharmaceutical applications.

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Chapter 3: LC-MS/MS for the analysis of highly polar aminoglycoside compounds

3.1 Introduction

The identification and separation of highly polar compounds from biomatrices are of great importance in pharmaceutical research and development [34,77]. However, LC-MS/MS determination of concentration levels of highly polar compounds in biological samples is very challenging for the following reasons. First, in sample preparation, the polar nature of these compounds makes it difficult for them to be recovered by liquid-liquid extraction [78]. Second, in order to obtain best response in MS using positive electrospray ionization, it is necessary to keep the mobile phase acidic, which causes ionization of basic, polar compounds. In chromatographic separation, it is very difficult to achieve proper retention of the ionized polar compounds on reversed phase columns; therefore the polar compounds cannot be clearly separated from other polar interference peaks and unresolved endogenous species in the sample matrix. These drawbacks lead to ion suppression, low sensitivity and unreliable quantitation on LC-MS/MS [79,80].

The aminoglycoside class of antibiotics is typical highly polar compound. This class contains a phamacophoric 1,3-di-aminoinositol moiety, whose alcoholic functions are substituted through glycosidic bonds with characteristic aminosugars to form pseudo-oligosaccharides [81]. They are basic and are free-water soluble at all pH levels. Examples of aminoglycosides are: gentamicin, kanamycin, tobramycin, and apramycin. These molecules are thermodynamically stable over a wide range of pH values and temperatures and have molecular weights ranging from 400 to 500 g/mol. The aminoglycosides are basic polycations with pK_a values that range from 7.2 to 8.8 [82]. Among them, gentamicin is one of the most commonly used aminoglycoside antibiotics that inhibits both Gram-positive and Gram-negative bacteria and is widely used in veterinary medicine to treat serious infections [83]. Their toxic effects are related to their levels in blood and are mediated by the special affinity of these aminoglycosides for kidney cells and sensory cells of the inner ear [84]. Because of the small differences between these compounds and the lack of chromophores, LC-MS is the analytical method of choice with a detection limits of approximately 400 ng once injected onto the column [85]. In order to meet the US Food and Drug Administration (FDA) and European Commission (EC)'s established limits of tolerance or maximum residue limits for antibiotics in food, an easy and robust

LC-MS/MS method must be developed for quantitative analysis of these highly polar compounds. This method should also satisfy the analytical need of combination antibiotic treatments, which involves aminoglycosides paired with compounds from another antibiotic series in order to treat severe pseudomonas infections. Several efforts have been made to solve the problems of low sample recovery and to achieve improved chromatography [33,78,79,86-99].

One way for polar compounds to achieve great retention is to use a hydrophilic interaction chromatographic (HILIC) column, which uses unbonded silica silanol or diol bonded stationary phases such as amino, anionic, amide, cationic and zwitterionic bonded phases. It was reported that using zwitterionic ZIC-HILIC columns interferes with consistent retention times for aminoglycoside samples [96]. A large fraction of the recently published work has used unmodified bare silica as the separation material (Betasil, Hypersil, Kromasil, Atlantis) [31,100]. The separation mechanism of HILIC column is that polar analyte partitions into and out of the adsorbed water layer allowing the charged polar analyte to undergo cation exchange with the charged silanol groups (or other functional groups coated on the silica surface). The advantages of underivatized bare silica columns are improved

retention of polar compounds, low back pressure with high organic mobile phases, and direct injection of organic solvent extracted samples [34]. Moreover, non-modified bare silica gel has some advantages for HILIC applications in comparison to the chemically bonded stationary phase, as it is not subject to bleeding of the bonded phase from the column [101]. It has been suggested that a layer of water on the silica surface can act as a deactivating reagent on the adsorption site. However, on occasion irreversible adsorption has been observed on bare silica in HILIC mode. In order to achieve a lower limit of quantitation of 100 ng/mL, 500 μL of sample had to be injected [96]. It was reported that the elution of some oligosaccharides from HILIC columns requires a significant level of salt in the mobile phase. The separation mechanism of the HILIC column is a superimposition of electrostatic attraction on hydrophilic interactions [29]. Based on the difference of the organic modifier, the pH level of the buffer, and ionic strength, the analyte gets retained by various mechanisms which could cause double peaks as well as increased carryover for highly polar molecules [102]. The combination of the mechanism has not been well studied in current literature.

Another way to increase retention times of polar compounds on RPLC is to derivatize the analyte or to add an ion pairing reagent to the

mobile phase. Several LC methods have been developed for the analysis of aminoglycosides from different biological matrices which involves sample derivatization [86,103,104] or addition of an ion pairing reagent to the mobile phase [85,87]. While derivatization is a tedious and laborious procedure, adding an ion pairing reagent to the mobile phases is a regularly practiced technique. With the better penetration and interaction of the hydrophobic moiety of the ion pairing reagent, the analyte has better retention. For MS purposes, the ion pairing reagents must be volatile and easily removed. The most commonly used ion pairing reagents are phosphoric acid (H_3PO_4), alkyl sulfonates, tetra-alkyl ammonium salts [105], trifluoroacetic acid (TFA) [106], TCA [91], and perfluorinated carboxylic acids with *n*-alkyl chains [107]. Counter ions can ion pair with basic functional groups and impart increased rigidity to the molecule and, in addition, exclude these basic groups from the hydrophobic surface of the column. The ion pairing reagents bind with the basic or acid functional groups and bring these charged basic or acid groups to the hydrophobic surface, leading to longer retention times than without ion pairing reagents. However, adding ion pairing reagents can often cause the pH of the local environment of the analyte to differ from the pH of the mobile phase. That change can lead to a more rigid structure of the analyte which leads to a peak

sharpening effect. The retention time of an analyte increases as the molecular weight of the ion pairing reagent increases. Zhu *et al.* used 5% TCA with 13 aminoglycoside compounds and used two HLB SPE cartridges at different pH levels to simultaneously purify the compounds and showed a detection limit for gentamicin from food of animal origin as 120-140 ng/g [99]. Heller *et al.* used 30% TCA to extract the sample and added 55 mM TFA as an ion pairing reagent to the mobile phase A. Using an injection volume of 60 µL, they got a 3.3 ng/mL LOQ for gentamicin from bovine plasma [91]. Using dual SPE cartridges at extreme pH levels to extract samples and 10 mM heptafluoroburyric acid (HFBA) in the mobile phase, Park *et al.* achieved similar detection limits to those demonstrated by Heller [108]. Adding an ion pairing reagent often times causes contamination of the ion source and a reduction in sensitivity [107]. For example, TFA [108], HFBA [78,89] or pentafluoropropionic acid (PFPA) [109] rapidly contaminated the ion source and a severe matrix effect was observed [95 ,108]. TCA was reported to be used in plasma precipitation at various concentrations and volume ratios to samples and also used as an ion pairing reagent in the mobile phase. However, when TCA was added to the mobile phase, a two-range standard curve had to be calibrated due to the ion suppression effect of TCA [110]. Moreover, the effects of TCA concentration on analyte recovery,

chromatographic behavior of the analyte, and separation mechanism on silica columns were poorly characterized. In our investigation, we studied the above issues and developed an easy, robust, and validated LC-MS/MS method for quantitative analysis of highly polar aminoglycoside compounds in rat plasma.

3.2 Experimental

3.2.1. Chemicals and reagents

All solvents used were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (88%) was supplied by J. T. Baker (Phillipsberg, NJ, USA). 2,2,2-trichloroacetic acid (TCA) (99+%) was purchased from Acros (Morris Plains, NJ, USA). Control Rat Plasma in K2EDTA (Individual MALE 031-APEK2-MI) was purchased from Bichemed (Wichester, MA, USA). Gentamicin, tobramycin, kanamycin, apramycin and ciprofloxacin were obtained from Pfizer Global Research & Development (Groton, CT, USA).

3.2.2 Equipment

A standard multi-tube vortex-mixer from VWR Scientific Products (West Chester, PA, USA) was used for vortex-mixing, and an Eppendorf centrifuge model 5810R from Brinkmann Instruments Inc. (Westbury, NY, USA) was used for centrifugation. An Applied Biosystems/MDS Sciex (Concord, ON, Canada) model API 4000 triple quadrupole mass spectrometer equipped with LC-10AD Prominence solvent delivery system, degasser and SCL-10 Avp system controller (Shimadzu, Columnbia, MD, USA) was used for LC-MS/MS analysis. A Leap Technologies CTC PAL autosampler with Shimadzu 10AD pump was used. A Harvard Apparatus (South Natick, MA, USA) syringe pump with a 500 µL syringe from Hamilton Co. (Reno, NE, USA) was employed for compound infusion.

3.2.3. Sample preparation using TCA or acetonitrile (ACN) induced plasma protein precipitation

Stock solutions of gentamicin, kanamycin, apramycin and tobramycin were prepared as 1 mg/mL in water. Stock solutions were spiked in rat plasma to final concentrations of 4000 ng/mL. TCA was diluted in water to obtain 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30% and 35%

concentration (w/v). For the TCA plasma crash, 30 µL TCA at various concentrations was added to 50 µL plasma samples and a white precipitation of protein was observed. Following that precipitation, 170 µL internal standard solution of tobramycin at a concentration of 500 ng/mL in water was added. Samples were centrifuged at 4000 rpm for 5 minutes before aliquoting. For ACN plasma crash, 50-400 µL ACN at various ratios was added to 50 µL of the plasma samples and a precipitation of protein was observed. Analyte recovery was calculated by peak areas counts ratios with samples recovered from plasma and samples from neat solutions. 200 µL samples were aliquoted into 1.2 mL polypropylene tubes (96-well format), then 10 µL was injected into LC-MS/MS. Samples at each concentration level were analyzed in triplicate over three independent batch runs.

3.2.4. Preparation of calibration standards and quality control samples

Stock solutions of gentamicin, kanamycin, apramycin, tobramycin were prepared as 1 mg/mL in water. Tobramycin was further diluted to a concentration of 500 ng/mL for using as an internal standard. Stock solutions were serially diluted with rat plasma or water. Analytical standards used to construct calibration curves were prepared separately for each type of extraction method. For plasma and neat samples, standards were prepared by

spiking known quantities of the standard solutions into rat plasma and water respectively. Serial dilutions were then carried out to achieve desired concentrations. Standard reference curves were prepared for analysis in the following concentrations: gentamicin, 1, 2, 5, 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/mL. For kanamycin and apramycin, 10, 25, 50, 100, 250, 500, 1000, 2500, 5000, and 10000 ng/mL. Accuracy (% RE) and precision (% CV) of the assay were assessed by analyzing 40, 400, 4000 or 80, 800, 8000 ng/mL quality control samples prepared identically to the analytical standards.

3.2.5. LC-MS/MS analysis

Gradient chromatography was performed using a Synergi 4 μm Max-RP 80 Å 50 X 2.00 mm C12 column (Phenomenex, Torrance, CA, USA) and an Atlantis HILIC Silica 5 μm 50 X 2.10 mm column (Waters, Milford, MA, USA). Gradient elution was applied with 0.1% formic acid in 100% water (A) and 0.1% formic acid in 100% ACN (B) at a flow rate of 0.25 mL/min. The gradient used for the Synergi column was 0.10 min, 5% B; 1.50 min, 70% B; 2.50 min, 90% B; 3.50 min, 5% B; 3.6 min, stop. The column was equilibrated for 1 minute before beginning the run. The gradient

used for the Atlantis column was 0.10 min, 95% B; 1.50 min, 30% B; 2.50 min, 10% B; 3.50 min, 95% B; 3.6 min, stop. The column was equilibrated for 1 minute before run.

Positive ion electrospray tandem mass spectra were recorded using an AB Sciex API 4000 triple quadrupole mass spectrometer with multiple reaction monitoring (MRM) detection mode equipped with Analyst (version 1.41) operating software. The ionspray voltage was set to 5000 V, and the probe temperature was set at 500 ℃. Nitrogen was used as the collision gas. The nebulizer (GS1), curtain, and turbo gas (GS2) were set to 40, 10, and 50 psi, respectively. MRM parameters of test compounds were set as described in Table 3.1. Dwell times were set to 200 ms for each transition.

3.2.6. Method validation

Recoveries of the analyte were determined by comparing the peak area of five extracted samples of 4000 ng/mL at TCA concentrations of 15%, 20%, 25%, 30%, 35% (w/v) using the mean peak area of recovery standards. Five replicates of each of the recovery standards were prepared by adding the analyte to water at the same TCA concentrations.

Batches, consisting of triplicate calibration standards at each concentration, were analyzed inter and intra day to complete the method validation. In each batch, quality control (QC) samples at 40, 400, 4000 or 80, 800, 8000 ng/mL were assayed in sets of three replicates to evaluate the inter and intra day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) were used as measure of accuracy and precision.

3.3 Results and discussion

3.3.1. TCA induced plasma protein precipitation

Plasma protein precipitation with organic solvents is commonly used for analyte recovery. However, because polar compounds have a low solubility in organic solvents, analyte recovery is very low. Figure 3.1 shows analyte recovery for gentamicin, tobramycin and ciprofloxacin versus ACN/water volume ratio. The analyte recovery was approximately 20% and dropped slightly as the volume ratio increased.

Figure 3.2 shows analyte recovery for gentamicin, tobramycin and ciprofloxacin when various TCA concentrations were used for plasma protein precipitation. Ciprofloxacin was used for comparison purposes

[111]. The data shows that analyte recovery increased as TCA concentration increased for gentamicin and tobramycin. Since ciprofloxacin is less polar (clogD = -0.78 at pH = 6.5) [112] than gentamicin and tobramycin (clogD = -8.39 and -9.58 at pH = 6.5) [32,113], its recovery is not as good and plateaued from 15 to 30% TCA.

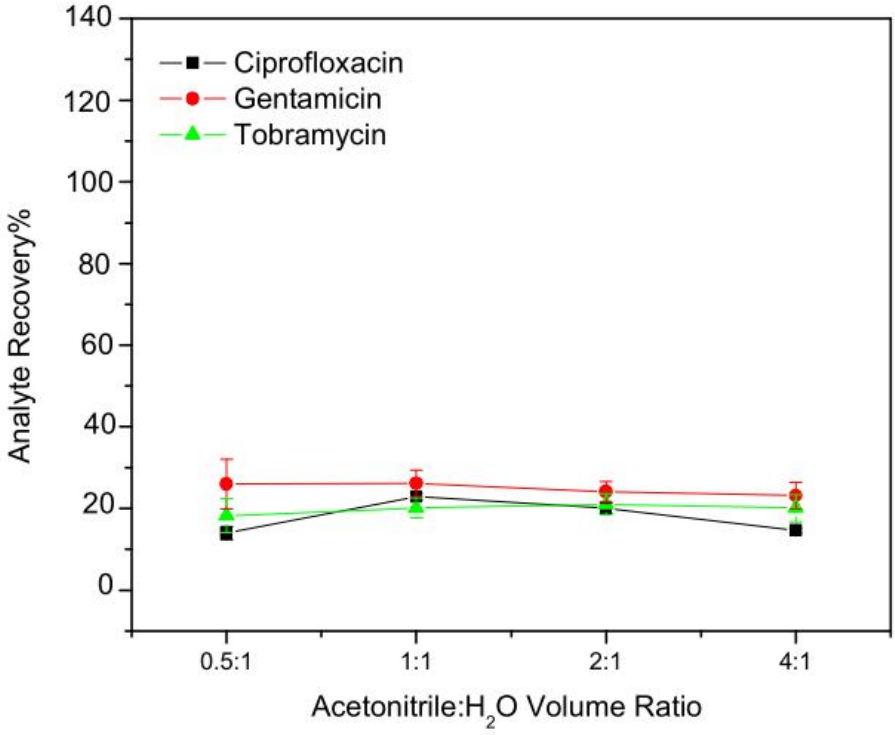


Figure 3.1 Analyte recovery versus ACN/water volume ratio for polar small molecules.

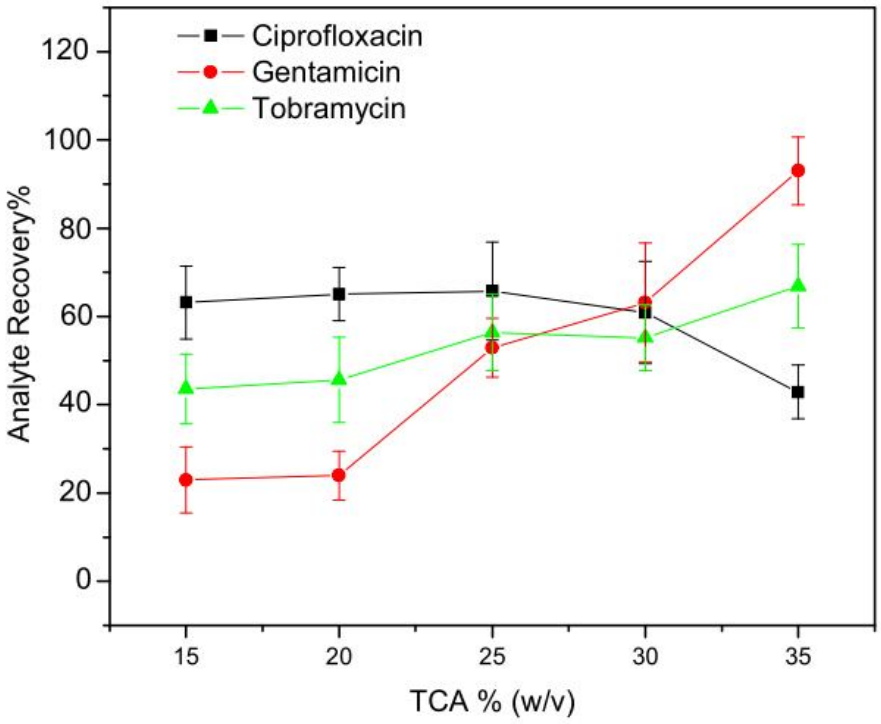
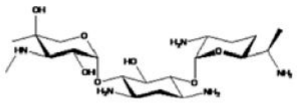
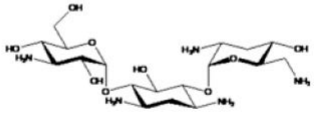
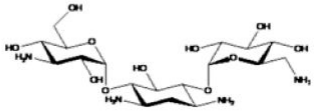
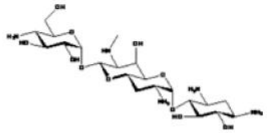
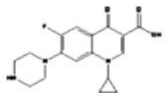


Figure 3.2 Analyte recovery versus TCA concentration for polar small molecules. Reprinted from [66] with permission.

Table 3.1 MRM parameter on MS analysis of aminoglycosides. Reprinted from [66] with permission.

Compound	Chemical structure	MW	Q1	Q3	DP	CE	EP	CXP
Gentamicin		463.6	464	323	70	20	10	10
Tobramycin		467.5	468	163	60	42	10	10
Kanamycin		484.2	485	163	90	36	10	10
Apramycin		539.3	540	217	70	38	10	10
Ciprofloxacin		331.4	332	288	70	24	10	10

Q1 molecular ion, Q3 daughter ion, DP declustering potential, CE collision energy, EP entrance potential, CXP collision cell exit potential

The mechanism of TCA-induced protein precipitation was studied by Sivaraman [112]. Protein-precipitating action of TCA tends to be independent of the nature of the proteins. Acid induced structural transitions occur during protein precipitation. The pH is not the dictating force in inducing protein precipitation. Acid induced protein precipitation is unique to TCA and strongly dependent on the trichloro group. Although blood plasma contains not only dissolved proteins, but also glucose, clotting factors, mineral ions, hormones and carbon dioxide [114], its TCA induced protein precipitation observes a similar phenomenon; at 0.1% and 1% TCA concentration, no precipitation was observed and the protein was partly changed to molten globule state. Protein precipitation started at 5% TCA, and reached a maximum of 30% TCA.

3.3.2 Retention time and hydrophilic interaction chromatographic mechanism study on Atlantis HILIC column

Hydrophilic interaction chromatography is orthogonal to reversed phase chromatography and is used to better retain polar compounds. Present HILIC theory dictates that HILIC retention is caused by a partitioning of the injected analyte solute molecules between the mobile phase eluent and a water-enriched layer in the hydrophilic HILIC stationary phase

[29-32,77,96,100,113]. The more hydrophilic the analyte is, the more the partitioning equilibrium shifts towards the immobilized water layer in the stationary phase, and thereby, the more the analyte is retained.

Figure 3.3 shows the chromatograms of gentamicin and tobramycin prepared in water with different percentages of TCA. The mobile phase A was pure water with 0.1 % formic acid (pH = 2.78) and the mobile phase B was 100% ACN with 0.1 % formic acid. Two peaks for each compound were observed at different TCA levels. As the percentage of TCA increased, the peak area for the first peak increased while the peak area for the second peak decreased. At the same time, the retention times decreased as well. These two peaks were due to two separation mechanisms. One separation mechanism is the electrostatic interaction or ion exchange between the positively charged samples and the negatively charged silanol groups, which is correspondent to the first peak. Another separation mechanism is hydrophilic interaction between the neural sample and the water layer surface which corresponds to the second peak. When increasing the percentage of TCA, the amount of positively charged molecules also increases.

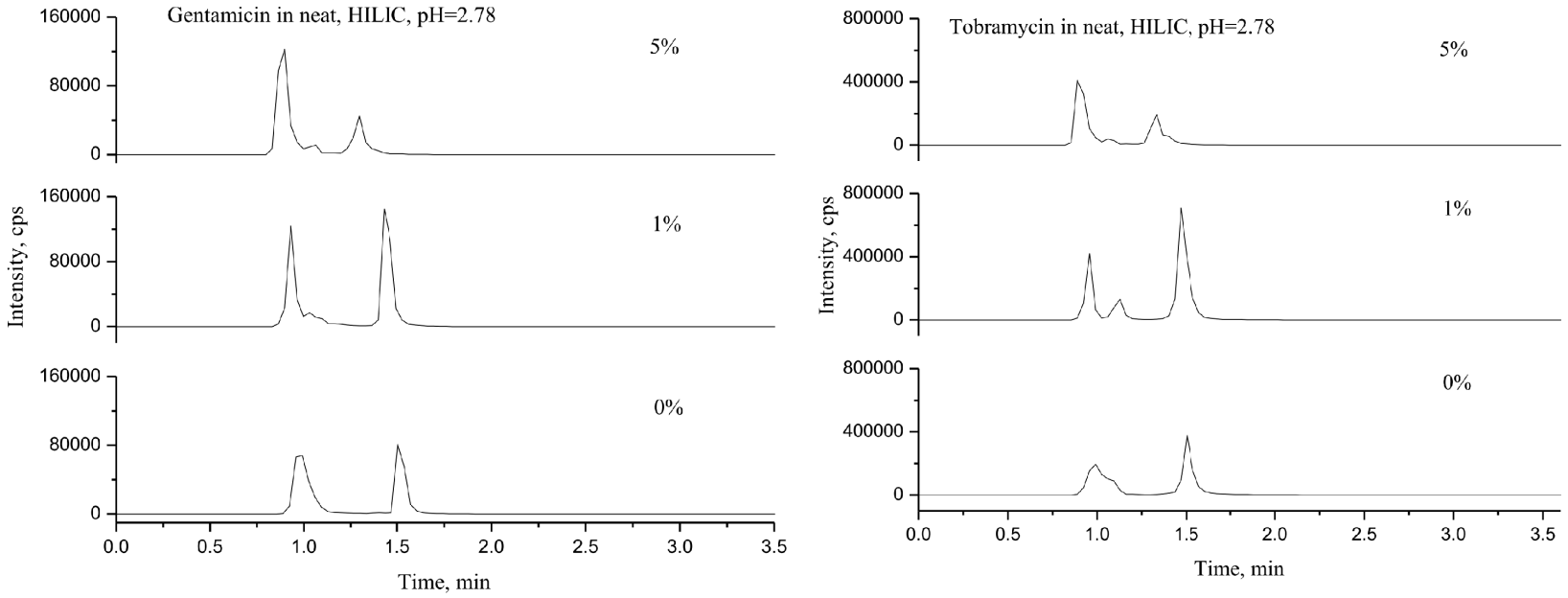


Figure 3.3 Chromatograms of Gentamicin/Tobramycin neat samples on HILIC column (pH=2.78), TCA Crash

This increase is explained by considering the apparent pK_a of gentamicin, which is 8.2, and the apparent pK_a of tobramycin at 8.0 [30], which made the height of the first peak increase. The charge status of the aminoglycoside molecules depends on the pK_a of amino groups which varies depending on their positions from 6.7 to 9.7 [115] as well as the local sample pH. With 0.1 % formic acid in the mobile phase, the ionization of silanol groups on the silica surface were partly, but not completely, suppressed [101], which was why there were electrostatic interactions. Increasing the percentage of TCA also increased the hydrophobicity of the samples, which was why the retention times decreased. Though TCA may form ion pairs with the sample molecules, it has no impact on retention time change on the HILIC column. The main impact of TCA is to change the local sample pH.

For gentamicin/tobramycin plasma samples , it is useful to consider that plasma actually is in a physiological buffer with a pH of 7.4 [116]. The charge status of the basic and polar aminoglycoside is determined by the pH of the buffer. At higher TCA levels, when the acidic effect of TCA surpassed the buffering capacity, a more of the molecules began to be positively charged and the above mentioned first peak began to appear. When mobile phase A changed to 10 mM of ammonium acetate in 100% water (pH = 6.86), retention times for both compounds shifted slightly to the

left. This indicates that dipole-dipole interaction was part of the hydrophilic interaction and was weakened by the addition of an electrolyte. The appearance of two peaks and the increase of the first peak and decrease of the second peak are similar to Figure 3.3, which indicated the same retention mechanism.

Figure 3.4 shows the chromatograms of gentamicin/tobramycin plasma samples on HILIC column with mobile phase A changed to 10 mM ammonium hydroxide in pure water (pH = 10.6). No good chromatograms were seen when TCA concentration was greater than 10%. It is seen that the retention times for both compounds increased. In general, the pH value of the running buffer affects the retention as an ionized molecule is more hydrophilic and is retained with more strength in HILIC, than compared to its neutral state. In this case, because the sample molecules are already very hydrophilic, their ionized form did not show any better hydrophilicity. On the other hand, the increase of water phase pH to 10.6 makes the majority of silanol groups on the silica surface ionized and negatively charged which caused more dipole-dipole interaction and increased the retention times. Increasing the level of TCA quickly decreased the peak intensity and caused the appearance of another peak which had a shorter retention time.

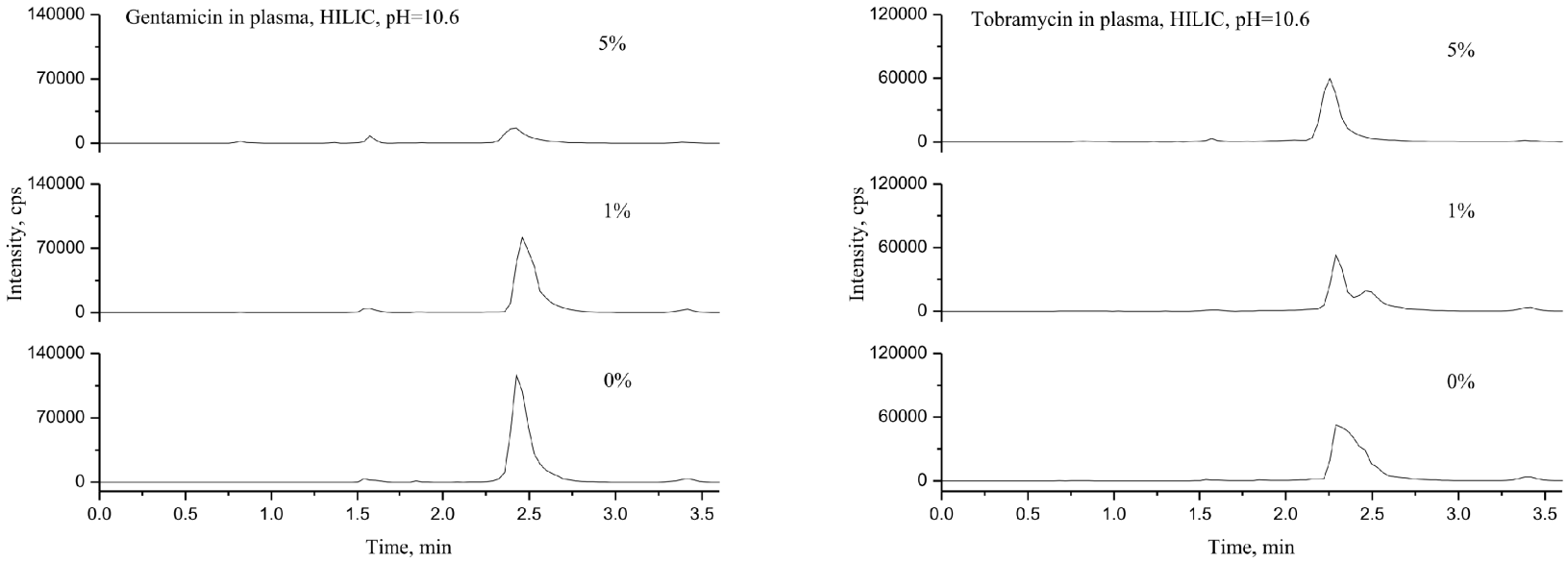


Figure 3.4 Chromatograms of Gentamicin/Tobramycin plasma samples on HILIC column (pH=10.6), TCA Crash

Though TCA was an ion paring reagent, the lack of carbon coating on the surface did not enable hydrophobic interaction and the separation was not based on ion pairing effect. Since there were no hydrophobic interactions between the samples and the stationary phase, the forming of ion pairs between TCA molecule and sample molecules have no impact on retention time change. The driving force for retention time change is pH change. The function of TCA was only to change the pH of the sample.

On the whole, the retention and selectivity by HILIC are affected by the fraction of organic solvent, the ionic strength and the pH of the buffer. Because the bare silica surface is easily charged and the charge status is dependent on the pH of the mobile phase, the strong electrostatic interactions and dipole-dipole interactions are part of the separation mechanism. The charge status of the samples is also a very important factor that determines the separation mechanism.

In order to achieve retention of highly polar analytes using RP chromatography, non-volatile highly aqueous mobile phases must be often used which are not ideal for compound ionization by ESI-MS. It is suggested that HILIC requires mobile phases that are highly volatile and

ideal for compound ionization by ESI-MS. However, this is only true when isocratic elution was used. In fact gradient elution often times has to be used to achieve good peak shape or even to achieve any peak. For RP chromatography the volatile component of the mobile phase often times reaches to 80-90% when analytes are ionized, while for HILIC it reaches 5-50%, which doesn't help ionization efficiency. Moreover, polar biological matrices are hard to separate from polar compounds, therefore HILIC chromatograph is more subject to potential matrix suppression.

Another issue encountered when using a bare silica column is carryover. The adsorption of basic polar analytes on the bare silica surface is so strong that the carryover could range from 2% to 50%. The adsorption of plasma matrix components to the bare silica surface could change the surface charge condition and cover silanol groups, causing complication of chromatography. It is well know that basic compounds can interact with the unmodified silanols of silica-based columns, resulting in band tailing, poor retention and peak shape [107].

3.3.3 Retention time and reversed phase chromatographic mechanism study on Synergi Max RP column

A Synergi Max RP column coated with C-12 was used for RP chromatography and to achieve maximum retention. When 0.1% formic acid was added to both of the mobile phases, the pH was lowered to 2.78, which controlled the ionization situation of the surface silanol groups. Even though the silica surface is coated with C-12, approximately 25-50% of the silanol groups present on the silica surface are bonded to silanes because of steric hindrance. Silanol groups are weakly acidic and depend on the surface silanol groups (free silanols, germinal silanols, vicinal silanols) with a pK_a range of 2-8 [117]. The effective surface charge is primarily controlled by the (de)protonation of the silanol groups [118]. When the pH is equal to 2.78 there were still parts of the silanol groups that were negatively charged.

The residual surface silanol groups are able to undergo hydrogen-bond and dipole-dipole interactions, and when negatively charged, electrostatic interactions with polar compounds, which causes peak tailing and decreased chromatographic resolutions. In contrast, the charge status of gentamicin and tobramycin molecules was controlled by the concentration of the TCA that was added to the sample preparation. Figure 3.5 shows the

chromatograms of gentamicin and tobramycin neat samples on Synergi column (pH = 2.78) with different percentages of TCA. It has been observed that when the percentage of TCA concentration is between 0 and 1, there was very little retention for gentamicin and tobramycin.

This is not unexpected because there was virtually no hydrophobic interaction between the C-12 alkyl chain and the analyte. The hydrogen bond and dipole-dipole interactions between the surface silanols and the analyte were reduced by the alkyl layer. When the TCA concentration reached 5%, some of the gentamicin and tobramycin molecules began to accumulate charges, and another peak that had a longer retention time began to appear. When the TCA concentration reached 15%, the retention time increased. When the percentage of TCA was greater than 20%, a single, sharp peak was observed for both gentamicin and tobramycin, indicating the ion pairing effect had reached its maximum.

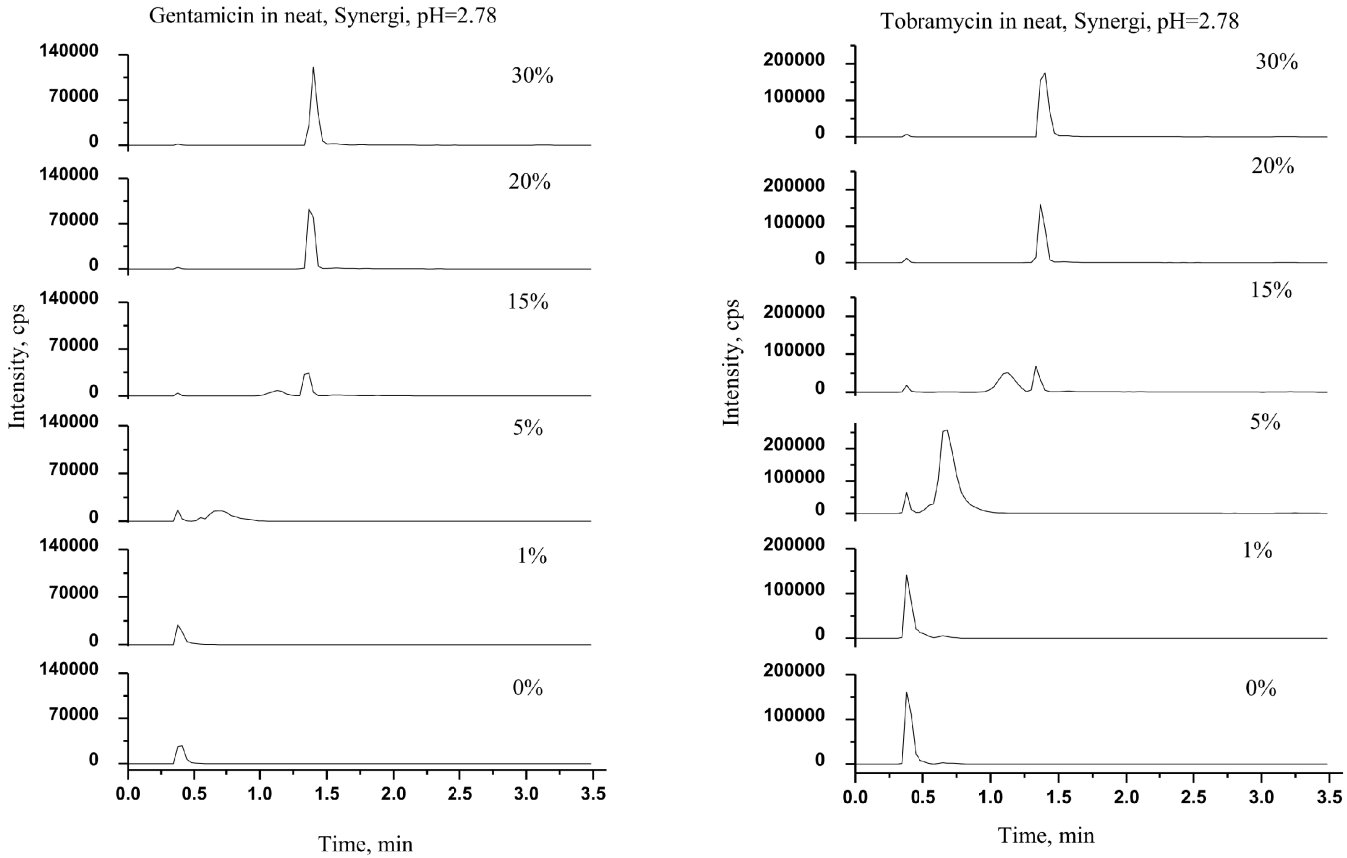


Figure 3.5 Chromatograms of Gentamicin/Tobramycin neat samples on Synergi column (pH=2.78), TCA Crash

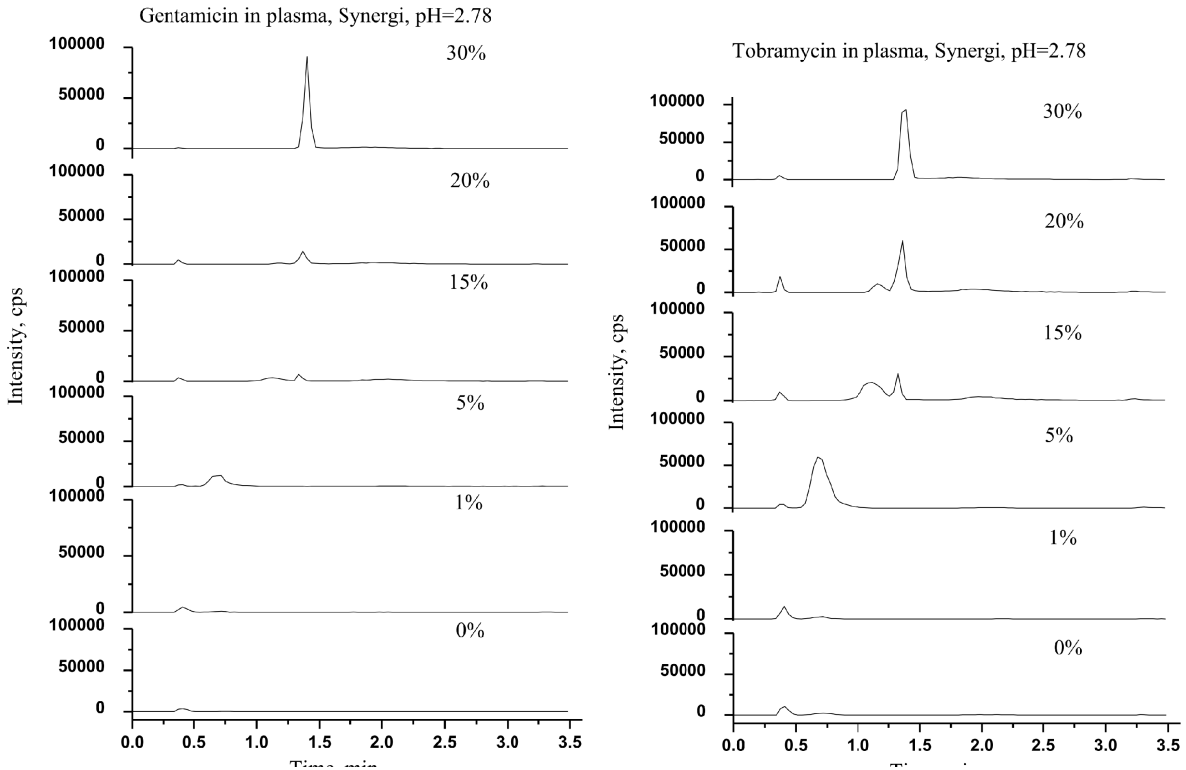


Figure 3.6 Chromatograms of Gentamicin/Tobramycin plasma samples on Synergi column (pH=2.78), TCA Crash. Reprinted from [66] with permission.

Figure 3.6 shows the same experiments related to gentamicin and tobramycin plasma samples. When the TCA concentration was higher than 10%, not only could it precipitate proteins in plasma, but also it had the same effect of increasing the retention time. When the retention time was increased, the peaks did not become wider but narrower; therefore TCA also had the effect of sharpening the peaks. Increasing retention time also made it possible to separate the analyte from the sample matrix, which was composed primarily of protein residues and salts. In contrast, when no TCA was used and the retention time was low, the analyte peak intensity was subject to the matrix effect, and was significantly lower. Because TCA was added to the samples but not the mobile phase, it was ion paired with the analyte and helped to increase the retention of the analyte but it didn't have the signal suppressing effect of ion pairing reagent added to the mobile phase. When compared with the commonly used perfluorinated carboxylic acids with alkyl chains, TCA disassociated easily with the analyte and as its concentration increased the signal counts increased as well. When no TCA was used but ACN was used for neat samples, no analyte precipitation was observed and the peak intensity counts for the analyte remained almost the same (data not shown). However, when ACN was used for plasma protein

precipitation as the ratio of ACN/water increased, the peak intensity counts decreased, which suggested that more analyte precipitated with proteins when the ratio of ACN increased.

3.3.4 Quantitation

3.3.4.1. HILIC

The calibration curve of gentamicin/tobramycin plasma samples over the range of 20-5000 ng/mL without use of an ion pairing reagent on the Atlantis HILIC column with 10 mM ammonium acetate buffer is shown in Figure 3.7. A non-linear fitting, as the concentration increases suggests that irreversible adsorption happened during the sample preparation and sample analysis. The adsorption to the silica column is the dominant reason since same samples produce a linear curve when analyzed with a reversed phase column. The LLOQ was 20 ng/mL, which was not as good as the LLOQ obtained from reversed phase chromatography as discussed below.

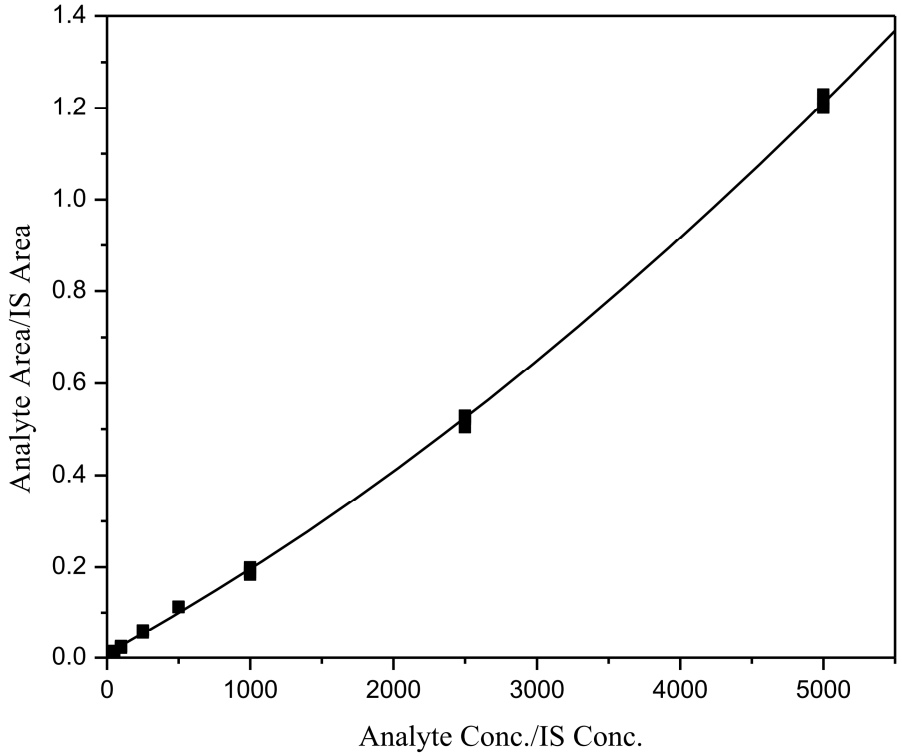


Figure 3.7 Calibration curve of Gentamicin plasma samples without ion pairing reagent on Atlantis HILIC column (10 mM ammonium acetate buffer)

3.3.4.2. Reversed phase chromatography:

The calibration graph shown in Figure 3.8 for gentamicin was generated from MRM analysis of five replicate rat plasma samples at the calibration standard concentration level over the range of 1-5000 ng/mL, with tobramycin as the internal standard at 500 ng/mL. Kanamycin and

apramycin were prepared in the same manner as gentamicin except the range was 10-10000 ng/mL.

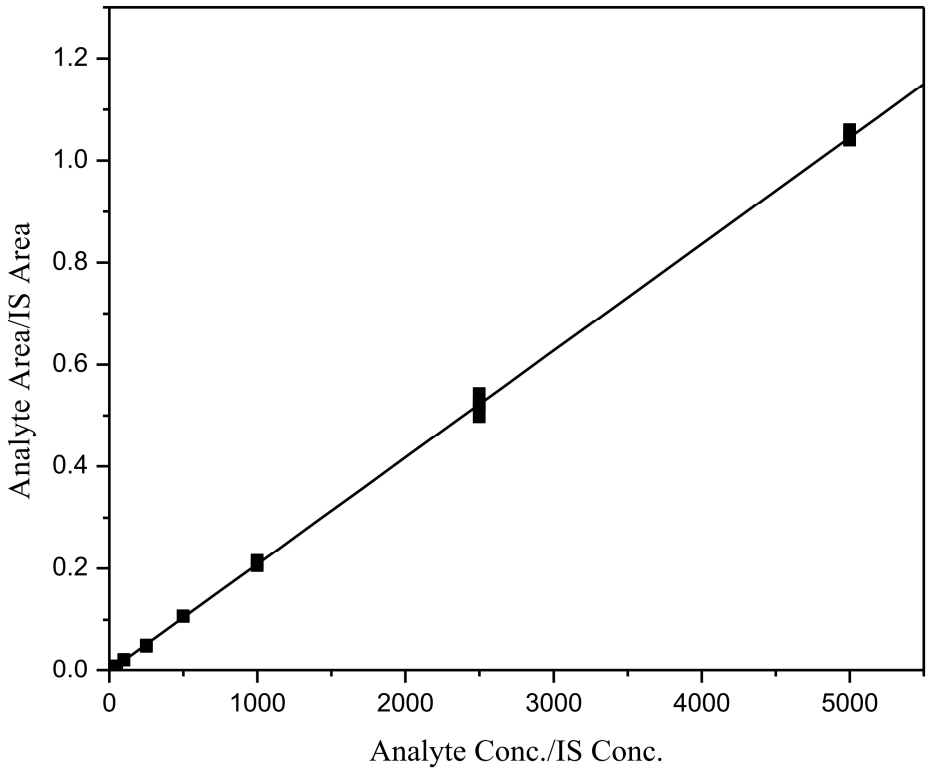


Figure 3.8 Calibration curve of Gentamicin plasma samples with 30%TCA on Phenomenex Max-RP column (0.1% formic acid in mobile phase)

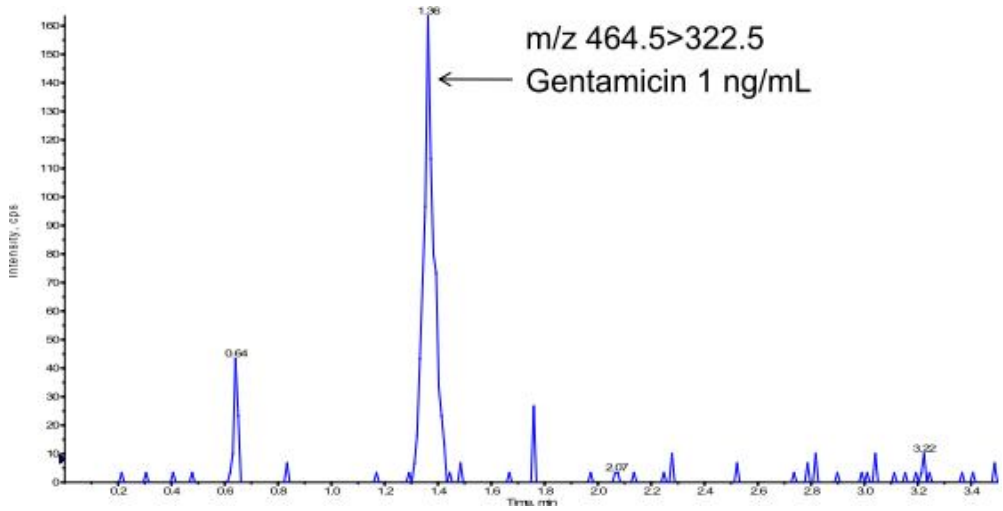


Figure 3.9 LLOQ of Gentamicin plasma sample is 1 ng/mL.

With an injection volume of 10 µL, good responses over the concentration ranges were obtained. Calibration curve regression was weighed as 1/x and performed using linear fit of quantities versus peak area ratios. Precision and accuracy data are shown in Table 3.2. The LLOQ corresponding with a coefficient of variation less than 20% was 1, 20 and 10 ng/mL for gentamicin, kanamycin and apramycin, respectively. Figure 3.9 shows the chromatogram of the LLOQ of gentamicin. The standard calibration curves were linear over the concentration range with a correlation coefficient better than 0.9993. The precision was over the range of 2.6-4.1, 3.3-5.0, 1.5-9.9%, and accuracy was 94.7-103.7, 87.9-104.9, 91.3-103.6% for gentamicin, kanamycin and apramycin respectively. Since TCA was only added to samples but not to the mobile phase, no ion

suppression was observed which would cause the non-linearity of the standard curve. The LLOQ is much lower than can be achieved with ion pairing chromatography using perfluorinated carboxylic acids ion-pairing reagent and HILIC columns.

Table 3.2 Precision and accuracy data for gentamicin, kanamycin and apramycin. Reprinted from [66] with permission.

Nomonal conc. (ng/mL)	Inter or intra day	Gentamicin			Kanamycin			Apramycin		
		Measured conc.(ng/mL)	%RSD	%RE	Measured conc.(ng/mL)	%RSD	%RE	Measured conc.(ng/mL)	%RSD	%RE
2 (LLOQ)	intraday	1.89			16.5			10.2		
		1.93			17.5			8.39		
		1.8			19.2			10.2		
		2.01			17.1			8.2		
	interday	1.84	4.07	-5.3	17.6	5.02	-12.1	8.67	9.89	-8.68
40 (LQC)	intraday	40.3			43.5			43.1		
		40.1			40.5			41.8		
		38			42			42.8		
		41.4			39.8			38.8		
	interday	38.8	3.33	-0.7	44.1	4.63	4.95	40.7	4.38	3.6
400 (MQC)	intraday	408			376			407		
		406			371			400		
		384			386			391		
		419			396			393		
	interday	392	3.46	0.45	410	3.92	-3.05	409	2.02	0
4000 (HQC)	intraday	4140			4250			4190		
		3990			4000			4100		
		4280			4090			4090		
		4190			4100			4100		
	interday	4130	2.64	3.65	4330	3.33	3.85	4220	1.51	3.5

Abbreviations: LLOQ, lower limit of quantitation; LQC, low quality control, MQC, medium quality control; HQC, high quality control; %RSD relative standard deviation; %RE, percent relative error

3.3.5 Real Sample Analysis

Aminoglycoside compounds alone or in combination with other antibacterial compounds were used for intravenous (IV) dose of bacterial infected rats to obtain their pharmacokinetic and pharmacodynamic (PK-PD) profiles and to discover their synergetic effect against Gram-negative bacteria. The dose level was 2 mg mL^{-1} kg^{-1} compound in normal saline. Whole blood samples were collected at 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr and 24 hr and processed to plasma by centrifugation. Figure 3.10 shows the chromatogram of gentamicin plasma sample collected at 4 hr time point.

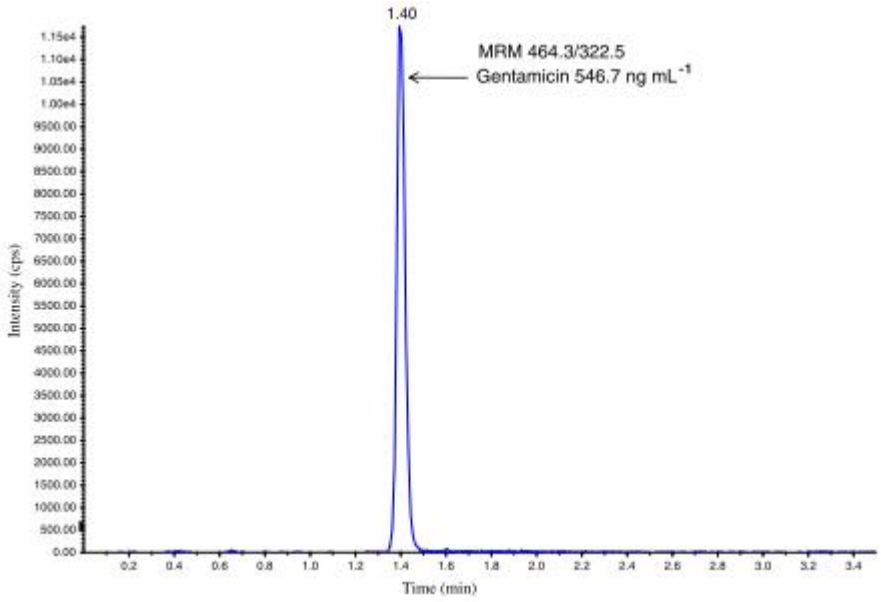


Figure 3.10 The chromatogram of gentamicin plasma sample collected at 4 h after IV dose of gentamicin at 2 mg/mL• kg. Reprinted from [66] with permission.

3.4. Conclusions

In this project, we have studied the effect of the concentration of TCA on plasma protein precipitation and sample recovery efficiency. It was found that the TCA sample crash method gives better sample recovery than the ACN sample crash method when the concentration of TCA reach 25-30% for polar small molecules. It can be concluded that the TCA sample crash method is a general sample preparation method for polar compounds such as aminoglycosides. Moreover, when TCA is used, it has the effect of increasing the retention of highly polar small molecules such as gentamicin and tobramycin as well as sharpening the elution peaks.

By studying the retention behavior of gentamicin and tobramycin on a HILIC column, the mechanism of analyte interaction with a silica surface was further understood. The charge status of the silica surface is dependent on the pH of the running buffer. The TCA concentration, on the other hand, determines the charge status of the analyte and the pH of the injected sample. The charge status of the analyte and the charge of silica surface together determine the chromatographic behavior.

An LC-MS/MS method has been developed and validated for the analysis of gentamicin, kanamycin and apramycin with tobramycin as the internal standard. The method used TCA protein precipitation, a reversed phase C-12 column and a very high aqueous content buffer to afford enough retention of gentamicin. A satisfactory LLOQ of 1 ng/mL and standard curve was obtained when injection volume is 10 μL. Compared with existing methods, our method avoided using ion pairing reagent in the

mobile phase, yet it yielded comparable or better sensitivity for the compounds studied than using various ion pairing agent in the mobile phase or using HILIC columns.

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Chapter 4: LC-MS/MS for the determination of polymyxins and vancomycin in rat plasma

4.1. Introduction

In the past 30 years, the emergence of multi-drug resistance (MDR) bacteria has created a situation in which there are few or no treatment options for infections by certain microorganisms. For example, the emerging MDR Gram-negative bacteria, *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* are resistant to all β-lactams, fluoroquinolones, and aminoglycosides [119,120]. Additionally, methicillin resistant S*taphylococcus aureus* (MRSA) has evolved into a significant pathogen among hospitalized patients around the world [121]. Lipopeptide Polymyxins (PMXs) and glycopeptide vancomycin (VCM) interact noncovalently to their target ligands, usually cell-wall or cell-membrane structures. As the noncovalent interactions are nonspecific than covalent interactions, it is more difficult for bacteria to develop resistance to these agents [122]. This mechanistic opportunity is used in developing antibacterial peptide drugs against MDR bacteria [123]. This has led to the resurgence in the use of PMX antibiotics which are active against a wide spectrum of Gram-negative bacteria despite their known nephrotoxicity [124,125]. Moreover, there exist renewed interests in the exploration of VCM and other glycopeptide modifications that are active against Gram-positive bacteria [126].

The two clinically used PMXs, PMB and PME (Structure shows in Table 4.1), are cyclic lipodecapeptides. In these peptides, the amino acid units 1-3 are linear and 4-10 form a 23-membered ring. Each molecule carries 5 free amino groups and, accordingly, 5 positive charges are present under physiological conditions [127]. The

main difference between PMB and PME is in the amino acid components. PMB is comprised mainly of PMB1 and PMB2 [128], and PME (also known as colistin), is comprised mainly of PME1 (colistin A) and PME2 (colistin B) [129,130]. The cationic molecules of PMX compete and displace Ca^{2+} and Mg^{2+} ions, and the hydrophobic segments of PMX microscopically form complexes with bacterial lipopolysaccharide, which causes local disturbance of the cell membrane, and increases cell permeability, cell lysis and death [131-134]. They display sub-micromolar minimum inhibitory concentration (MIC) values against a variety of Gram-negative bacteria [120,124,126,127,135,136].

Currently there is a lack of reliable information concerning the pharmacokinetic data for PMXs in humans [120,128]. PMXs are highly soluble in water and poorly soluble in organic solvents [137]. The unique molecular properties of PMXs present chromatographic challenges with a variety of conventional reverse phase LC columns. Since all the main components of the PMXs possess five free amino groups which tend to adsorb onto silica surface [138], severe peak tailing is observed for untreated PMX samples with LC. Therefore, either derivatization [139] or further purification are required for optimal bioanalysis. Bioanalytical methods such as capillary zone electrophoresis (CZE) [140,141], high-performance liquid chromatography (HPLC) with fluorescence detection, UV spectrophotometric detection or scanning fluorescence detection [139,142,143], and LC-MS/MS [144,145] have been used for quantitative analysis. Since CZE and LC with UV and fluorescence detection lack structure-specific selection [142], and fluorescence detection requires compound derivatization for a sensitive and specific method [102,139,142,143], LC-MS/MS is the choice for pharmaceutical industry because of

its high sensitivity and structural specificity. The reappraisal of PMXs as the only available active antibiotics for some bacteria species as well as the combined-drug synergy study of PMXs with other antibacterial compounds [120] demand a simple and accurate analytical method with adequate dynamic range and sensitivity for the determination of PMXs in biological samples.

Recently, LC-MS/MS methods have been developed for quantification of PME (colistin) in milk and animal tissues [145,146]. The methods required the use of strong and highly concentrated acids for sample recovery followed by laborious sample clean-up, preconcentration, and long separation time. LC-MS/MS methods have also been reported for the analysis of PME in human plasma and urine [144,147]. These methods are unsatisfactory since they require a long and expensive procedure of SPE, consumption and injection of a large volume of samples (100-200 μL), and long separation time with poor chromatography. The reported sensitivity for PME in any matrices ranges from 30-300 ng/mL (g) per 10 μL injection.

Another class of antibiotic peptide drugs is glycopeptide antibiotics. This class is composed of glycosylated cyclic or polycyclic nonribosomal peptides, neutral sugars and an amino sugar. The peptides consist of cross-linked unusual aromatic amino acids and conventional amino acids such as aspartic acid [148]. Significant glycopeptide antibiotics include VCM, dalbavancin, teicoplanin, telavancin, bleomycin, ramoplanin, and decaplanin [149,150]. They are soluble in aqueous solvent but not in nonpolar organic solvents. This class of drugs inhibits the synthesis of cell walls in susceptible microbes by inhibiting peptidoglycan synthesis. They bind

to the amino acids within the cell wall, preventing the addition of new units to the peptidoglycan.

VCM is a benchmark compound for various preclinical pharmacology models treating endocarditis [151,152]. However, the unique molecular properties of VCM presented similar bioanalytical challenges as PMXs. Current LC-MS methods include using strong cation exchange SPE for sample preparation from serum followed by LC-full scan Fourier transform MS [153], online sample extraction followed by column switching technique [154], and an offline sample extraction technique using TFA and methanol [155]. The LLOQ obtained ranged from 1 to 10 ng/mL. The above methods require complicated extraction procedure, large injection volume and long separation time. Moreover, the previous studies didn't apply sample recovery optimization.

The two classes of antibiotics, lipopeptide PMXs and glycopeptide VCM, actually have similarities. They are both peptide drugs of similar molecular weight range; the sizes of the peptide parts are dominant in either the lipopeptide molecules or in the glycopeptide molecule. The purpose of the study is to develop and validate a general bioanalytical method based on the same principle for the above antibacterial peptide compounds.

4.2. Experimental

4.2.1 Chemicals and reagents

All solvents used were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (88%) was supplied by J. T. Baker (Phillipsberg,

NJ, USA). TCA (99+%) was purchased from Acros (Morris Plains, NJ, USA). Control Rat Plasma in EDTA K2 (Individual MALE 031-APEK2-MI) was purchased from Bichemed (Wichester, MA, USA). PMB (polymyxin B sulfate), PME (colistin methanesulfonate), VCM and dalbavancin were obtained from Pfizer Global Research & Development (Groton, CT, USA). [Glu^1]-Fibrinopeptide B human (⩾97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2.2 Equipment

A standard multitube vortex-mixer from VWR Scientific Products (West Chester, PA, USA) was used for vortex-mixing, and an Eppendorf centrifuge model 5810R from Brinkmann Instruments Inc. (Westbury, NY, USA) was used for centrifugation. An Applied Biosystems/MDS Sciex (Concord, ON, Canada) model API 4000 triple quadrupole mass spectrometer equipped with a Shimadzu LC-10AD Prominence solvent delivery system, degasser and SCL-10 Avp system controller (Columnbia, MD, USA) was used for LC-MS/MS analysis. A Leap Technologies CTC PAL autosampler with Shimadzu 10AD pump was used. A Harvard Apparatus (South Natick, MA, USA) syringe pump with a 500 μL syringe from Hamilton Co. (Reno, NE, USA) was employed for compound infusion.

4.2.3 Sample preparation using TCA or acetonitrile (ACN) induced plasma protein precipitation

For TCA induced plasma precipitation, TCA was diluted in water to obtain 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30% and 35% concentration (*w/v*). To 50 μL plasma samples, 30 μL TCA at various concentrations were added; white protein precipitation was observed; then 170 μL water was added. Samples were centrifuged

at 4000 rpm for 5 minutes, and 50 μL of the supernatants were aliquoted into a 1.2 mL polypropylene 96-well plates for sample analysis. For ACN induced plasma precipitation, to 50 μL plasma samples, 25-200 μL ACN (at various ACN/water ratios) were added to 50 μL of plasma samples, and protein precipitation was observed. 50 μL of the supernatants were aliquoted and reconstituted in 10% ACN for sample analysis. Analyte recovery was calculated by peak areas count ratios of samples recovered from plasma and samples from water solutions at correspondent TCA concentrations. Samples at each concentration level were analyzed in triplicate over three independent batch runs.

4.2.4 Preparation of calibration standards

Stock solutions of PMB (containing PMB1 and PMB2), PME (containing PME1 and PME2), Fibrinopeptide B, VCM and dalbavancin were prepared as 1 mg/mL concentration in water with their purity factors considered. Fibrinopeptide B and dalbavancin were further diluted to 500 ng/mL for use as internal standards for PMXs and VCM, respectively. Stock solutions were serially diluted with rat plasma or water. Analytical standards used to construct calibration curves were prepared separately for each type of extraction method. The stock solutions of the compounds were prepared in water and the stock standard solutions were carried out by serial dilutions of the stock solutions to desired concentrations. Plasma and neat solvent working standards were prepared by spiking known quantities of the stock standard solutions to the blank rat plasma and water, respectively. The final concentrations for PMXs working standards are: 5, 10, 25, 50, 100, 250, 500, 1000, 2500, 5000 ng/mL; for VCM: 1, 2, 5, 10, 25, 50,100, 250, 500, 1000, 2500, 5000 ng/mL. Accuracy (% RE) and precision (% CV) of the assay were assessed by analyzing quality control

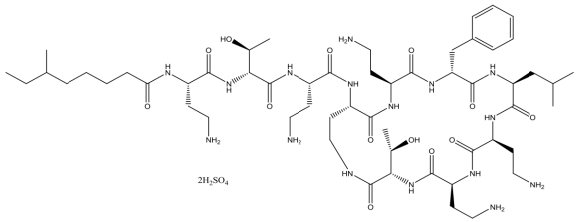
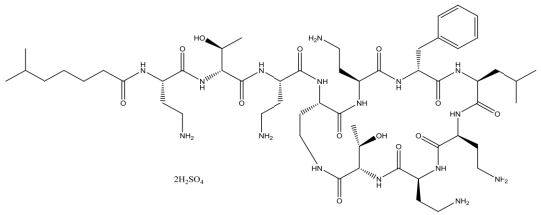
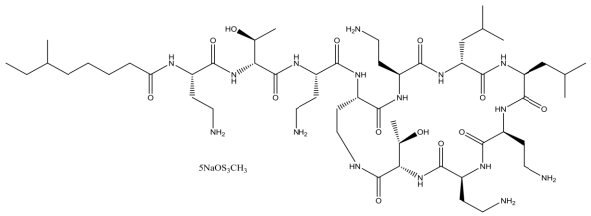
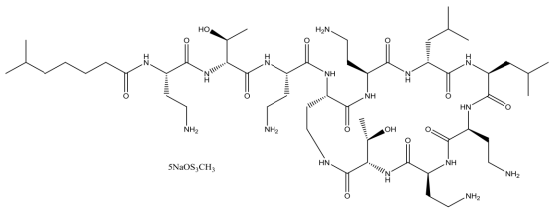
samples of 19.5, 156, 1250 ng/mL for PMB1 and PME1 and 39.1, 313, 2500 ng/mL concentration for PMB2, PME2 and VCM. Quality control samples were prepared identically to the analytical standards.

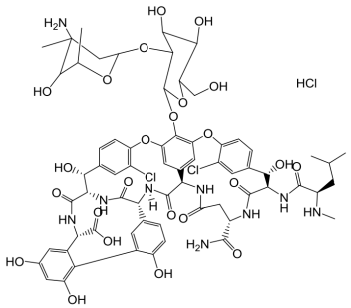
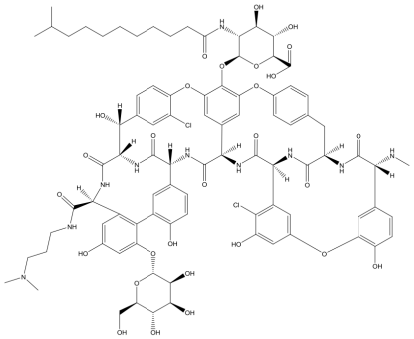
4.2.5 LC-MS/MS analysis

A Phenomenex Jupiter C18 5μ 300 Å 50x2 mm column (Torrance, CA, USA) was used for the analysis. Gradient chromatography was performed with 0.1% formic acid in 100% water (A) and 0.1% formic acid in 100% ACN (B) at a flow rate of 0.25 mL/min. The gradient used was 0-0.5 min, 5% B; 1.5 min, 70% B; 2.5 min, 90% B; 3.0-3.5 min, 5% B; 3.6 min, stop. The injection volume was 10 μL. To test the impact of the silica pore size of the column with VCM, isocratic elution was applied at 6% B with 0.1 % acetic acid. The column was equilibrated for 1 min before each run.

Positive ion electrospray tandem mass spectra were recorded using an AB Sciex API 4000 triple quadrupole mass spectrometer with multiple reaction monitoring (MRM) detection mode controlled by Analyst (version 1.41) operating software. The ionspray voltage was set to 5000 V, and the probe temperature was set at 500 ℃. Nitrogen was used as the collision gas. And the nebulizer (GS1), curtain, and turbo gas (GS2) were set to 40, 10, and 50 psi, respectively. MRM parameters of test compounds were set as described in Table 4.1. Dwell times were set to 200 ms for each transition.

Table 4.1 Compound structures and MRM parameters for MS analysis. Reprinted from [67] with permission.

Commercial Name	Chemical Structure	FW	FW of Salt	Salt Factor	purity %	Q1	Q3	DP	CE	CXP	EP
Polymyxin B1		1203.5	1399.6	1.16	95	602.5	241.2	70	34	10	10
Polymyxin B2		1189.5	1385.6	1.16	95	595.6	227.5	70	38	10	10
Polymyxin E1		1169.5	1759.9	1.50	95	578.5	227.2	70	36	10	10
Polymyxin E2		1155.4	1745.9	1.51	95	585.6	241.3	70	34	10	10

Vancomycin		1449.3	1485.7	1.03	90%	725.5	144.0	70	46	10	10
Dalbavancin		1816.7	1816.7	1.00	100%	909.5	340.6	110	40	10	10
[Glu1]-Fibrinopeptide B human	Glu-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg	1570.6	1570.6	1.00	90%	786.3	187.3	70	52	10	10

Abbreviations: Q1, molecular ion; Q3, daughter ion; DP, declustering potential (V); CE, collision energy (eV); EP, entrance potential; (V) CXP, collision cell exit potential (V)

4.3. Result and discussion

4.3.1 TCA Induced Plasma Protein Precipitation

Plasma protein precipitation with organic solvents is commonly used for analyte recovery. However, because of the very low solubility of the peptide compounds in organic solvents, their analyte recoveries were less than 20% at various ACN/water volume ratios (0.5:1, 1:1, 2:1, 4:1). In order to obtain better recoveries for high throughput liquid-liquid extraction methods, chlorine-containing acid induced protein precipitation had been used. In our research, we choose TCA over HCl or $HClO_4$ since it was studied that protein precipitation is not dictated by pH but is strongly dependent on the trichloro group [112]. No precipitation was observed at 0.1% and 1% TCA concentration, but the protein was partly changed to a molten globule state. Protein precipitation initiated at 5% TCA, and reached a maximum at about 30% TCA. Figure 4.1 shows analyte recovery for PMB, PME, Fibrinopeptide B and VCM when various TCA concentrations were used for plasma protein precipitation. The data shows that analyte recoveries increased as TCA concentration increased for all the peptides. Since VCM is less polar than PMX, its recovery plateaued about 15 to 35% TCA. For PMX the analyte recoveries surpassed 100% when TCA concentration was higher than 20%, which indicated that adding TCA not only helped protein precipitation but also helped to increase the mass spectrometric response of PMX. It was determined 30% TCA was the concentration to optimize analyte recoveries.

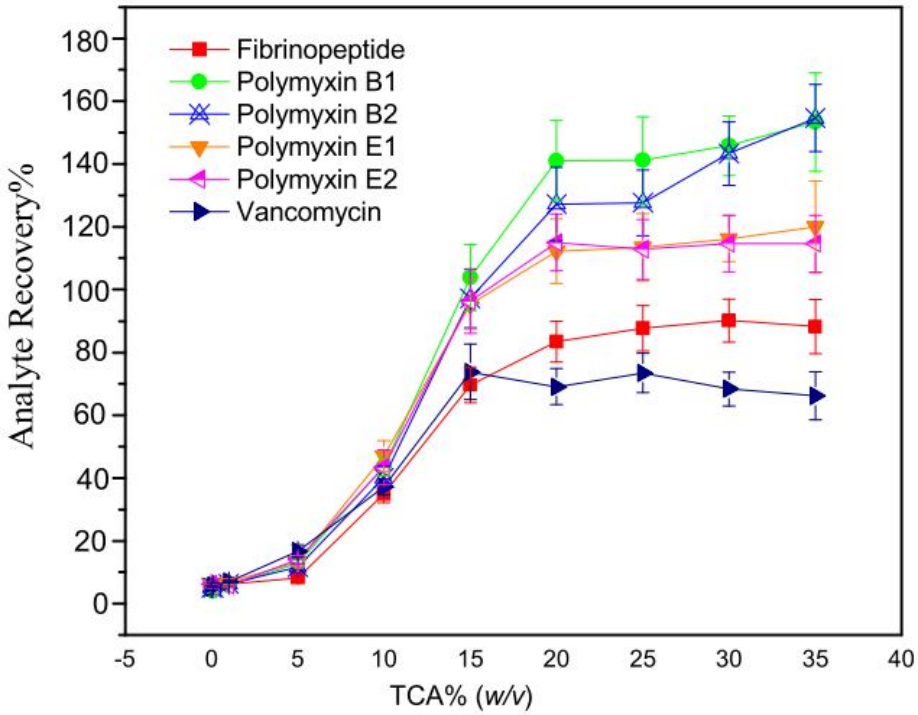


Figure 4.1 Analyte recovery versus TCA concentration for peptide molecules. Reprinted from [67] with permission.

4.3.2 Chromatographic Conditions Optimization

One important aspect of liquid chromatography separation involves matching the pore size of the packed silica with the size of the analyte molecules. Several columns with different pore sizes had been tested with VCM as shown in Table 2. It was found that amongst important column retention parameters such as carbon load, surface area, coverage, and pore size, increasing pore size can improve peptide retention while holding other parameters constant. The molecular weights (MW) of the antibacterial peptide compounds are greater than 1000 Da and the Phenomenex Jupiter C18 5µ 300 Å 50x2 mm column was selected for LC-MS/MS analysis. PMX molecules were also tested, and the Phonomenex column was found to offer the best performance.

Table 4.2 Column parameters versus retention times for VCM. Reprinted from [67] with permission.

Column	Retention Time (min)	Surface Area (m^2/g)	Carbon Load (%)	Bonded Phase Coverage (µmol/m^2)	Bonded Phase Coverage (µmol/g)	Pore Size (Å)
Phenomenex Jupiter 5µ C18 50x2 mm	3.0	170	13.3	5.50	935	300
Varian Intersil 5µ ODS 3 100x2 mm	2.6	320	15.0	3.23	1034	100
Phenomenex Lunar C(18)2 5µ C18 50x2 mm	1.6	400	13.5	5.50	2200	100
Varian MetaSil AQ 3µ C18 50x2 mm	1.1	220	12.0	2.52	554	80

For PMX, the chromatography was optimized by employing a gradient elution that started at a very low ACN percentage (5%) where it was held for 0.5 min to allow the analyte to achieve good retention. After 1.5 min, ACN percentage was increased to 90%, as high organic content helped the analyte achieve better ionization efficiency. Figure 4.2 shows the effects of TCA concentration on retention of PMB1, PME1 and VCM (the data for PMB2 and PME2 were very similar to PMB1 and PME1 and are not shown) in both neat (water) solution [Figure 4.2(a)] and in rat plasma [Figure 4.2(b)]. In both matrices, increasing the TCA percentage (only added in the samples) from 5% to 20% quickly increased the retention time of the PMXs and VCM until the retention time remained steady after 20% TCA. The increase of the retention time is derived from the ion-pairing effect of TCA. When TCA was added to the samples during sample preparation, TCA formed ion pairs with the polar molecules, increased their hydrophobicity, changed their charge status, and changed the interaction between the analyte and the column surface. When the percentage of

TCA was greater than 20%, a single, sharper peak was observed for both PMB1 and PMB2 compared to not adding TCA, indicating the ion pairing effect had reached its maximum. It was also seen that the buffering capacity of the plasma supernatant had reduced the retention differences between PMX and VCM.

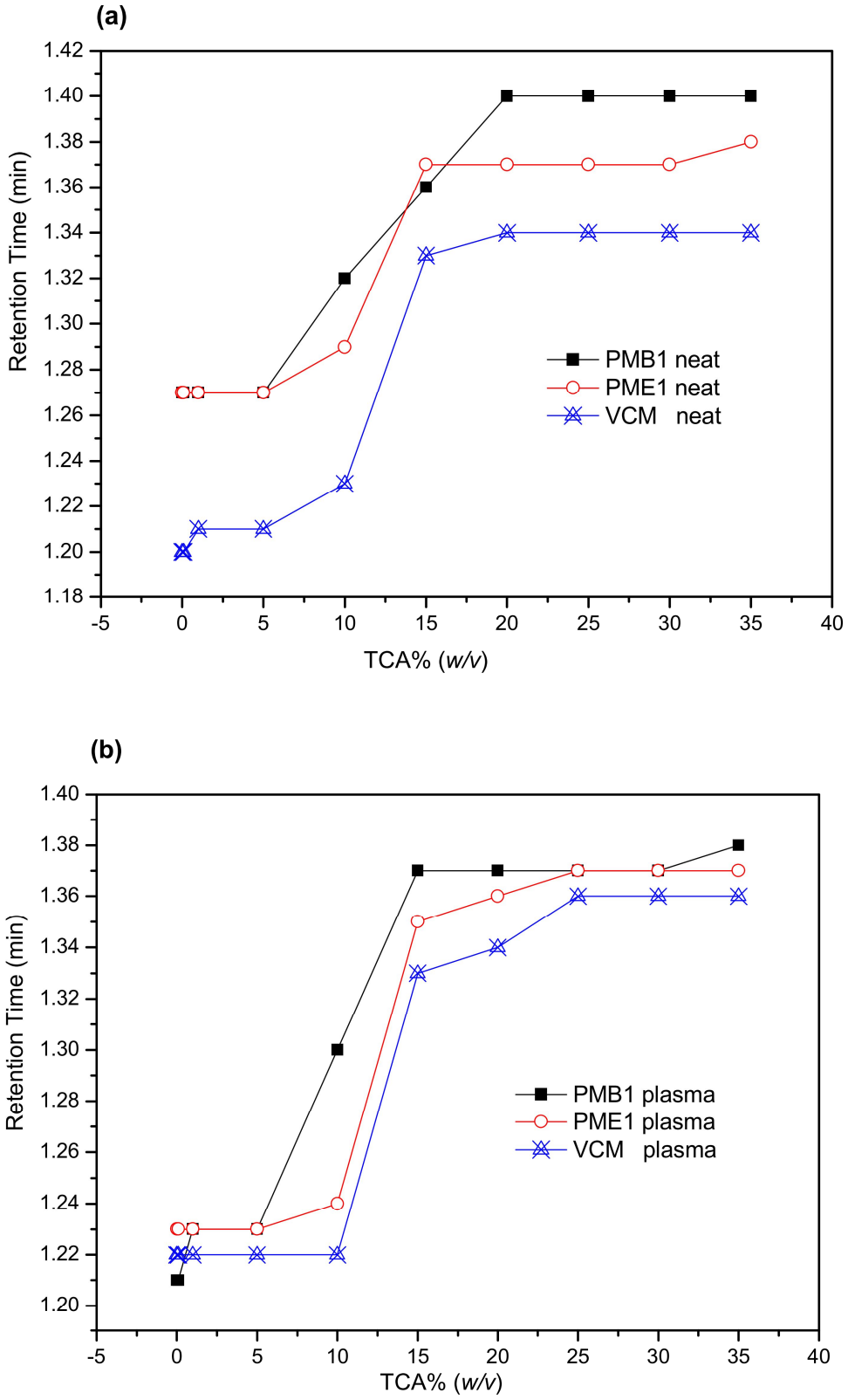


Figure 4.2 The effect of TCA concentration on retention of PMB1, PME1 and VCM tested with gradient elution in (a) neat solution; (b) rat plasma. Reprinted from [67] with permission.

To determine the ratios of PMB1 to PMB2 and PME1 to PME2, it was assumed that analyte pairs have the same response factor for MRM detection. This is reasonable since the molecules differ by a single CH_2 group [144,145,156]. As purified PMB1, PMB2, PME1 and PME2 are unavailable and their compositions differ between manufacturers and batches, the percentage of the components were determined by their peak area ratios with respect to the total peak area. The percentage of PMB1 and PMB2 was found to be 78.0 ± 0.8% and 17.0 ± 0.8%. The percentage of PME1 and PME2 was found to be 71.0 ± 1.1% and 24.0 ± 1.1%.

4.3.3 Quantitation

The calibration curves for PMXs were generated from MRM analysis of five replicate rat plasma samples at the calibration standard concentration level covering the range of 5-5000 ng/mL, with Fibrinopeptide B as the internal standard at 500 ng/mL and an injection volume of 10 µL. VCM was prepared in the same manner as PMXs except the concentration range was 1-5000 ng/mL, with dalbavancin as the internal standard. Good responses over the concentration ranges were obtained. Calibration curve regression was weighted as 1/x and analyzed using linear fit of quantities versus peak area ratios. Precision and accuracy data are shown in Table 3. The standard calibration curves were linear over the concentration range with a correlation coefficient better than 0.9989. The method validation yielded precision results of: 4.3-7.4, 2.3-9.2, 5.1-10.8, 3.8-9.4, and 7.8-10.3% and accuracy results of: 91.7-104.2, 91.7-105.1, 94.9-104.8, 94.3-107.4%, and 96.2-102.0% for PMB1, PMB2, PME1, PME2, and VCM, respectively. The LLOQs corresponding to a coefficient of variation less than 20% were 7.5, 18.1, 7.3, 5.0 and 1.0 ng/mL for

PMB1, PMB2, PME1, PME2 and VCM, respectively. Since TCA was only added to the samples but not to the mobile phases, the ion suppression which would cause the non-linear standard curves was not observed. The data demonstrated that good accuracy and precision of this assay was developed for rat plasma samples. This is a significant improvement over assays reported in the literature in terms of sensitivity, simplicity and understanding of the chromatography challenges for both PMXs [145,146] and VCM [153]. For PMXs the reported methods require a long and expensive procedure of SPE, consumption and injection of a large volume of samples (100-200 μL), and long separation time (7-16 mins) with poor chromatography (peak tailing and peak fronting) and low sensitivity (For PME in any matrices ranges from 30-300 ng/mL (g) per 10 μL injection).Similarly for VCM our method avoided complicated extraction procedure, large injection volume, or long separation time, yet it achieved better or similar sensitivity than the literature reports (1-10 ng/mL).

Table 4.3 Precision and accuracy data for PMXs and VCM. Reprinted from [67] with permission.

Polymyxcin B1					Polymyxcin B2				
Nomonal Conc. (ng/mL)	Inter or Intraday	Measured Conc. (ng/mL)	%RSD	%RE	Nomonal Conc. (ng/mL)	Inter or Intraday	Measured Conc. (ng/mL)	%RSD	%RE
7.5 (LLOQ)	Intraday	7.47			18.1 (LLOQ)	Intraday	21.5		
	Intraday	7.62				Intraday	18.5		
	Intraday	7.23				Intraday	17.1		
	Interday	8.05				Interday	19.8		
	Interday	8.44	6.4%	103.5%		Interday	18.2	9.2%	105.1%
19.5 (LQC)	Intraday	17.0			39.1 (LQC)	Intraday	38.2		
	Intraday	17.5				Intraday	39.8		
	Intraday	19.0				Intraday	35.2		
	Interday	18.2				Interday	34.7		
	Interday	20.7	7.4%	94.8%		Interday	41.5	7.5%	96.9%
156 (MQC)	Intraday	135			313 (MQC)	Intraday	295.9		
	Intraday	143				Intraday	279.6		
	Intraday	150				Intraday	293.6		
	Interday	137				Interday	281.9		
	Interday	150	4.5%	91.7%		Interday	284.3	2.3%	91.7%
1250 (HQC)	Intraday	1300			2500 (HQC)	Intraday	2469.8		
	Intraday	1250				Intraday	2376.6		
	Intraday	1370				Intraday	2399.9		
	Interday	1250				Interday	2423.2		
	Interday	1340	4.3%	104.2%		Interday	2353.3	3.6%	96.2%

Polymyxcin E1					Polymyxcin E2				
Nomonal Conc. (ng/mL)	Inter or Intraday	Measured Conc. (ng/mL)	%RSD	%RE	Nomonal Conc. (ng/mL)	Inter or Intraday	Measured Conc. (ng/mL)	%RSD	%RE
7.3 (LLOQ)	Intraday	6.91			5.0 (LLOQ)	Intraday	5.79		
	Intraday	7.55				Intraday	5.72		
	Intraday	6.87				Intraday	4.67		
	Interday	8.43				Interday	5.11		
	Interday	8.50	10.8%	104.8%		Interday	5.56	9.4%	107.4%
19.5(LQC)	Intraday	19.1			39.1 (LQC)	Intraday	44.4		
	Intraday	20.0				Intraday	43.1		
	Intraday	17.9				Intraday	36.8		
	Interday	20.3				Interday	42.3		
	Interday	18.6	5.1%	98.4%		Interday	40.0	7.7%	105.7%
156 (MQC)	Intraday	159			313 (MQC)	Intraday	294		
	Intraday	151				Intraday	290		
	Intraday	149				Intraday	281		
	Interday	135				Interday	313		
	Interday	146	5.6%	94.9%		Interday	298	3.8%	94.3%
1250 (HQC)	Intraday	1280			2500 (HQC)	Intraday	2450		
	Intraday	1240				Intraday	2520		
	Intraday	1230				Intraday	2460		
	Interday	1230				Interday	2560		
	Interday	1390	5.4%	101.9%		Interday	2430	4.3%	99.4%

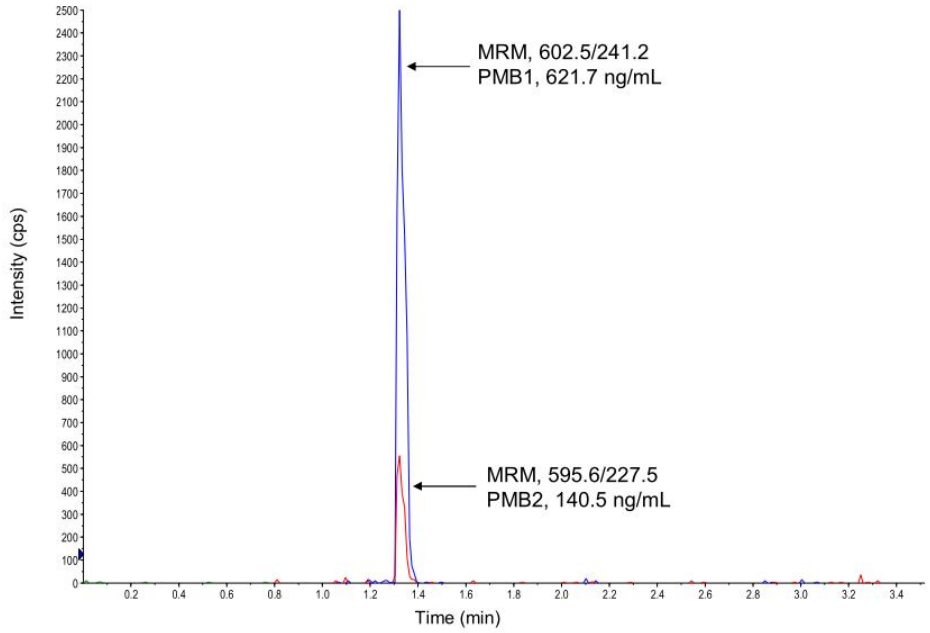
Vancomycin				
Nominal Conc. (ng/mL)	Inter or Intraday	Measured Conc. (ng/mL)	%RSD	%RE
1.0(LLOQ)	Intraday	0.909		
	Intraday	1.02		
	Intraday	1.04		
	Interday	0.900		
	Interday	1.08	8.1%	99.0%
39.1(LQC)	Intraday	35.0		
	Intraday	42.7		
	Intraday	36.1		
	Interday	35.5		
	Interday	38.9	8.2%	96.2%
313 (MQC)	Intraday	270		
	Intraday	356		
	Intraday	323		
	Interday	329		
	Interday	302	10.3%	101.0%
2500(HQC)	Intraday	2530		
	Intraday	2600		
	Intraday	2590		
	Interday	2640		
	Interday	2390	7.8%	102.0%

Abbreviations: LLOQ, lower limit of quantitation; LQC, low quality control, MQC, medium quality control; HQC, high quality control; %RSD relative standard deviation; %RE, percent relative error

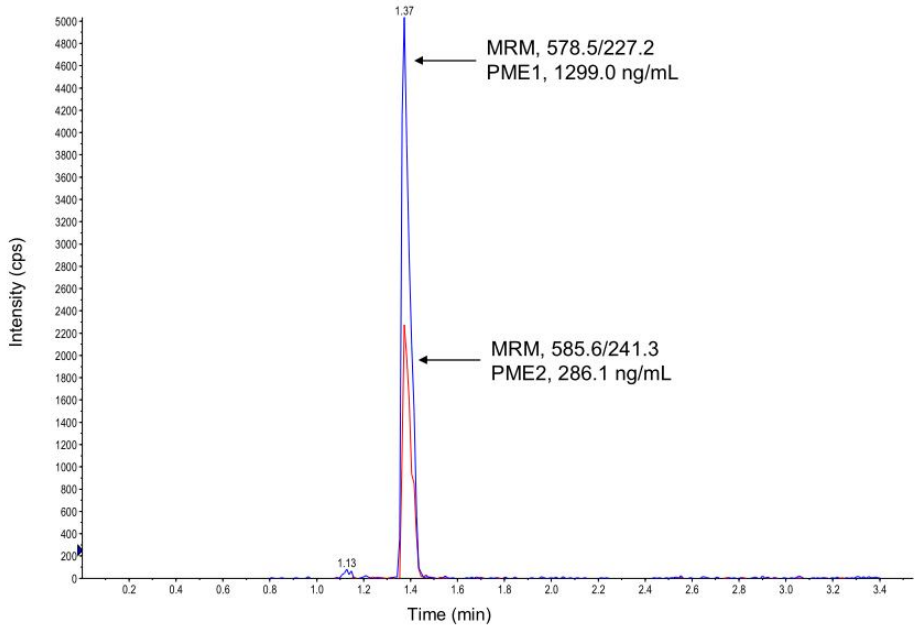
4.3.4 Real Sample Analysis

PMB and PME were administrated individually though intravenous (IV) route into the tail vein of Sprague-Dawley (SD) rats to obtain their pharmacokinetic profiles. The dosed amounts were 0.2, 0.4 and 2 mg/kg of compound in sterile saline. VCM were administrated individually via subcutaneous (SC) route into bacterially infected Sprague-Dawley (SD) rats in order to obtain the pharmacokinetic and pharmacodynamic (PK-PD) profiles. The dose amounts were 20, 60, and 200 mg/kg in sterile saline. Whole blood samples were collected at 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr and 24 hr and processed by centrifugation to obtain plasma. Figure 4.3(a) shows the chromatograms for PMB1 and PMB2, Figure 4.3(b) for PME1 and PME2, and Figure 4.3(c) for VCM plasma sample collected at the 4 hr time point when the dose amounts for PMB and PME were 2 mg/kg and for VCM was 200 mg/kg.

(a)


MRM, 602.5/241.2
PMB1, 621.7 ng/mL
MRM, 595.6/227.5
PMB2, 140.5 ng/mL
Intensity (cps)
Time (min)

(b)


1.37
MRM, 578.5/227.2
PME1, 1299.0 ng/mL
MRM, 585.6/241.3
PME2, 286.1 ng/mL
1.13
Intensity (cps)
Time (min)

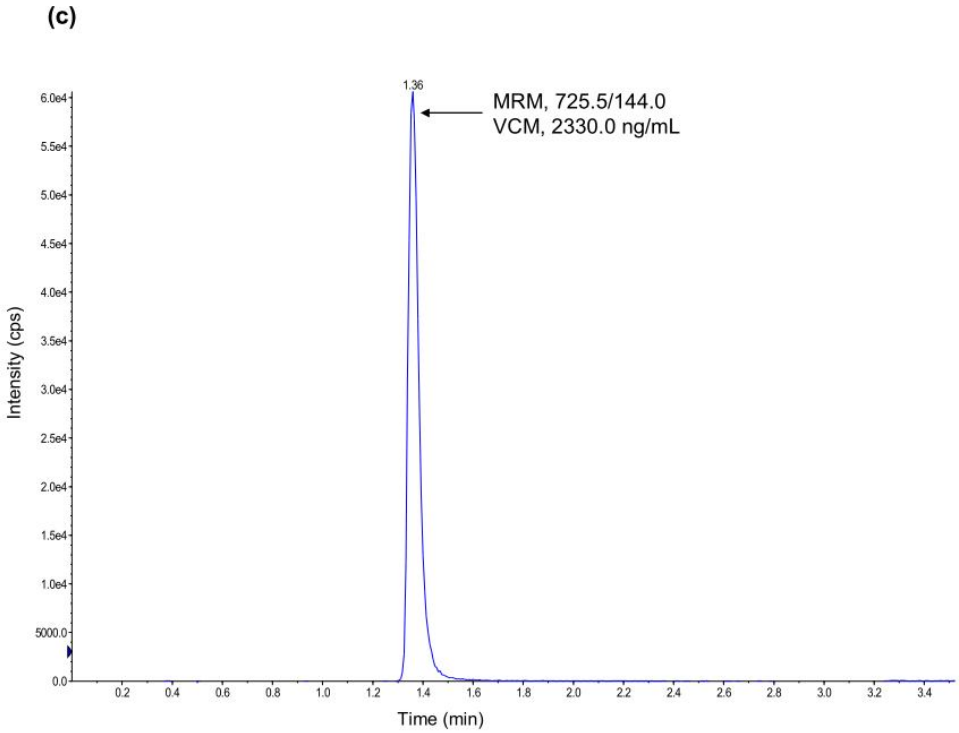


Figure 4.3 The chromatograms of peptide drugs rat plasma sample collected at 4 hr (a) PMB1 and PMB2, after IV dose of PMB at 2 mg/kg; (b) PME1 and PME2, after IV dose of PME at 2 mg/kg; (c) VCM, after SC dose of VCM at 200 mg/k. Reprinted from [67] with permission.

4.4. Conclusions

In the present work, we have studied the effect of the concentration of TCA on plasma protein precipitation and sample recovery efficiency for antibacterial peptide compounds. It was found that the TCA sample precipitation method gave better sample recovery than the ACN sample precipitation method when the concentration of TCA reached 25-30% for

these polar peptide molecules. It can be concluded that the TCA sample precipitation method is a general sample preparation method for hydrophilic peptide compound with MW less than 2000 Da. Moreover, when TCA is used, it has the effect of increasing the retention of the peptide molecules as well as sharpening the elution peaks. LC-MS/MS methods have been developed and validated for the analysis of PMB and PME with Fibrinopeptide B as the internal standard and VCM with dalbavancin as the internal standard. The method used TCA protein precipitation, a reversed phase C-18 column with pore size of 300 Å, and a very high aqueous content buffer to afford acceptable retention. Satisfactory LLOQs of 7.5, 18.1, 7.3, 5.0 and 1.0 ng/mL for PMB1, PMB2, PME1, PME2 and VCM, respectively, were obtained using an injection volume of 10 μL. Compared with existing methods, the method detailed in this paper avoided using ion pairing reagents in the mobile phase, derivatization, SPE, organic solvent extraction and long separation time, yet it yielded similar or better sensitivity for the compounds studied.

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Chapter 5: Development and validation of an efficient liquid chromatography-tandem mass spectrometry method for determination of fifteen estrogens and metabolites in human serum

5.1 Introduction

Endogenous female hormones are important indicators in human physiology and pathology. Determination of estrogens and metabolites is one of the most critical steps in human physiological and pathological diagnosis, especially in risk assessment of certain cancers. Bioanalytical method development and validation plays an essential role in analyzing female hormones, e.g. estrogens and metabolites in human blood, urine and tissues, because it is very challenge to determine endogenous estrogens and metabolites accurately at extremely low levels, e.g. pg/mL or pmol/L. Varieties of bioanalytical techniques or methodologies have been developed and applied for analyzing estrogens and metabolites, such as radioimmunoassay, LC-MS/MS, GC-MS/MS [157-160], and liquid chromatography with electrochemical detection (HPLC/ECD) [161]. The method specificity and sensitivity are the major advantages of LC-MS/MS and GC-MS/MS over radioimmunoassay and HPLC/ECD.

A large number of LC-MS, GC-MS, LC-MS/MS and GC-MS/MS methods for determination estrogens and metabolites have been published in the past. The bioanalytical methods developed in recent years focused more on LC-MS/MS and GC-MS/MS techniques, because the earlier studies demonstrated that LC-MS and GC-MS were significantly less sensitive in analyzing estrogens and metabolites than LC-MS/MS and GC-MS/MS [159,162]. It was obvious that those LC-MS/MS methods directly analyzing biological samples containing estrogens and metabolites were simple and straightforward [163-167]. However, a number of studies demonstrated that the LC-MS/MS methods directly analyzing estrogens and metabolites were significantly less sensitive than those methods analyzing chemically derivatized estrogens and metabolites [157,168-170], because the neutral molecules of estrogens and metabolites might not be effectively ionized under electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) modes. Therefore, chemical derivatization became an important sample preparation procedure for estrogens and metabolites before LC-MS/MS analysis.

An ideal derivatization reagent should react with estrogens and metabolites selectively and quantitatively under mild conditions within a

short time, and those estrogen derivatives should be stable and easily ionized during LC-MS/MS analysis. There were mainly five classes of reagents used for derivatizating estrogens and metabolites, including: 1) sulfonyl cholride, e.g. dansyl chloride, 1,2-dimethylimidazole-4-chloride and pyridine-3-sulfonyl chloride; 4-(1-H-pyrazol-1-yl)benzenesulfonyl chloride [171]; 2) carbonyl chloride or carboxylic acid N-hydroxysuccinimide ester, e.g. picolinoyl chloride [172] and N-methyl-nicotinic acid N-hydroxysuccinimide ester [170]; 3) benzyl bromide, e.g. pentafluorobenzyl bromide [169,173] and 4-nitrobenzyl bromide [174]; 4) fluorobenzene or fluoropyridine, e.g. 2,4-dinitro-5-fluorobenzene analogues [168] and 2-fluoro-1-methyl-2-pyridinium p-toluensulfonate [43] ; and 5) hydrazide, e.g. (Carboxymethyl)trimethylammonium chloride hydrazide (Girard T reagent) [157,175], and p-tolune sulfonhydrazide [176].

In contrast to the sulfonyl chloride, carbonyl chloride, benzyl bromide and fluorobenzene reagents, the hydrazide reagents reacted only with ketolic estrogens and metabolites. They seemed suitable for certain estrogens, but not for determining all the estrogens and metabolites at the same time, because those alcoholic estrogens and metabolites, e.g. estradiol and estriol, were excluded from the related analytical methods

[157,175,176]. Pentafluorobenzyl bromide estrogen derivatives were sensitive to both ESI^+ [173] and $APCI^-$ [43,169] modes, and these derivatives had lower limits of quantitation (LOQ) values under $APCI^-$ mode than the LOQ values of derivatives of dansyl chloride and 2-fluoro-1-methyl-pyridinium p-toluenesulfonate under ESI^+ mode, because there were less interferences from analogue compounds and the matrix background under $APCI^-$ mode. Nevertheless, the dervatization reaction of estrogens with pentafluorobenzyl bromide was ten times longer than the dervatization reaction with dansyl chloride (30 min vs. 3 min at 60 ºC) [43]. A study by Higashi *et al.* indicted that the derivatization reaction of estrogens with 4-nitrobenzene sulfonyl chloride was the most complete and quantitative in comparison to those reactions with 4-nitrobenzoyl chloride, 4-nitrobenzyl bromide, 2,4-dinitro-fluorobenzene. In addition, the reaction with 4-nitrobenzoyl chloride was lack of selectivity, because it could react with both phenolic and alcoholic hydroxyl groups of estrogens at the same time, whereas 4-nitrobenzyl bromide, 2,4-dinitro-fluorobenzene and 4-nitrobenzene sulfonyl chloride reacted with phenolic hydroxyl group only [174]. These results implied that a sulfonyl chloride was a preferred reagent for derivatizing estrogens and metabolites, due to its reaction completeness and selectivity. Further, a sulfonyl chloride reagent containing

a basic or preionzed nitrogen atom, e.g. on dansyl molecule or on a pyridine, imidazole, pyrazole or piperizine ring, could significantly enhance the ionization of estrogen derivatives under ESI^+ mode, and increase the detection sensitivity [157,168,171].

Dansyl chloride was a typical sulfonyl chloride reagent used for derivatizing estrogens and metabolites from varieties of matrix, such as river water [43], charcoal-stripped fetal bovine serum [171], mouse plasma and brain [177], human urine [178,179], breast tissue [180], and serum [181]. Since most of the endogenous estrogens and metabolites exist as glucuronide and sulfate conjugates, and these conjugates should be hydrolyzed by β-glucuronidase and sulfatase before derivatization. Xu and colleagues published a number of LC-MS/MS methods for determination of fifteen dansylated unconjugated estrogens and metabolites in urine and serum [8,179-181]. However, these methods had a very long elution time, 100 minutes, which significantly affected the method throughput. In addition, even the 75 min gradient for the Phenomenex Synergy Hydro-RP 4 μm column (150 x 2.0 mm) was insufficient to separate all the fifteen dansylated estrogens and metabolites.

In this study, we developed a method providing a better separation with a significantly shorter elution time. The method eluted a Phenomenex Synergi Hydro-RP 2.5 μm column (100 x 2.0 mm) at a higher temperature with the mobile phases consisting of acetonitrile, methanol, water and formic acid at a faster flow rate. We also attempted to optimize the dansyl derivatization procedures and the detection sensitivity at pg/mL level in human serum. The method was validated using the optimized LC-MS/MS parameters.

5.2 Experimental

5.2.1 Reagents

Dichloromethane and formic acid were obtained from EMD Chemical Inc. (Gibbstown, NJ, USA). Methanol and acetonitrile were obtained from Pharmco (Brookfield, CT, USA). Dansyl chloride (reagent grade) and β-Glucuronidase/sulfatase from *Helix pomatia* (Type H-2) were purchased from Sigma Chemical co. (St. Louis, MO, USA). Sodium bicarbonate, glacial acetic acid and L-ascorbic acid were purchased from J. T. Baker (Phillipsburg, NJ, USA). Sodium hydroxide and sodium acetate were purchased from Fisher Scientific (Fairlawn, NJ, USA). Delipidized

double charcoal stripped human serum was purchased from Golden West Biologicals (Temecula, CA, USA). Fifteen estrogens and metabolites (see Figure 5.1), including estrone (E_1), estradiol (E_2), estriol (E_3), 16-epiestriol (16-epiE_3), 17-epiestriol (17-epiE_3), 16-ketoestradiol (16-ketoE_2), 16α-hydroxyestrone (16α-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 4-methoxyestrone (4-$MeOE_1$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$),

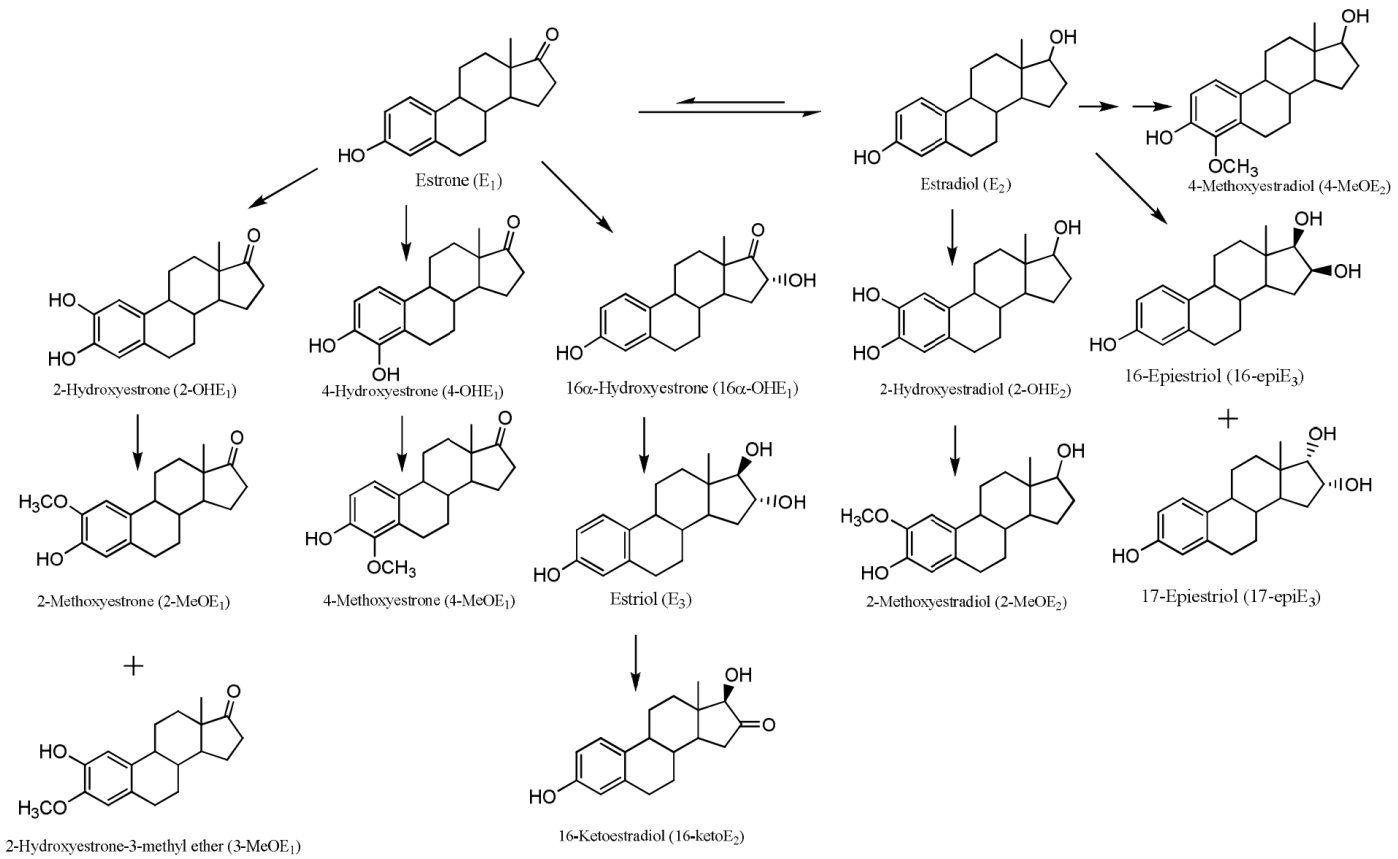


Figure 5.1 Structures of endogenous estrogen metabolism in humans.

2-methoxyestradiol (2-$MeOE_2$), 4-methoxyestradiol (4-$MeOE_2$), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1) and 2-hydroxyestradiol (2-OHE_2), were purchased from Steraloids, Inc. (Newport, RI, USA). Deuterium-labeled estrogens and metabolites, including estradiol-2,4,16,16-d_4 (d_4-E_2), estriol-2,4,17-d_3 (d_3-E_3), 2-hydroxyestradiol-1,4,16,16,17-d5 (d_5-2-OHE_2) and 2-methoxyestradiol-1,4,16,16,17-d_5 (d_5-2-$MeOE_2$), were obtained from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). The estrogens, metabolites and the deuterium-labeled analytical standards were ≥98% pure.

5.2.2 Instruments

A vortex-mixer (Model: 37600) and a dri-bath (Model: DB-16525) from Thermolyne Corporation (Dubuque, IW, USA) were used for vortex-mixing and heating of the derivatization reaction. A zymark turbovap LV evaporator (Model: ZW700) from Sotax Corp (Horsham, PA, USA) was used for evaporating the solvents from the samples. The LC-MS/MS system consisted of a Shimadzu SIL HTc auto sampler, two Shimadzu LC-10AD VP series pumps, a degasser, a SCL-10 Avp system controller, a CTO-10AS column oven (Columnbia, MD, USA), and an Applied Biosystems/Sciex (Concord, ON, Canada) model API 5000 triple quadrupole mass

spectrometer controlled by Analyst software. A Harvard Apparatus (South Nathick, MA, USA) syringe pump with a 500 μL syringe from Hamilton Co. (Reno, NE, USA) was employed for compound infusion. A Synergi Hydro-RP 2.5 μm 80 Å column, 100 x 2.0 mm, a Kinetex 2.6 μm C_{18} column, 100 x 2.1 mm, and a Synergy Hydro-RP 4 μm 100 Å column, 150 x 2.0 mm, were purchased from Phenomenex (Torrance, CA, USA). An Asentis Express 2.7 μm C_{18} column, 100 x 2.1 mm, was purchased from Sigma–Aldrich/Supelco (St. Louis, MO, USA).

5.2.3 Standard preparation

5.2.3.1 Stock and working standard solutions

Each stock solution of the estrogens, metabolites or the deuterium-labeled analytic standards was prepared at 80-200 μg/mL by dissolving an accurate weighed standard with methanol containing 0.1% (w/v) L-ascorbic acid in a volumetric flask. The working standard solutions of estrogens and metabolites at 400-4000 ng/mL and the deuterium-labeled standards at 100 ng/mL were prepared by diluting the stock solutions with methanol containing 0.1% (w/v) L-ascorbic acid. The stock and working standard solutions were stored at –20ºC, and were equilibrated at room temperature before analysis.

5.2.3.2 Calibration standard and quality control samples

The calibration standards of the fifteen estrogens and metabolites were prepared in a range of 12-10980 pg/mL by sequentially diluting working standard solutions with charcoal stripped human serum containing 0.1% (w/v) L-ascorbic acid to 10 concentration levels. The quality control standards were prepared at four levels: limit of quantitation (LOQ, 12-87 pg/mL), low quality control (LQC, 30-210 pg/mL), medium quality control (MQC, 75-801 pg/mL) and high quality control (HQC, 761-8465 pg/mL) of the estrogens and metabolites. The deuterium-labeled internal standard (100 ng/mL), 20 μL, was added to each of the calibration standard solutions and the quality control solutions.

5.2.3.3 Sample Preparation

The deuterium-labeled internal standard (100 ng/mL), 20 μL, the enzymatic hydrolysis buffer containing 2 mg of L-ascorbic acid, 0.5 mL, the β-glucuronidase/sulfatase solution, 5 μL, and 0.15 M sodium acetate buffer (pH=4.1), 0.5 mL, were added to 0.5 mL of each serum sample. This sample mixture was incubated at 37 ºC for 20 hours. Then the sample mixture was extracted with 8 mL of dichloromethane for 10 minutes. The aqueous phase

was discarded, while the organic phase was transferred into a test tube, and was evaporated at 60 ºC under nitrogen flow to dryness. The dried sample was mixed with 150 µL of 0.1 M sodium bicarbonate buffer (pH=9.0) and 150 µL of dansyl chloride solution (5 mg/mL in acetonitrile) and vortexed for 1 minute. This mixture was transferred into a 400-µL glass insert in a 2-mL HPLC sample vial, and the vial was sealed by an HPLC vial cap. After the vial was heated at 60 ºC for 15 minutes, it was cooled down to the room temperature, and was analyzed by LC-MS/MS. The same preparation procedures of hydrolysis, extraction and derivatization were used for all of the standard and the serum samples.

5.2.4 Analytical procedures

5.2.4.1 Method development

The method development was performed using a Phenomenex Synergi Hydro-RP 2.5 µm column, a Phenomenex Kenetex 2.6 µm C_{18} column and a Supelco Asentis Express 2.7 µm C_{18} column. The LC-MS/MS parameters listed in Tables 5.1 and 5.2 were evaluated in order to optimize sample derivatization procedures, LC separation efficiency and MS/MS detection sensitivity, e.g. derivatization temperatures and reaction time, column temperature, mobile phases (buffers at difference pHs and

different organic phase gradients at different flow rates), injection volume, and MS/MS conditions (gas temperature, voltage, collision energy, etc.). The MS instrument was tuned with the optimized parameters using the dansyl derivatized estrogens and metabolites before method validation.

Table 5.1 MRM parameters for MS/MS analysis of estrogens and metabolites.

Compound	Molecular weight (g/mol)	Dansyl molecular ion (m/z)	Daughter ion (m/z)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)	Entrance potential (V)
E_1	270.4	504.3	171.1	136	52	8	10
E_2	272.4	506.3	171.2	141	53	10	10
E_3	288.4	522.4	171.1	136	52	10	10
16-epiE_3	288.4	522.4	171.1	136	52	10	10
17-epiE_3	288.4	522.4	171.1	136	52	10	10
16-ketoE_2	286.4	520.3	171.1	136	52	10	10
16α-OHE_1	286.4	520.3	171.1	136	52	10	10
3-$MeOE_1$	300.4	534.3	171.1	136	52	10	10
2-$MeOE_1$	300.4	534.3	171.1	136	52	10	10
4-$MeOE_1$	300.4	534.3	171.1	136	52	10	10
2-$MeOE_2$	302.4	536.3	171.1	136	52	10	10
4-$MeOE_2$	302.4	536.3	171.1	136	52	10	10
2-OHE_1	286.4	753.3	170.1	136	52	10	10
4-OHE_1	286.4	753.3	170.1	136	52	10	10
2-OHE_2	288.4	755.3	170.1	136	52	10	10

Table 5.2 LC-MS/MS parameters for new method development and validation.

Parameter	Evaluated settings for development	Optimized settings for validation
Derivatization		
Reaction time	3, 5, 7, 10, 15, 20, 25, 30 min	15 min
Dansyl chloride concentration	1, 3, 5 mg/mL	5 mg/mL
HPLC		
Column temperature	40, 50, 60 °C	60 °C
Mobile phase A	0.1% formic acid in water, pH2.7 25 mM ammonia formate, pH3.0 25 mM ammonia acetate, pH4.7	0.1% formic acid in water, pH2.7
Mobile phase B	isopropanol: acetonitrile (v/v) methanol : acetonitrile (v/v) tetrahydrofuran: methanol: acetonitrile (v/v) (with 0.1% formic acid) 0, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 75:25, 80:20, 85:15, 90:10, 100	methanol : acetonitrile:formic acid 85:15:0.1(v/v)
HPLC Gradient	many different conditions	0 min 33% A/67% B 30 min 28% A/72% B 30.1-35 min 33% A/67% B
Flow rate	0.2, 0.3, 0.4, 0.5 mL/min	0.4 mL/min
Injection volume	10, 20, 40, 80 µL	20 µL
MS/MS		
Curtain gas	30, 35, 40, 45, 50 psi	40 psi
Gas1	35, 40, 45, 50 psi	50 psi
Gas2	40, 45, 50, 55 psi	50 psi
Ionspray voltage	4000, 4500, 5000, 5500 volt	5500 volt
Gas temperature	400, 450, 500, 550, 600 °C	550 °C
Collision gas	6, 7, 8, 9, 10, 12 psi	12 psi

5.2.4.2 Method validation

After the LC-MS/MS parameters listed in Tables 5.1 and 5. 2 were optimized during the method development, the method was validated to confirm the specificity, accuracy, precision, linearity, sensitivity, recovery of sample hydrolysis and derivatization, and sample stability using a Phenomenex Synergi Hydro-RP 2.5 μm column. The four quality control standard solutions (LOQ, LQC, MQC and HQC) were injected six times each on the first day, and six times on each of the following two days to assess accuracy and precision. The ten calibration standard solutions were injected for evaluating the linearity of each estrogen or metabolite. To evaluate the sample stability, the serum samples were kept on bunch at ambient temperature for 4 hours, and were allowed to go through three freeze (-80 ºC)/thaw (room temperature) cycles in three consecutive days. Then the serum samples were hydrolyzed, derivatized and analyzed by LC-MS/MS. In order to compare our method with a typical published method in sample preparation, LC separation efficiency and MS detection sensitivity, we evaluated the recovery of sample hydrolysis and extraction, and analyzed one set of dansylated estrogens and metabolites at LOQ level using both our method and the published method [181].

5.3 Results and discussion

5.3.1 Method development

The previous published dansyl chloride derivatization procedures were mixing the dried estrogen sample with 50-100 μL of dansyl chloride at 1 mg/mL in acetone, and heating at 60 ºC for 3 min [43], 5 min [181] or 15 min [171]. In our experiment, the derivatization reaction was evaluated with 150 μL of dansyl chloride at different concentrations, i.e. 1, 3 or 5 mg/mL in acetonitrile; and with different reaction times at 60 ºC, i.e. 3, 5, 7, 10, 15, 20, 25 or 30 min. The reason of changing solvent from acetone to acetonitrile was that dansyl chloride had a higher solubility in acetonitrile than in acetone. The results indicated that the derivatization reaction was complete enough when the extracted and dried sample from 0.5 mL of serum reacted with 150 μL dansyl chloride (5 mg/mL) at 60 ºC for 15 minutes. A lower dansyl chloride concentration or a shorter reaction time led to an incomplete derivatization, while a longer reaction time resulted in degradation of the derivatized products, e.g. a darker reaction solution and higher baseline noises during LC-MS/MS analysis.

As sown in Table 5.2, those mobiles phases, e.g. 25 mM ammonium formate at pH 3.0, 25 mM ammonium acetate at pH 4.7, 0.1% formic acid, and different ratios of methanol, acetonitrile, isopropanol and tetrahydrofuran at different flow rates, e.g. 0.2, 0.3, 0.4 and 0.5 mL/min were assessed to obtain the most efficient separation for the fifteen estrogens and metabolites. The final choice of mobile phases were 0.1% formic acid in water as Mobile phase A, and 0.1% formic acid in a premixed mixture of 85% methanol and 15% acetonitrile (v/v) as Mobile phase B at a flow rate of 0.4 mL/min, because the mobile phases containing ammonium formate, ammonium acetate, isopropanol and tetrahydrofuran, and the other flow rates reduced the separation efficiency. The column temperature at 60 °C provided a lower column pressure and better separation than at 40-50 °C. The injection volume, 20 µL, gave in an appropriate sensitivity for estrogens and metabolites, because a lower injection volume, e.g. 15 µL or less, reduced the analyte signals, while a higher injection volume, e.g. 40-80 µL, elevated baseline noises. A Phenomenex Synergi Hydro-RP 2.5 µm column became the final choice of our method, because it provided a better separation for the fifteen dansylated estrogens than a Phenomenex Kenetex 2.6 µm C_{18} column and a Supelco Asentis Express 2.7 µm C_{18} column.

In comparison to the typical published method [181], our method derivatized the serum sample with dansyl chloride at a higher concentration (5 mg/mL vs. 1 mg/mL) and for a longer time (15 min vs. 3 min), and improved the separation of the fifteen estrogens and metabolites by a smaller particle size column (2.5 μm vs. 4.0 μm) eluted with mobile phases containing water, methanol, acetonitrile and formic acid. The shorter column (100 mm vs. 150 mm), higher column temperature (60 °C vs. 40 °C) and faster mobile phase flow rate (0.4 ml/mL vs. 0.2 ml/mL) significantly reduced the method run time from 100 minutes to 35 minutes, as summarized in Table 5.3. The typical overlays of MRM chromatographic profiles of dansylated estrogens and metabolites from the published method and from our gradient method are shown in Figure 5.2 and Figure 5.3, respectively. These figures and the retention times of the fifteen estrogens and metabolites listed in Table 5.4 demonstrated that the two pairs of E_3/16-ketoE_2 and 2-MeOE_2/4-MeOE_1 peaks were overlapped in the published method, while they were separated by our gradient method with the retention time differences between peaks ≥ 0.2 min.

Table 5.3 Comparison of two HPLC-MS/MS methods in derivatization and HPLC conditions.

Parameters	Published method*	New method
Dansyl chloride	1 mg/mL	5 mg/mL
Reaction at 60°C	5 min	15 min
Column	Synergi Hydro-RP, 150 x 2.0 mm, 4 µm, 100 Å	Synergi Hydro-RP, 100 x 2.0 mm, 2.5 µm, 80 Å
Column temperature	40 °C	60 °C
Mobile phases	A: formic acid 0.1/water 100 B: methanol	A: formic acid 0.1/water 100 (v/v) B: formic acid 0.1/methanol 85/acetonitrile 15 (v/v)
Gradient	A/B: 28/72 to 15/85 in 75 min (100 min run)	33/67 to 28/72 in 30 min (35 min run)
Flow rate	0.2 mL/min	0.4 mL/min
Injection volume	20 µL	20 µL

* publish method by Xu *et al.*

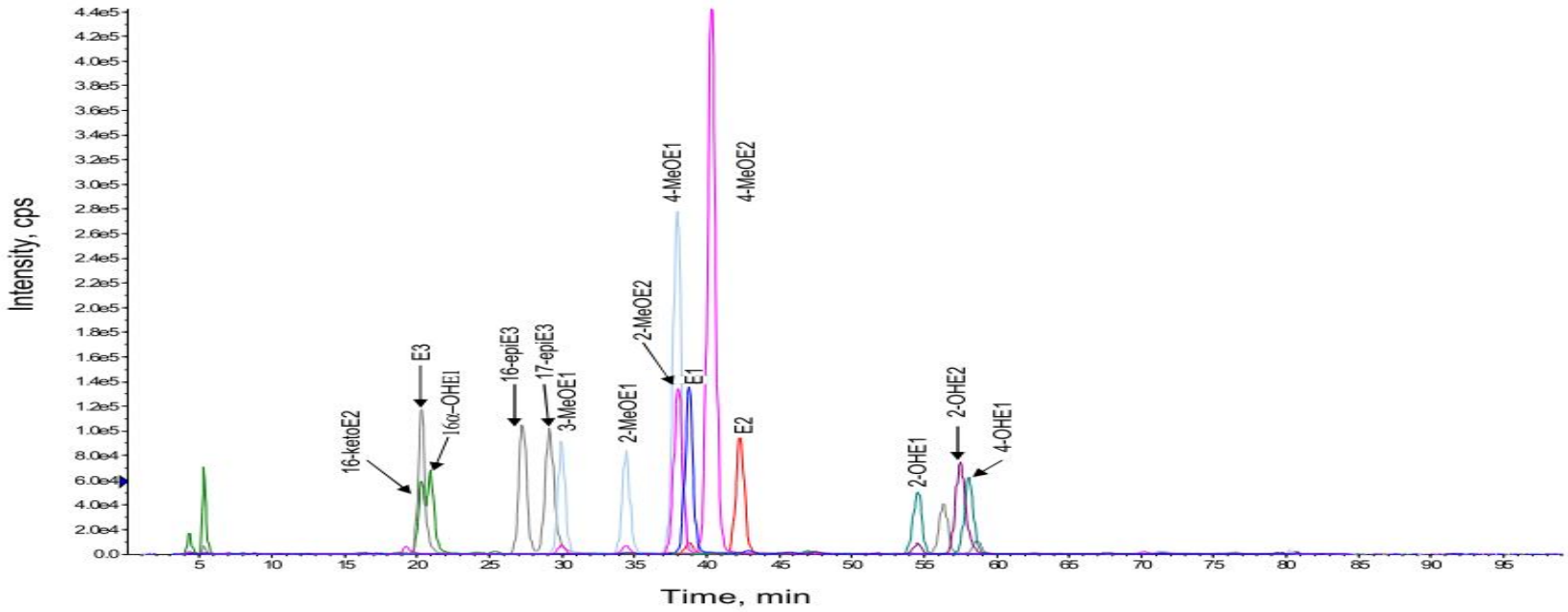


Figure 5.2 Overlay of typical MRM chromatographic profiles of dansyl derivatives of estrogens metabolites for the published method by Xu *et al.*

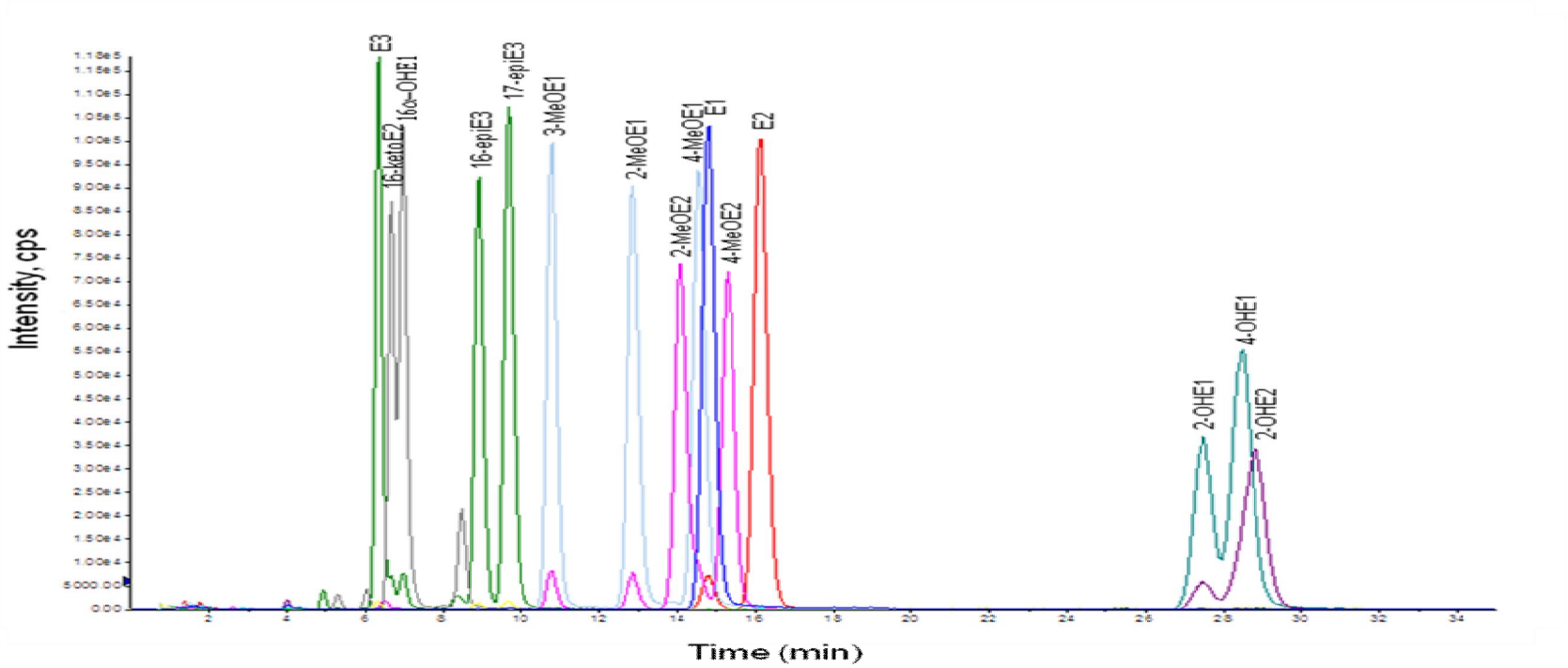


Figure 5.3 Overlay of typical MRM chromatographic profiles of dansyl derivatives of estrogens and metabolites for the new gradient method.

Table 5.4 Comparison of two LC-MS/MS methods in separation and sensitivity

Compound	Published method[a]		New Method	
	RT[b] (min)	LOQ (pg/mL)	RT[b] (min)	LOQ (pg/mL)
E_3	20.4[c]	6.5[d]	6.6	5.3[d]
16-ketoE$_2$	20.3[c]	25.2	7.0	13.1
16α-OHE$_1$	20.9	19.1	7.3	11.9
16-epiE$_3$	27.3	11.4	9.3	8.5
17-epiE$_3$	29.1	19.8	10.0	13.6
3-MeOE$_1$	30.0	19.2	11.1	21.0
2-MeOE$_1$	34.5	36.2	13.2	36.8
2-MeOE$_2$	38.0[c]	26.7	14.5	36.0
4-MeOE$_1$	37.9[c]	28.4	14.9	31.8
E_1	38.8	8.7	15.1	6.6
4-MeOE$_2$	40.3	30.2	15.7	38.1
E_2	42.3	11.0	16.5	11.7
2-OHE$_1$	54.6	74.1	27.8	71.1
4-OHE$_1$	58.1	66.7	28.8	39.2
2-OHE$_2$	57.5	57.1	29.3	53.8

[a] data acquired using the publish method by Xu *et al.*
[b] Retention time
[c] Overlapped peaks
[d] Calculated based on compound in water

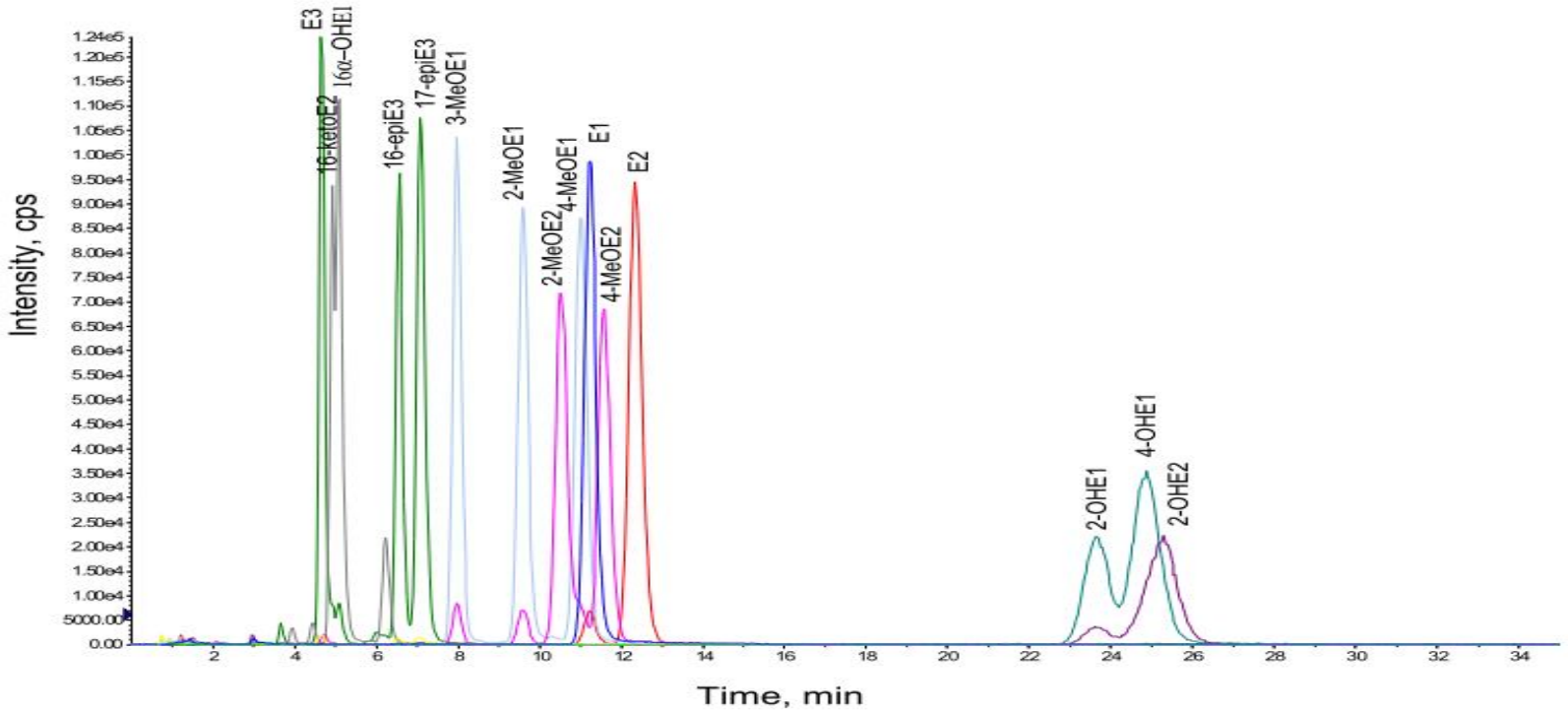


Figure 5.4 Overlay of typical MRM chromatographic profiles of dansyl derivatives of estrogens and metabolites for the new isocratic method

When we eluted the Hydro-RP 2.5 μm column with an isocratic mobile phase consisting of 30% water , 70% organic phase (methanol/acetonitrile=85/15) and 0.1% formic acid, the separation of the fifteen estrogens and metabolites was similar as that from the gradient method, as shown in Figure 5.4. However, the peak heights of three peaks between 23 and 26 minutes were much lower than those from the gradient method, leading to decreased method sensitivity. Therefore, the gradient method was selected for our method validation.

5.3.2 Method validation

5.3.2.1 Specificity

As shown in Table 5.4 and the typical overlay of selected ion chromatograms of fifteen dansylated estrogens and metabolites at HQC level in Figure 5.3, most of the estrogens and metabolites were well separated, except that 4-MeOE_1/E_1 and 4-OHE_1/2-OHE_2 were partially overlapped. Since dansylated 4-MeOE_1, E_1, 4-OHE_1 and 2-OHE_2 had different molecule ions, there were no cross interferences among these compounds in accuracy, precision, linearity and sensitivity. The only significant interference from

blank serum was observed for E_3 at the LQC and LOQ levels. These results indicated that the method was specific for these estrogens and metabolites.

So far, the most efficient LC-MS/MS method was reported by Yang *et al.*, which could separate fifteen (out of sixteen) N-methyl nicotinic acid ester derivatized estrogens and metabolites in seven minutes using an Agilent XDB-C18, 50 x 2.1 mm, 1.8 μ column [170]. However, that method had a limit of detection (LOD) within a range of 0.36-2.34 ng/mL, far higher than the LOQ range (5.3-71.1 pg/mL) of our method. The separation efficiencies of other published methods for varieties of derivatized estrogens and metabolites were not so good, although they had comparable sensitivities [169,171-173,181].

5.3.2.2 Method sensitivity

The sensitivity of our method is presented as LOQ with a signal to noise ratio of 10 to 1. In general, the LOQ values listed in Table 5.4 are in a range of 5.3-71.1 pg/mL (6.6 pg/mL for E_1, 11.7 pg/mL for E_2 and 5.3 pg/mL for E_3), and are comparable to those LOQ values acquired using the typical published method by Xu *et al.* (LOQ = 8 pg/mL reported in the original article [181]), and to those LOQ values (0.4-10.0 pg/mL) from other

published LC-MS/MS and GC-MS/MS methods using varieties of derivatization reagents [157,169,171-173]. Nevertheless, the LOQ values in Table 5.4 seem having an increasing trend following the retention time. This might be because the longer the retention time, the broader and shorter the peaks, leading to lower detection sensitivities. In addition, the differences in ESI^+ ionization and fragmentation in MS/MS stage for different compounds might also cause the variations in the LOQ values. On the other hand, detection of dansyl derivatives of estrogens and metabolites using ESI^+ mode might be less selective than detection of pentafluorobenzyl estrogen derivatives under $APCI^-$ mode [43], because most of the dansylated estrogens and metabolites had the same daughter ions of m/z 171^+, as shown in Table 5.1. This suggested that an optimal sulfonyl chloride derivatisation reagent should have more specific fragment ions for the derivatized estrogens and metabolites, and the sensitivity should be enhanced more dramatically if the fragment ions contain an isotope(s), e.g. a chlorine or bromine atom(s).

5.3.2.3 Accuracy

Accuracy of this method was determined by analyzing duplicate sample preparations of the estrogens and metabolites at the four quality

control levels, HQC, MQC, LQC and LOQ, and the measure results were compared with the theoretical values. As shown in Tables 5.5 and 5.6, the accuracy values at all these levels were within a range of 93.1-112.9% for intraday results, and within a range of 91.7-109.8% for interday results, except those for E_3 at LQC and LOQ levels due to interferences from the blank serum. These results were comparable to those from the methods reported before [171,181].

5.3.2.4 Precision

The precision of the method was assessed by evaluating both method precision (intraday precision) and system repeatability (interday precision). The method precision for the estrogens and metabolites was presented by the relative standard deviation of the response of six sample preparations (RSD%, n=6) at the same levels of LOQ, LQC, MQC and HQC on the same day. The relative standard deviations of six sample preparations (RSD%) of estrogens and metabolites on the same day (intraday) were in a range of 1.7-13.2% within the concentration range of 12-8465 pg/mL, as shown in Table 5.5. Similarly the relative standard deviations of six sample preparations (RSD%) of the estrogens and metabolites in three consecutive days (interday) were in a range of 2.3-16.9% within the concentration range

of 12-8465 pg/mL, as shown in Table 5.6. These results demonstrated that the method had suitable precision and system repeatability within the determination ranges.

5.3.2.5 Linearity and recovery of sample derivatization

As shown in Table 5.7, the LC/MS/MS system had linear responses to the fifteen dansylated estrogens and metabolites in the range of 12-10980 pg/mL with regression coefficients $r^2 \geq 0.9934$. Since a large portion of the fifteen estrogens and metabolites exist as glucuronide and sulfate in human body fluids, e.g. serum and urine, the conjugated estrogens and metabolites need to be hydrolyzed with glucuronidase and sulfatase before dansyl derivatization. When we followed glucuronidase/sulfatase hydrolysis procedures from the published method [181], and derivatized the unconjugated fifteen estrogens and metabolites with dansyl chloride under our optimized conditions, the total recovered estrogens and metabolites were within a range of 74.4-95.6% at a concentration range of 30-801 pg/mL, as shown in Table 5.7. This range of recovery ratio of sample hydrolysis and derivatization was proved to be adequate for sample analysis by the results of accuracy, precision, linearity and sensitivity. In order to demonstrate the suitability of the method for real human serum sample analysis, three batches

of unknown human serum samples were analyzed. As shown Table 5.7, the levels of the determined estrogens and metabolites by our method were close to those from the typical method reported before [181].

5.3.2.6. Sample stability

The sample stability was evaluated by allowing the serum samples spiked with estrogens and metabolites to stay at the room temperature for four hours, or to go through three freeze/thaw cycles in three consecutive days. Then, these samples underwent hydrolysis, derivatization and LC-MS/MS analysis. The accuracy (82.3-118.1%) and precision (2.0-9.0%RSD) results demonstrated that the samples were stable during the stability testing, and suitable for sample analysis under the assigned storage conditions, as shown in Table 5.8.

Table 5.5 Accuracy and intraday precision for estrogens and metabolites in serum.

Compound	LOQ (12-87 pg/mL, n=6)		LQC(30-210 pg/mL, n=6)		MQC (75-801 pg/mL, n=6)		HQC (761-8465 pg/mL, n=6)	
	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%
E_1	101.7	11.3	99.4	8.3	109.6	4.6	105.4	3.2
E_2	93.1	11.3	95.5	7.2	102.7	2.5	109.2	3.3
E_3	N/A*	N/A	N/A	N/A	100.5	6.4	101.5	4.6
16-epiE_3	96.4	13.2	100.1	7.7	101.6	3.3	108.7	6.0
17-epiE_3	103.3	11.7	108.8	8.5	100.6	5.8	108.4	7.4
16-ketoE_2	105.6	10.8	106.0	8.4	104.9	3.3	100.0	6.4
16α-OHE_1	106.6	11.1	110.7	5.9	105.0	5.3	108.4	2.1
3-$MeOE_1$	105.3	12.2	101.8	7.6	101.4	4.0	101.4	4.0
2-$MeOE_1$	101.1	8.5	103.9	6.1	105.5	1.7	104.2	5.7
4-$MeOE_1$	102.6	8.7	98.1	3.8	97.5	3.1	97.5	3.2
2-$MeOE_2$	101.5	8.8	104.6	5.6	107.2	2.6	106.5	2.7
4-$MeOE_2$	108.2	7.3	101.6	6.5	95.7	1.8	94.2	5.1
2-OHE_1	106.4	8.5	105.3	6.5	102.1	10.5	105.0	9.5
4-OHE_1	97.6	10.4	96.7	6.9	106.7	7.6	110.3	7.7
2-OHE_2	98.9	12.1	94.9	9.3	97.9	8.3	112.9	3.3

* Interference from blank serum

Table 5.6 Accuracy and interday precision for estrogens and metabolites in serum.

Compound	LOQ(12-87 pg/mL, n=18)		LQC (30-210 pg/mL, n=18)		MQC (75-801 pg/mL, n=18)		HQC (761-8465 pg/mL, n=18)	
	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%
E_1	102.0	16.9	93.8	11.8	103.9	7.4	101.5	6.7
E_2	98.6	9.2	95.4	7.8	102.7	3.6	107.0	3.5
E_3	N/A*	N/A	N/A	N/A	100.2	6.7	97.9	7.3
16-epiE_3	98.9	15.5	98.4	8.0	98.4	7.0	101.9	9.7
17-epiE_3	109.8	15.9	106.1	9.5	97.2	8.0	100.1	9.5
16-ketoE_2	104.5	11.6	109.6	12.6	104.6	4.7	95.2	9.8
16α-OHE_1	102.8	10.4	104.9	8.7	102.7	6.9	98.2	9.8
3-$MeOE_1$	105.7	10.4	103.5	7.3	100.9	3.5	97.1	5.4
2-$MeOE_1$	101.3	8.4	104.5	5.8	104.9	2.7	100.8	7.3
4-$MeOE_1$	102.5	7.0	98.0	3.3	97.0	2.7	95.6	4.6
2-$MeOE_2$	102.3	9.1	105.6	6.5	107.0	2.9	105.6	2.3
4-$MeOE_2$	105.6	8.0	102.9	5.8	96.5	3.1	96.1	5.6
2-OHE_1	103.0	9.8	99.1	9.8	95.9	10.2	95.8	8.7
4-OHE_1	97.2	10.0	91.7	9.0	99.8	11.4	102.2	9.0
2-OHE_2	97.1	8.3	96.5	6.4	96.1	6.7	100.7	10.0

* Interference from blank serum

Table 5.7 Linearity, recovery of hydrolysis/derivatization and sample analysis of estrogens and metabolites in human serum.

Compound	Linearity (pg/mL)	r^2	MQC Recovery% (75-801 pg/mL)	LQC Recovery% (30-210 pg/mL)	Serum 1 (pg/mL)	Serum 2 (pg/mL)	Serum 3 (pg/mL)
E_1	12-1486	0.9982	84.9	74.4	233	264	209
E_2	17-1785	0.9963	84.8	75.6	23.1*	24.3*	19.8*
E_3	48-931	0.9953	86.3	76.9	ND	ND	ND
16-epiE_3	12-1429	0.9980	92.1	83.1	ND	ND	ND
17-epiE_3	24-2804	0.9971	88.0	82.0	10.1*	ND	ND
16-ketoE_2	12-1501	0.9951	95.6	79.2	12.8	27.9	25.2
16α-OHE_1	12-1438	0.996	88.2	78.3	ND	33.9	ND
3-MeOE_1	12-1413	0.9964	85.0	83.6	ND	ND	ND
2-MeOE_1	25-2844	0.9983	89.0	89.2	3.8*	39.3	45.2
4-MeOE_1	65-7082	0.996	87.5	81.3	29.1	ND	36.4
2-MeOE_2	31-3530	0.9976	85.8	80.6	ND	ND	ND
4-MeOE_2	84-7341	0.997	86.4	84.3	ND	ND	ND
2-OHE_1	78-10035	0.9940	86.2	76.5	22.0*	48.9*	33.4*
4-OHE_1	81-10980	0.9934	81.9	79.4	ND	31.3*	30.8*
2-OHE_2	79-9073	0.9950	83.8	82.8	ND	ND	ND

ND: not detected.

* The estimated concentration is below the LOQ but above LOD (S/N>3)

Table 5.8 Bench top and freeze thaw stability for estrogens and metabolites in serum.

Compound	Bench top stability[a] (75-801 pg/mL, n=6)		Freeze thaw stability[b] (75-801 pg/mL, n=6)	
	Accuracy%	Precision%	Accuracy%	Precision%
E_1	105.0	3.1	104.6	4.2
E_2	102.4	3.0	101.2	2.3
E_3	90.2	5.1	93.2	5.0
16-epiE_3	87.4	4.9	90.0	6.7
17-epiE_3	86.4	7.0	87.5	9.0
16-ketoE_2	88.7	7.9	92.5	5.6
16α-OHE_1	84.9	8.9	82.3	7.8
3-MeOE_1	96.8	4.4	96.0	2.9
2-MeOE_1	102.8	3.8	103.0	3.8
4-MeOE_1	96.4	5.4	98.0	2.0
2-MeOE_2	106.7	3.3	106.3	2.7
4-MeOE_2	94.1	7.8	97.4	2.9
2-OHE_1	103.5	8.8	115.8	8.4
4-OHE_1	110.0	6.7	118.1	4.8
2-OHE_2	100.0	9.2	109.0	5.4

[a] Sample solutions on bunch at 4 hours

[b] After 3 freeze and thaw cycles in three consecutive days

5.4 Conclusions

An efficient LC-MS/MS method was successfully developed and validated for determination of fifteen estrogens and metabolites in human serum. The sample derivatization procedures were optimized, and sample stability was assessed. The method was specific, accurate, precise, sensitive and linear within the calibration range. It had a comparable sensitivity to those from the typical published LC-MS/MS methods, while it had a much better LC separation efficiency, i.e. separating all of the fifteen dansylated estrogens and metabolites with a significantly reduced elution time.

The information in Chapter 5 has been adapted from a manuscript which is in preparation for publication in J. Chromatogr. B.

Chapter 6: Facilitating the Hyphenation of CIEF and MALDI-MS for Two-Dimensional Separation of Proteins

6.1 Introduction

Isoelectric focusing (IEF) is one of the most popularly used techniques for protein separations. In IEF, proteins are self-focused into narrow zones at positions corresponding to their *p*I values and the widths of these zones are inversely proportional to the square root of the focusing electric field strength. Theoretically, any protein zone can be compressed into a line-like band as long as the electric field strength is sufficiently high. In practice, however, the magnitude of the electric field strength is constrained by Joule heating. To overcome this problem, IEF is performed in a narrow-bore capillary (capillary isoelectric focusing or CIEF for short [182,183] in which excess Joule heat can be effectively dissipated through the wall of the capillary due to the increase surface-to-volume ratio.

The operation of CIEF consists of two major steps. In the first step, a mixture of carrier ampholytes and proteins is introduced into a capillary, and a DC voltage is applied to form a pH gradient and focus proteins inside the

capillary. In the second step, the focused protein zones are mobilized passing through a detector for measurement. The mobilization can be executed hydrodynamically [184], electroosmotically [185], or chemically [186]. Usually, the separated proteins are detected using a fixed-point UV absorbance or fluorescence detector. A whole-column detection approach has also been used recently to detect focused proteins without the mobilization step [187,188]. While these detectors work well to monitor the separations, they are incapable of identifying the separated proteins. Incorporation of CIEF with a mass spectrometer (MS) can potentially address this issue.

Coupling of CIEF with electrospray (ESI) MS was accomplished in the 1990's [19,58,189-191], and is capable of providing attomole sensitivity due to the concentration effect associated with CIEF [192]. CIEF-ESI-MS has been successfully applied for the analysis of a single protein (e.g., hemoglobin [58], alcohol dehydrogenase isoenzymes [193] and complex cell lysates [194,195]. Matrix-assisted laser desorption/ionization (MALDI) MS, introduced in 1988 [196,197], is another MS technique that is widely utilized for protein analysis. MALDI-MS is capable of analyzing large intact proteins with molecular mass in excess of 100 kDa [198]. However,

CIEF-MALDI-MS attracted much less attention than CIEF-ESI-MS, presumably due to the challenges of coupling CIEF with MALDI-MS.

In 1995, Foret *et al.*. [59] demonstrated the feasibility of off-line coupling of CIEF with MALDI-MS. In Foret's apparatus, a fiber-optic UV detector was attached to a CIEF capillary to determine the mobilization speeds and measure the bandwidths of separated proteins. With these parameters, every separated protein band was precisely fractionated. A sheath flow unit was incorporated at the exit of the capillary to facilitate the fractionation and distribution of these bands to a parallel-glass-tube collection interface [190]. An aliquot (2 μL) of each collected sample was then deposited onto a MALDI target. After the solvent was evaporated, 2 μL of a matrix solution was added. Evaporation of the matrix solvent resulted in the formation of protein-matrix crystals on the sample spot. This sample was then analyzed by a MALDI-MS. More recently [199,200], CIEF-separated proteins, along with the focusing medium, were fractionated via a sheath flow unit and deposited directly onto a MALDI-MS target. Peak resolution of this method increased with the decreasing deposition times. Under optimized conditions, most of the CIEF resolution was retained [199]. However, the additives (ampholytes and surfactants) in the focusing medium

reduce the MS signals considerably. In our lab, the similar effect was observed: Pharmalyte™ and 3-[(3-Cholamidopropyl)dimethylammonio] -1-propanesulfonate (CHAPS) severely suppressed the MS sensitivity. Because adequate additives are required to achieve proper CIEF separations, minimizing the signal suppression effect of these additives is therefore important.

In this work, we report a simple means to mitigate the above adverse effect. We first dropped a small volume (~1 μL) of water onto a MALDI-MS target. We then distributed a fraction of the CIEF-separated sample (~0.1 μL) to the center region and close to the bottom of the droplet. Likely because small additive molecules (carrier ampholytes, detergent and other salts) diffused faster than proteins, more protein molecules remained in the center region of the sample spot after the solvent was evaporated. By directing the laser to this region to ablate the sample, we improved the MS signal to noise ratio (S/N). We optimized the droplet volume and the laser-ablation region to maximize the S/N. We also applied this method for analysis of Apolipoprotein A-I (*apoA-I*, a membrane protein) expressed in *E. Coli* cells.

6.2 Materials and methods

6.2.1 Materials

Ribonuclease A (13.7 kDa, *p*I 9.60), horse myoglobin (16.9 kDa, *p*I 7.35 and 6.85), *β*-lactoglobulin B (18.3 kDa, *p*I 5.30), *β*-lactoglobulin A (18.4 kDa, *p*I 5.15), soybean trypsin inhibitor (20.1 kDa, *p*I 4.55), 3-(trimethoxysilyl)propyl methacrylate, *α*-cyano-4-hydroxycinnamic acid and cellulose acetate (CA) (39.7 wt%, average MW 50 kDa) were purchased from Sigma (St. Louis, MO). Pharmalyte (36% w/v, pH 3-10) was purchased from Amersham Bioscience (Piscataway, NJ). Acrylamide (AA), N,N'-methylene-bisacrylamide (Bis), ammonium persulfate (APS), and N,N,N',N'-tetramethylethylenediamine (TEMED) were bought from Bio-Rad Laboratories (Hercules, CA). CHAPS was obtained from Acros Organics (Morris Plains, NJ). Ammonia acetate was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Phosphoric acid (85%), sodium hydroxide, acetic acid, acetone, trifluoroacetic acid (TFA), methanol, and acetonitrile were bought from Fisher Scientific (Pittsburgh, PA). All solutions were prepared with ultrapure water purified by a NANOpure infinity ultrapure water system (Barnstead, Newton, WA). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ).

6.2.2 Preparation of cross-linked polyacryamide coated capillary

The coating procedure was similar to that reported previously [201], with slight modifications. Briefly, a fused-silica capillary (60 cm long × 150 μm i.d. × 375 μm o.d.) was washed with 1.0 M NaOH for 45 min, rinsed with DI water and acetonitrile each for 15 min, and then dried by flowing helium at 5 psi for 20 min. A solution of 0.40% (v/v) of 3-(trimethoxysilyl) propyl methacrylate and 0.20% (v/v) acetic acid in acetonitrile was flushed into the capillary for 1 hour. The capillary was then rinsed with acetonitrile for 15 min and dried by flowing helium at 5 psi through the capillary for 20 min. After 2.0 mL solution containing 4.0% (w/v) of AA and 0.024% Bis was purged with helium at 5 psi at room temperature for 1 hour, 1.0 μL of 10% APS and 10 μL of TEMED were added to the solution. This solution was immediately pressured into the capillary. After 1.5 min, the solution was pushed out with pressurized helium at 60 psi, and the helium was allowed to continuously blow through the capillary for 1 hour. The capillary coating set-up was shown in Figure 6.1. The capillary was ready to use after it was rinsed with water for ~10 min.

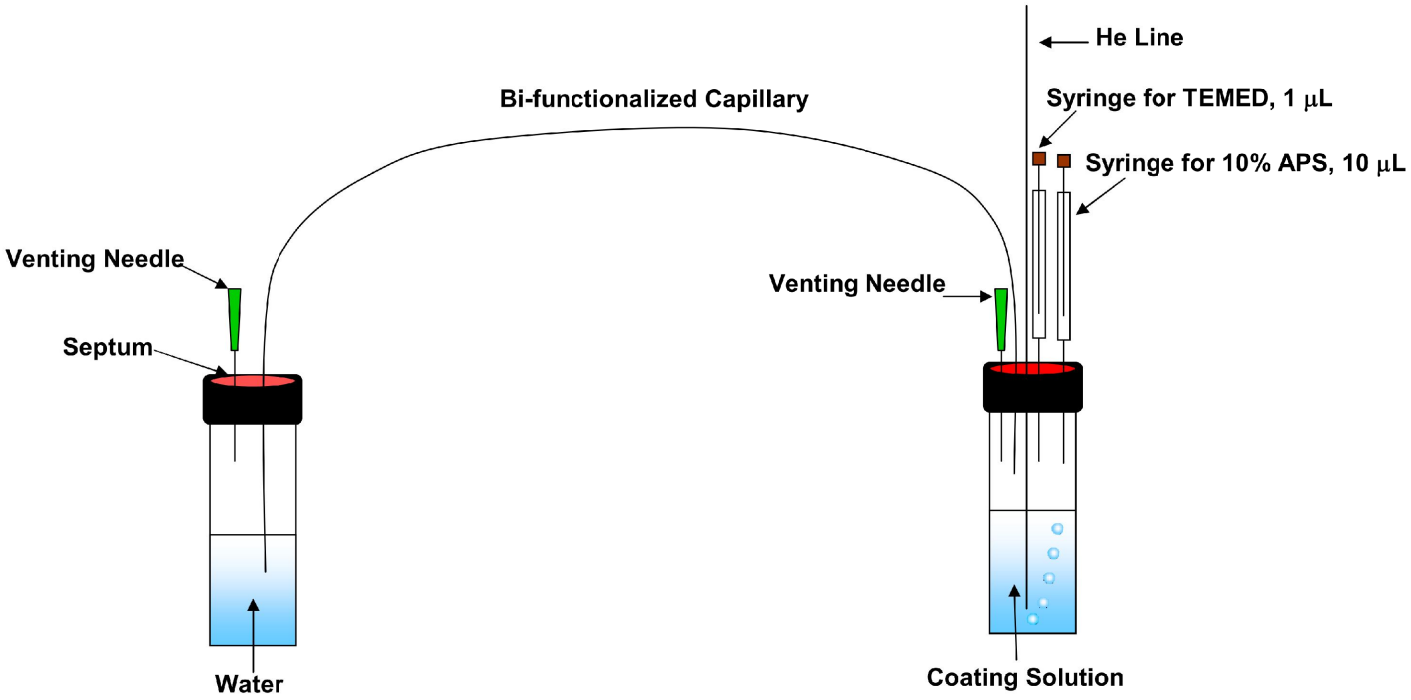


Figure 6.1 Diagram of capillary coating set-up.

6.2.3 Apolipoprotein A-I sample

ApoA-I sample was kindly provided by Ms. Shou Lu in Professor Zgurskaya's group in the Department of Chemistry and Biochemistry at University of Oklahoma. The sample was prepared and quantitated according to a previously published procedure [202].

6.2.4 Construction of cellulose acetate membrane grounding interface

The construction procedure was similar to that described by Whang [203] and Chen and Wang [64], with minor modifications. Briefly, a fracture was first produced at ~1.5 cm from one end of a cross-linked polyacryamide (CPA)-coated capillary, and a tiny drop of 12% (w/v) CA solution in acetone was applied to the fracture to evenly cover it. After the solvent was evaporated, a CA membrane was formed around the fracture. A small hole was then created at the bottom of a 0.65 mL plastic vial (Fisher Scientific, Pittsburgh, PA), and the vial was affixed to the CPA coated capillary with CA-covered fracture inside it (see Figure 6.2). Epoxy (Devcon, Riviera Beach, FL) was used to secure the vial and the capillary in position and seal the hole.

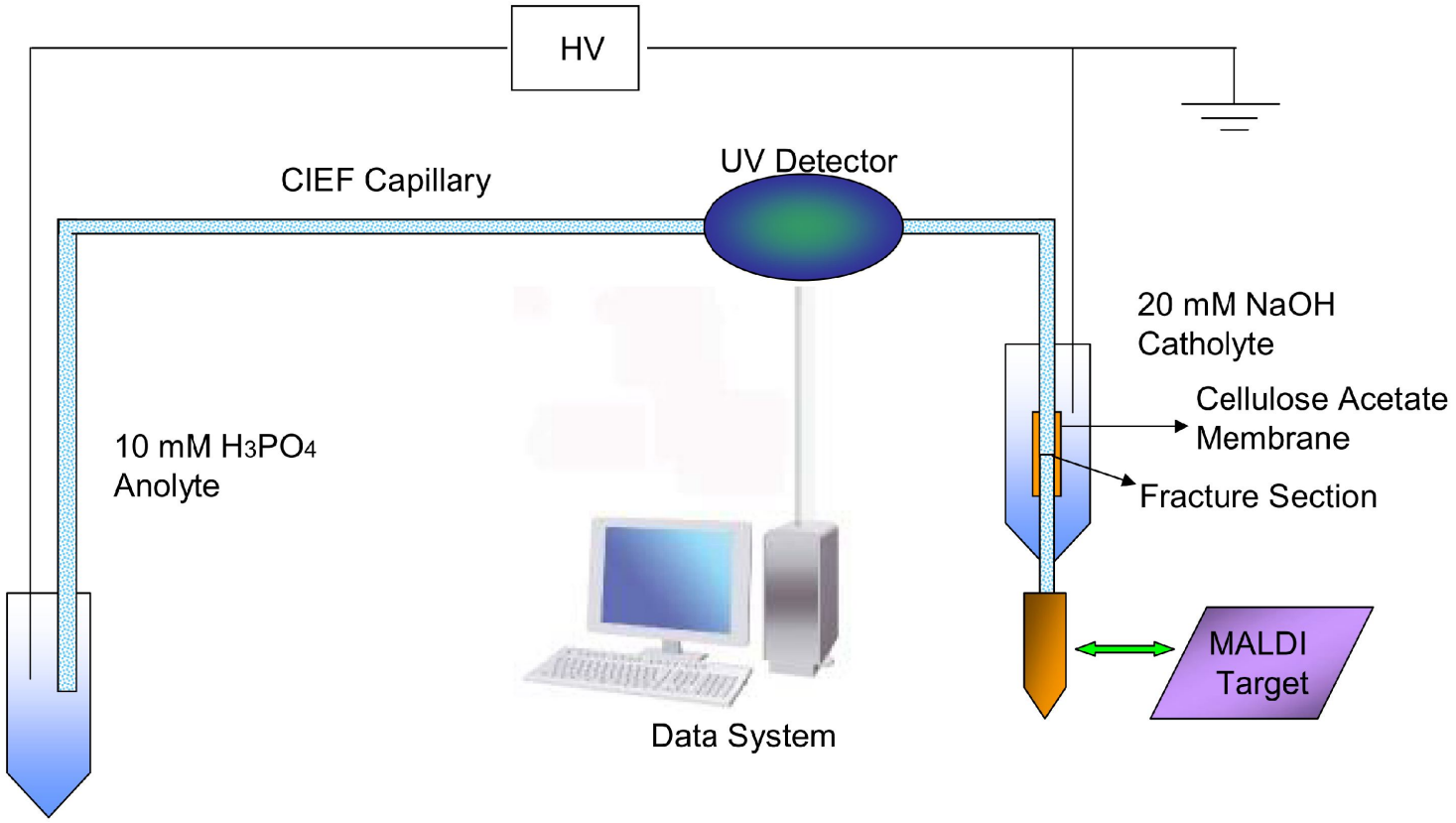


Figure 6.2 Schematic diagram of apparatus for CIEF separation and fractionation. Reprinted from [68] with permission.

6.2.5 Apparatus

Figure 6.2 presents a schematic diagram of the experimental apparatus. The above CPA coated capillary with a CA membrane grounding interface was used to perform CIEF separation, and the focusing voltage was provided by a Glassman high-voltage power supply (High Bridge, NJ). The anode was inserted in the anolyte and the cathode in the catholyte. The exit end of the capillary was immersed in the water in the auxiliary reservoir. As the separated proteins were mobilized from anode to cathode, they were monitored by a Linear-200 UV/visible detector (Linear Instruments Corp., Reno, NV) at 280 nm. The absorbance signal was acquired by an NI multifunctional card DAQCard-6062e (National Instruments, Austin, TX), and processed with an in-house-developed LabView program.

6.2.6 CIEF

To prepare for CIEF, the separation capillary along with the CA membrane grounding interface were rinsed with DI water, and the vial of the interface was loaded with a catholyte solution (20 mM of sodium hydroxide). After the capillary was filled with a mixture of protein(s) and focusing medium (Pharmalyte, CHAPS, and ammonia acetate), its exit end was inserted into a small container containing DI water (see Figure 6.2). The other end of the capillary was inserted into a container containing an anolyte

solution (10 mM of Phosphoric acid). Isoelectric focusing was initiated by applying a high voltage (20 kV) from the anolyte solution to the catholyte solution, and took ~20 min to complete. For absorbance detection of CIEF separated proteins, the focused bands were hydrodynamically mobilized to a UV/visible detector by lifting the anolyte solution by 2 cm relative to the water in the auxiliary reservoir at the exit end of the capillary while the high voltage was maintained during the entire mobilization process.

6.2.7 Protein fractionation/deposition and MALDI-MS identification

To prepare for protein fractionation/deposition, 1.0 μL of water was deposited at designated spot on a MALDI-MS target plate in the ambient environment a 3-5 seconds before the completion of CIEF focusing. After the auxiliary reservoir hosting the exit end of the capillary was removed, the MALDI-MS target plate with the water droplet was lifted (in the *z*-axis via a translation stage) so that the capillary tip was inserted into the water droplet with the capillary tip virtually touching the target plate. By raising the anolyte solution by 2 cm, the solution inside the capillary was delivered to the water droplet. During this delivering process, 1.0 μL of water was deposited at another spot on the target plate. After 30 second delivery, the target plate was dropped by 2-3 mm in the *z*-axis, shifted 4.5 mm in the *x*- or *y*-axis and lifted 2-3 mm in the *z*-axis for deposition/delivery to the next spot. This operation was repeated until all the focused proteins inside the capillary

were delivered to the target plate. During this process, the high voltage was applied across the capillary continuously.

After the above fractionation process was complete, the solvent in the water droplets were allowed to evaporate. It took ~9 min for each 1.0 μL water-droplet to get dried under the ambient conditions. Then, 0.5 μL MALDI matrix [10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% (v/v) water-acetonitrile with 0.1% (v/v) TFA] was added to each spot and allowed to dry again (which took ~5 min). Finally, the target plate was loaded into an Applied Biosystems 4800 Proteomics Analyzer for MALDI-MS identification. The m/z range of the system was set to 11 kDa-22 kDa or 35 kDa with a focus m/z of 16 kDa or 23 kDa in linear mode. MALDI-TOF spectra were analyzed using Data Explorer software Version 3.0 (Applied Biosystems).

6.3 Results and discussion

6.3.1 Effects of additives on MS signal-to-noise ratio

To examine the effect of additives on MS signal to noise (peak-to-peak noise) ratio, we mixed Pharmalyte or CHAPS (not both) at varying concentrations with a protein, and deposit this solution (~0.2 μL per spot) either directly onto a MALDI-MS target plate or into a 1 μL water droplet on the target plate. After the solvent was evaporated, we added 0.5

μL of MALDI-MS matrix (10 mg/mL α-cyano-4-hydroxycinnamic acid) to each sample spot and allowed the sample to dry again.

As shown in Figure 6.3, in this experiment, the sample contained 0.05 μg/μL horse myoglobin and varying concentrations of Pharmalyte or CHAPS (not both). 0.2 μL of this sample was delivered either directly to a MALDI target plate (for Figures 6.3A and 6.3C) or to 1 μL of water pre-deposited on the target plate (for Figure 6.3B and 6.3D). The sample was allowed to dry, and 0.5 μL of a matrix solution containing 10 mg/mL of α-cyano-4-hydroxycinnamic acid and 0.1% TFA in 1:1 acetonitrile-water was added to the sample spot. After the matrix solvent was evaporated, the target plate was transferred to an Applied Biosystems 4800 Proteomics Analyzer. The MS spectra were measured at an m/z range of 11 kDa-22 kDa or 35 kDa with a focus m/z of 16 kDa or 23 kDa in a linear mode. Spectra in Figures 6.3A and 6.3B were obtained from the protein-Pharmalyte mixtures, while spectra in Figures 6.3C and 6.3D were obtained from the protein-CHAPS mixtures.

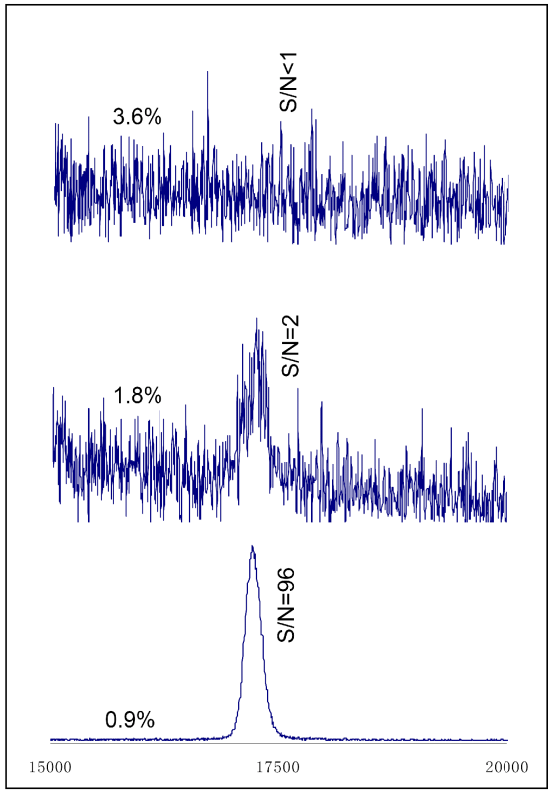
Figure 6.3 exhibits the effect of Pharmalyte and CHAPS concentration on the MS signal. Apparently (see Figure 6.3A and 6.3C), these additives severely suppress the MS signal. At 3.6% Pharmalyte, no MS signal could be detected. Interestingly (see Figure 6.3B and 6.3D), this effect

can be alleviated considerably by depositing the protein mixture to a 1 μL water droplet pre-loaded on the target plate, with a S/N improvement of 2-10 fold.

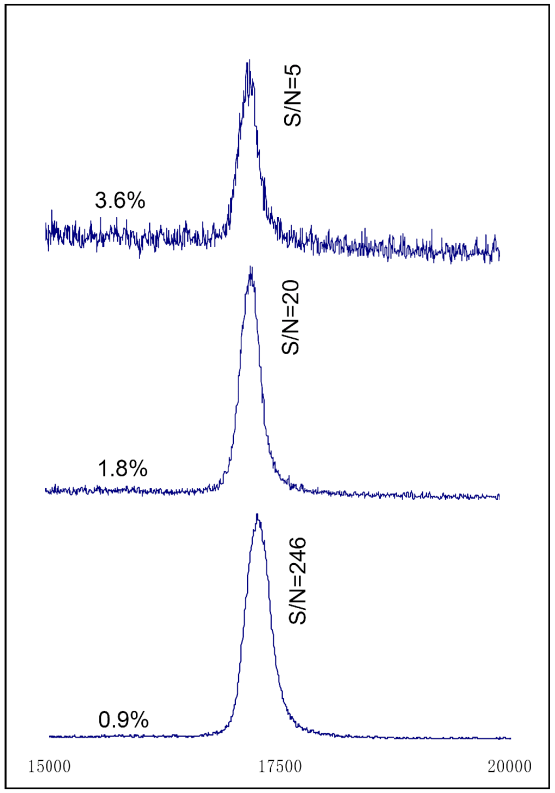
In the above tests, we simply added Pharmalyte or CHAPS to the protein for MS measurements. To make the test more representative to the experimental protocol for CIEF-MALDI-MS, we performed CIEF with different concentrations of Pharmalyte and CHAPS, fractionated the CIEF-separated proteins and deposited them (along with the focusing medium) into 1-μL-water droplets pre-loaded on the target plate. The remaining steps of the operations were identical to those in Figure 6.3B. Similar results were obtained, which confirmed the severe suppression of the MS signal by the additives.

We also tried to deposit the CIEF-separated proteins directly to the target plate. This experiment failed because we could not deposit the solution to the target plate owing to the solvent evaporation when the solution moved out of at the capillary tip.

Since a MALDI matrix solution was utilized to facilitate the protein ionization, the question arose if it was possible to use this solution to replace the water on the target plate. According to the experimental results the matrix solution exacerbated the signal suppression effect.


3.6%
S/N<1
1.8%
S/N=2
0.9%
S/N=96
15000
17500
20000

A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1.8%
S/N=20
0.9%
S/N=246
15000
17500
20000

B

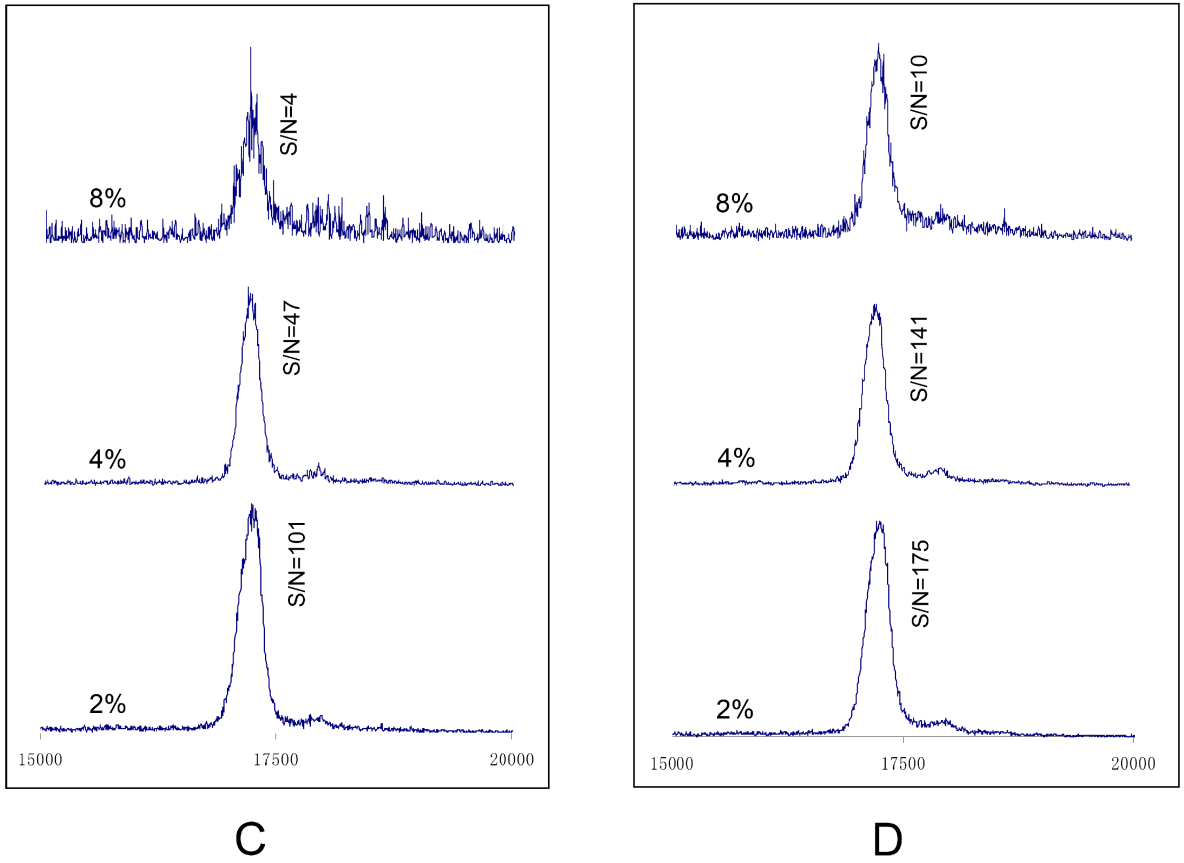


Figure 6.3 Effect of Pharmalyte and CHAPS on MS signal. Spectra in A and B were obtained from the protein-Pharmalyte mixtures, whereas spectra in C and D were obtained from the protein-CHAPS mixtures. Reprinted from [68] with permission.

The detailed mechanism of how the water droplet reduces the signal-suppression effect has not been systematically investigated. Presumably, the water droplet might have facilitated a "separation" of the additives from the proteins. According to the literature [204,205] the diffusion coefficient of a molecular is proportionally to the square root [204] or the cubic root of its weight [205]. Since the molecular weights of Pharmalytes and CHAPS are close to or less than 600 Da while those of the proteins used in this experiment are from 14 to 20 kDa, the diffusion coefficient of a protein is 2~6-fold smaller than that of an additive. As the mixture of additives and proteins was introduced to the middle of the water droplet, small additives diffused rapidly outwards while large proteins stayed where they were (diffused slowly). As the solvent was evaporated, the additives and proteins were somehow "separated".

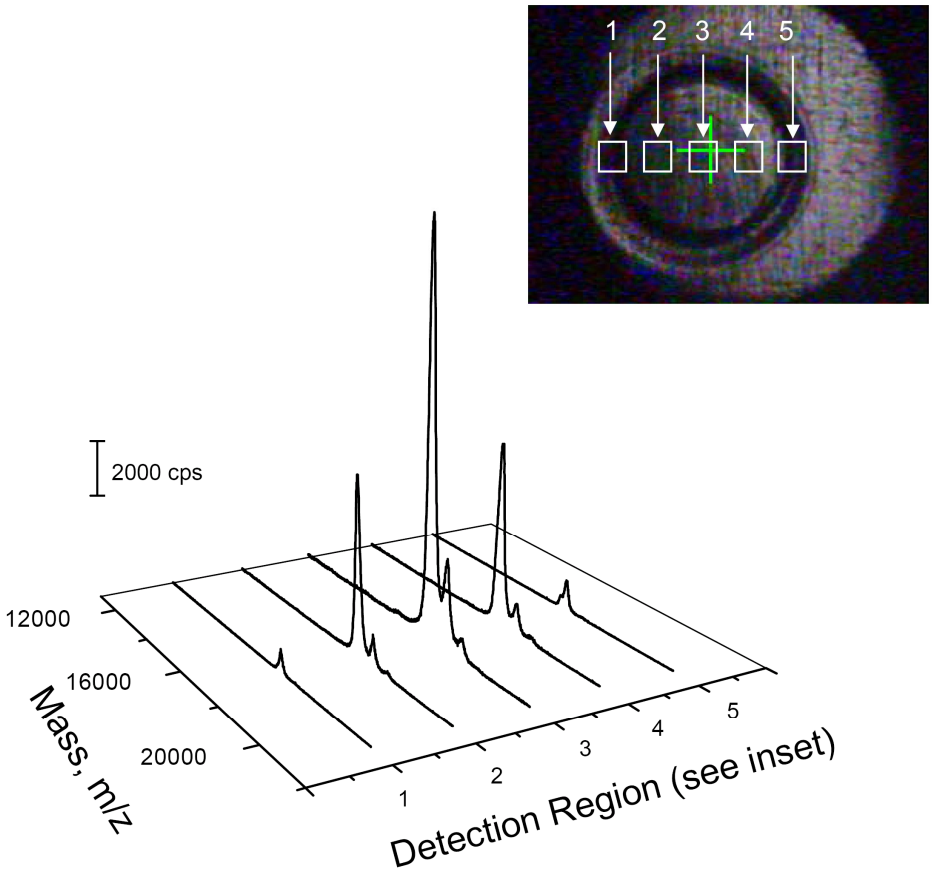


Figure 6.4 Effect of detection region on MS signal. Reprinted from [68] with permission.

6.3.2 Effects of detection region on MS signal

After a dry sample spot was produced on the target plate, we focused the laser in different regions of the sample spot (see the inset in Figure 6.4) and measured the MS spectra. The MS spectra were obtained by moving the detection region from the left side to the right side of the sample spot (see inset). The sample contained 0.05 μg/μL horse myoglobin, 0.9% Pharmalyte, 2% CHAPS and 0.5 mM ammonia acetate. The sample was loaded into a

cross-linked polyacryamide coated capillary (60 cm long × 150 μm i.d. × 375 μm o.d.) with a CA membrane grounding interface. A high voltage (20 kV) was applied across the capillary for 20 minutes to focus the protein. The focused protein was hydraudynamically mobilized by raising the anolyte reservoir by 2 cm. The sample exiting the capillary was delivered to 1 μL of water pre-deposited on the MALDI target plate. After 30 seconds (~0.1 μL sample collection), the sample was delivered to another water droplet. This operation was repeated until all the sample was mobilized out of the capillary. The sample was dried, and 0.5 μL of the matrix solution was added to the sample spot. After the matrix solvent was evaporated, the target plate was transferred to Applied Biosystems 4800 Proteomics Analyzer for mass spectra measurements.

Figure 6.4 presents the results as the laser was moved from one side the sample spot to the other. The highest S/N was obtained from the center region, which supported our hypothesis that most of the proteins remained in this region while the additives diffused to the edge.

6.3.3 Optimization of water droplet size

In the above test, the MS signal was likely affected by two parameters – the degree of the Pharmalyte and CHAPS being separated from the protein and the dilution of the protein. For example, if a large water

droplet was used, it should facilitate the protein-additive separation (to enhance the MS signal), but it should also dilute/spread the proteins (to reduce the MS signal). How will the water droplet size affect the MS signal? Figure 6.5 presents the MS signal as a function of water droplet size.

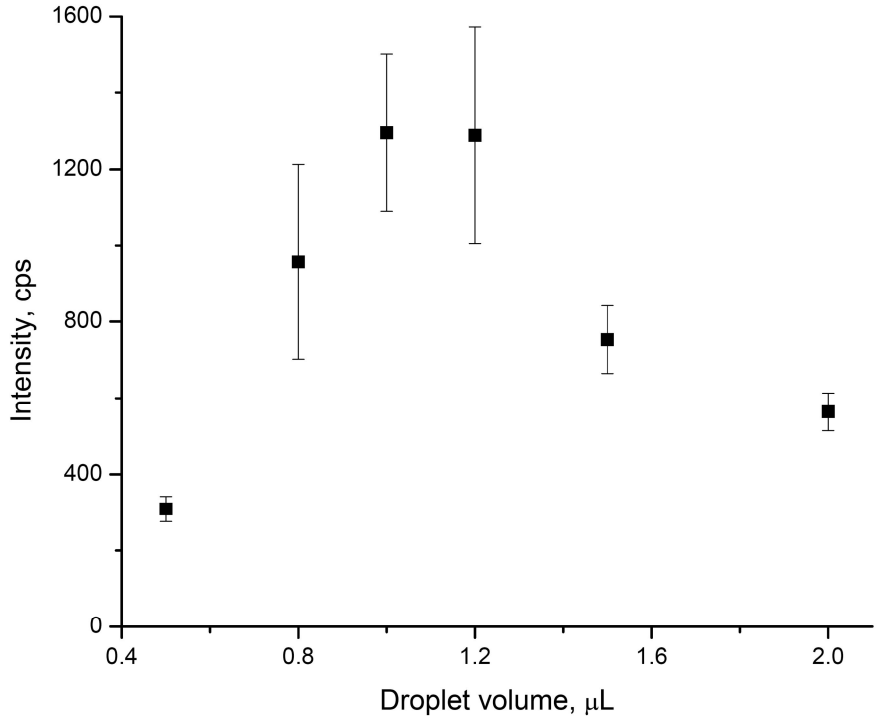


Figure 6.5 Optimization of water droplet size. Reprinted from [68] with permission.

The volume of the water droplet pre-deposited on the MALDI target plate changed from 0.5 μL to 2.0 μL. The sample contained 0.05 μg/μL horse myoglobin, 0.9% Pharmalyte, 2% CHAPS and 0.5 mM ammonia acetate. ~0.1 μL of the sample was delivered to the water droplet. All other conditions were the same as in Figures 6.3A and 6.3C. The error bars were

obtained by repeating the same tests for three times, from CIEF separation to MS measurement.

The signal increased with the droplet size as it changed from 0.5 μL to 1.2 μL, and then decreased from 1.2 μL to 2.0 μL. Overfilling was also observed when the droplet size was larger than 1.2 μL. In this experiment, we selected 1.0 μL droplet size throughout this work.

To validate the above mechanism hypothesis, we deliberately mixed the droplet solutions after aliquots of a CIEF-separated protein sample were delivered to them. The MS signal-suppression data were comparable to those in Figure 6.3B. Although these results cannot validate our hypothesis, they suggest that the differential diffusion could be one of the mechanisms which had contributed to the de-suppression of the MS signal. A systematic investigation of the mechanism is in progress in our lab, and the results will be reported elsewhere.

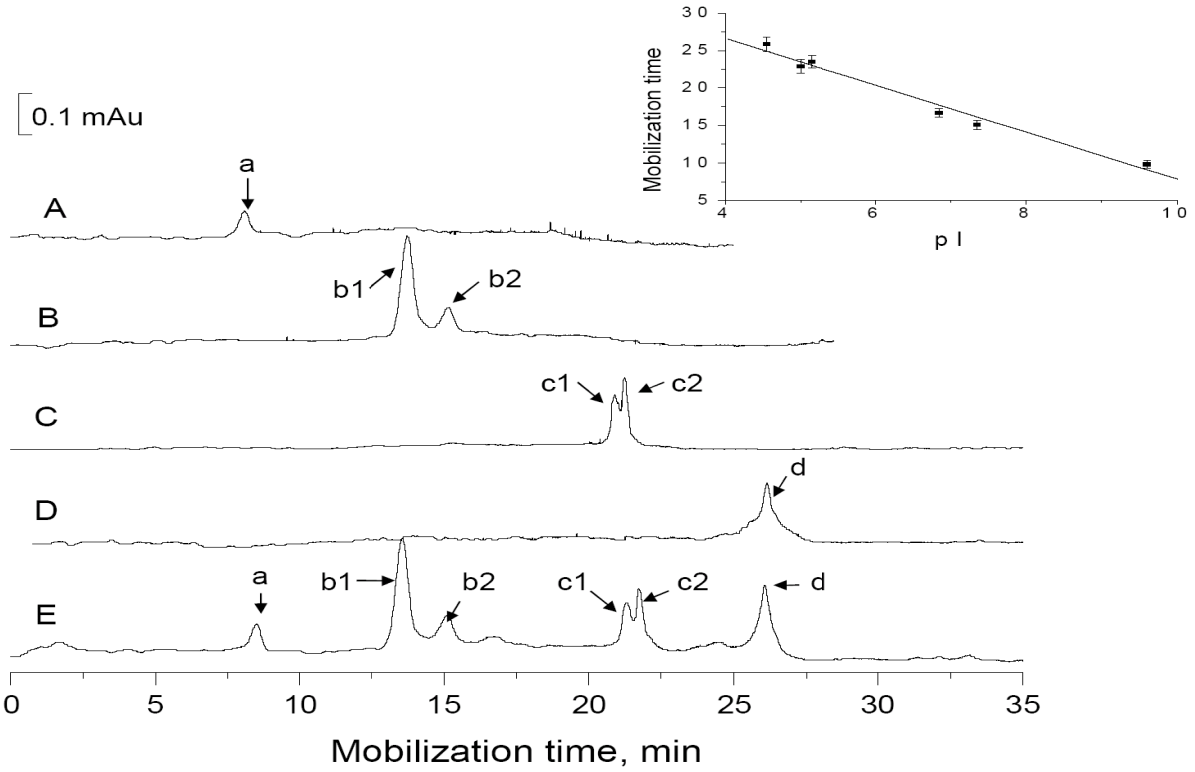


Figure 6.6 CIEF and mobilization of standard proteins. a) ribonuclease A, *p*I 9.60; b1, 2) myoglobin, *p*I 7.35 and 6.85; c1, 2) *β*-lactoglobulin A and B, *p*I 5.15 and 5.30; d) soybean trypsin inhibitor, *p*I 4.55. Reprinted from [68] with permission.

6.3.4 CIEF separation of standard proteins

Figure 6.6 presents the traces of CIEF separations of these proteins. The CIEF separations were performed in a cross-linked polyacrylamide coated capillary (60 cm long × 150 μm i.d. × 375 μm o.d.) with a focusing medium containing 0.9% Pharmalyte, 2% CHAPS and 0.5 mM ammonia acetate. We employed a 60 cm long and 150 μm i.d. (versus commonly 50 μm i.d.) capillary to perform the CIEF separation, because we could load more proteins inside the capillary to facilitate the following MALDI-MS detection. The mobilized proteins were monitored using an absorbance detector at 280 nm. Traces A-D were obtained from individual proteins, and trace E was obtained from a mixture of all these proteins. The inset shows the linear relationship between mobilization time and *p*I value. The protein positions correlate well with their *p*I values, evidenced by a good linear relationship (R^2 = 0.975, see the inset) between mobilization time and protein *p*I.

6.3.5 2-D (CIEF-MALDI-MS) separation of standard proteins

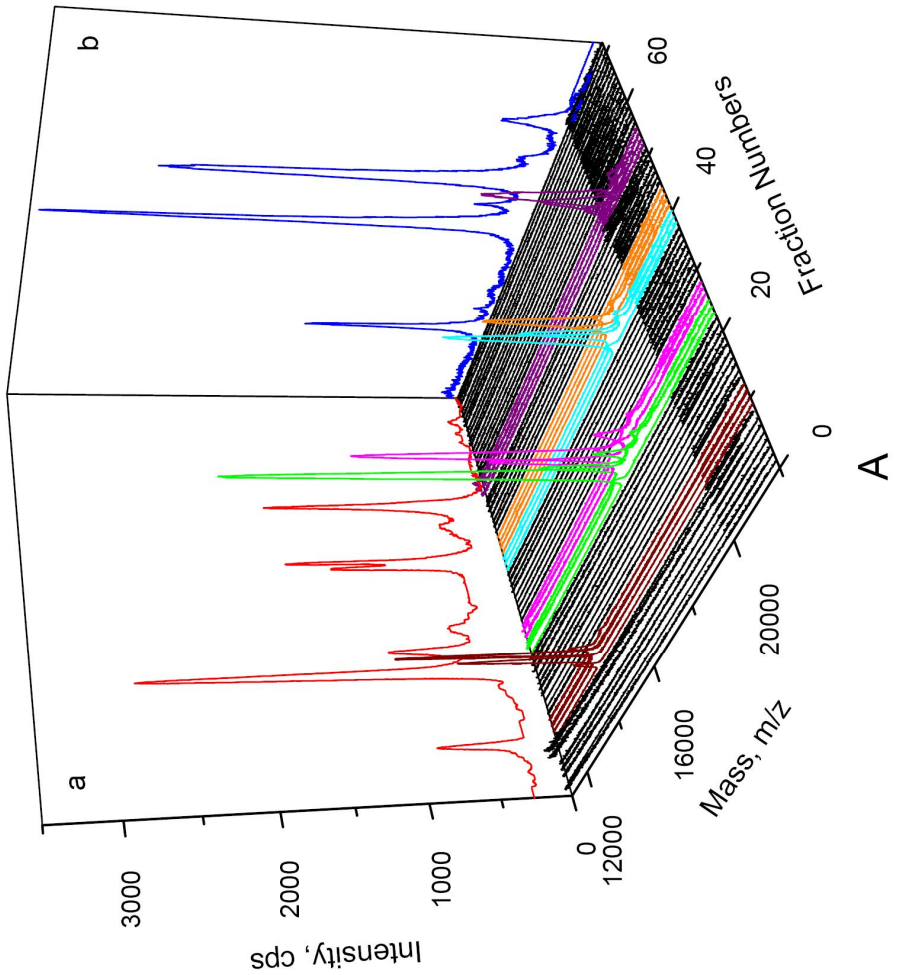
To demonstrate the fractionation of CIEF-separated proteins for MALDI-MS detection, we used the same four proteins as a model sample. The sample contained 0.05 μg/μL ribonuclease A (peak a), 0.0065 μg/μL horse myoglobin (peaks b1 and b2), 0.003 μg/μL β-lactoglobulin B & A

(peaks c1 and c2) and 0.05 μg/μL soybean trypsin inhibitor (peak d). After CIEF, the separated proteins were fractionated and deposited onto a MALDI target plate and MS spectrum of each fraction was measured, following the procedure as described in the experimental section. The CIEF separation results with UV absorbance detection (the trace in plane *a* of Figure 6.7A and the trace at the very top of Figure 6.7B) was obtained following the procedure as described in Figure 6.6. The MS spectrum of the standard protein mixture (the trace in plane *b* of Figure 6.7A and the trace on the left side of Figure 6.7B) was obtained using proteins without additives. All other spectra were obtained following the procedure as described in Figure 6.4, with detection at the central region.

All spectra were reconstructed into Figure 6.7A, representing a 2-dimensional (2-D, CIEF and MALDI-MS) separation in a 3-dimension format. In addition, we added the CIEF trace (with UV detection) on plane *a* and MALDI-TOF-MS spectrum of the same mixture on plane *b* in Figure 6.7A to assist the identification of all peaks from the 2-D separation. Figure 6.7B is another representation of the same set of data, from which we can see the 2-D separation peaks more clearly.

In this experiment, we used a collection time of 30 seconds for each fraction. Based on the results shown in Figure 6.7B, 90% of the CIEF resolution was retained. Obviously, when the CIEF resolution is high and the

proteins have very close *p*I values, one should reduce the collection time to retain the CIEF resolution. In doing so, one should always keep in mind that adequate protein(s) are deposited in the sample spot for MALDI-MS detection.


a
b
Intensity, cps
3000
2000
1000
0
12000
16000
20000
Mass, m/z
0
20
40
60
Fraction Numbers
A

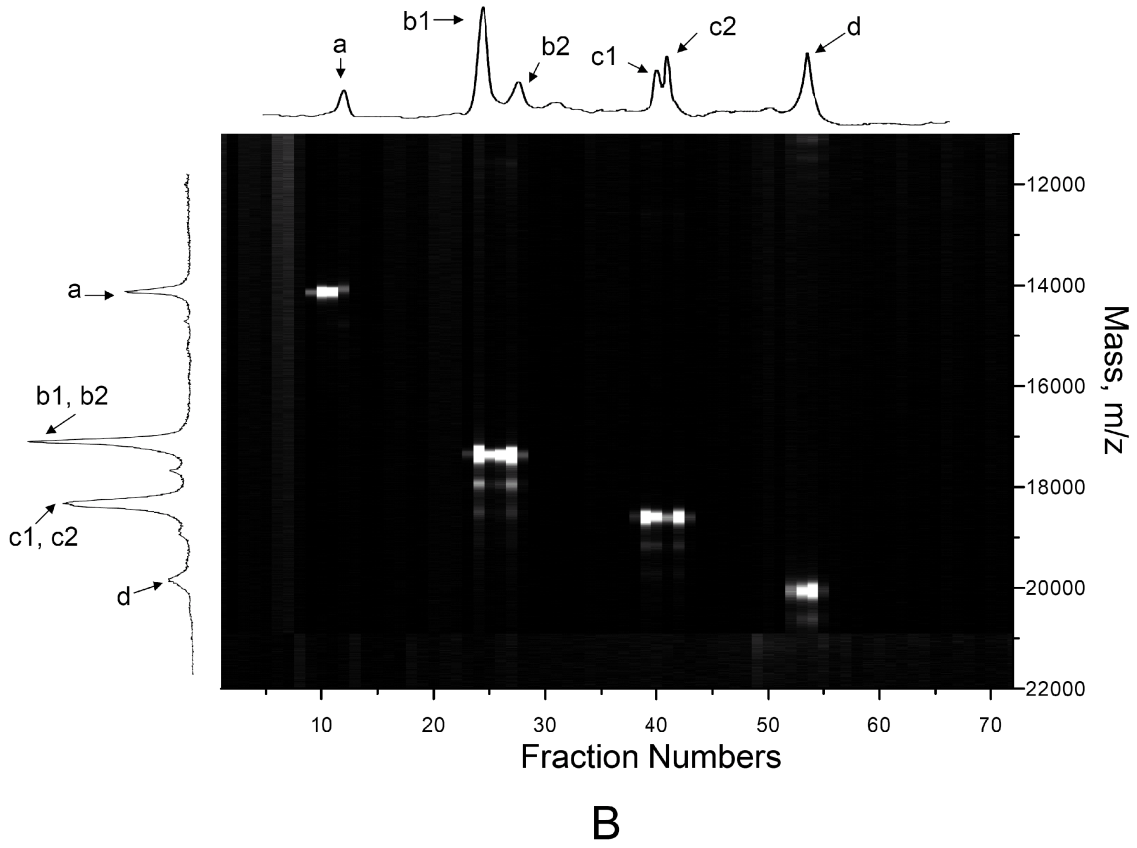


Figure 6.7. Two-dimensional (CIEF-MALDI-MS) separation of standard proteins. Reprinted from [68] with permission.

To test the limit of detection (LOD) of this method, we performed the same tests using more dilute standards, and obtained these LODs (S/N=3): ribonuclease A – 7.7 pmol, myoglobin horse – 0.82 pmol, and β -lactoglobulin A & B – 0.35 pmol. These numbers are several times higher than those of MALDI-MS analysis of pure proteins.

6.3.6 2-D (CIEF-MALDI-MS) separation of *ApoA-I*

The practical application potential of this 2-D separation approach was demonstrated by analysis of *apoA-I*, a multifunctional exchangeable apolipoprotein whose plasma concentration is inversely correlated with the incidence of cardiovascular disease [202]. The sample consisted of 0.05 μg/μL ribonuclease A (peak a), 0.124 μg/μL *apoA-I* (peak b), and 0.025 μg/μL *β*-lactoglobulin B & A (peak c1 and c2). Peaks d and e were from small molecule impurities. All other conditions were the same as described in Figure 6.7B. *ApoA-I* consists of 243-amino acids and has a molecular weight of 28.0 kDa. Figure 7 shows the 2-D separation of *apoA-I* mixed with ribonuclease A and *β*-lactoglobulin A & B. From fractions 40-41, we observed two mass peaks, one at ~28.0 kDa and the other at ~14.0 kDa. The latter was from the double charged *apoA-I*. Although *apoA-I* and *β*-lactoglobulin B were not well separated in the CIEF, and the double charged *apoA-1* and ribonuclease A were not well separated in the MALDI-MS, these proteins were well separated in the 2-D separations.

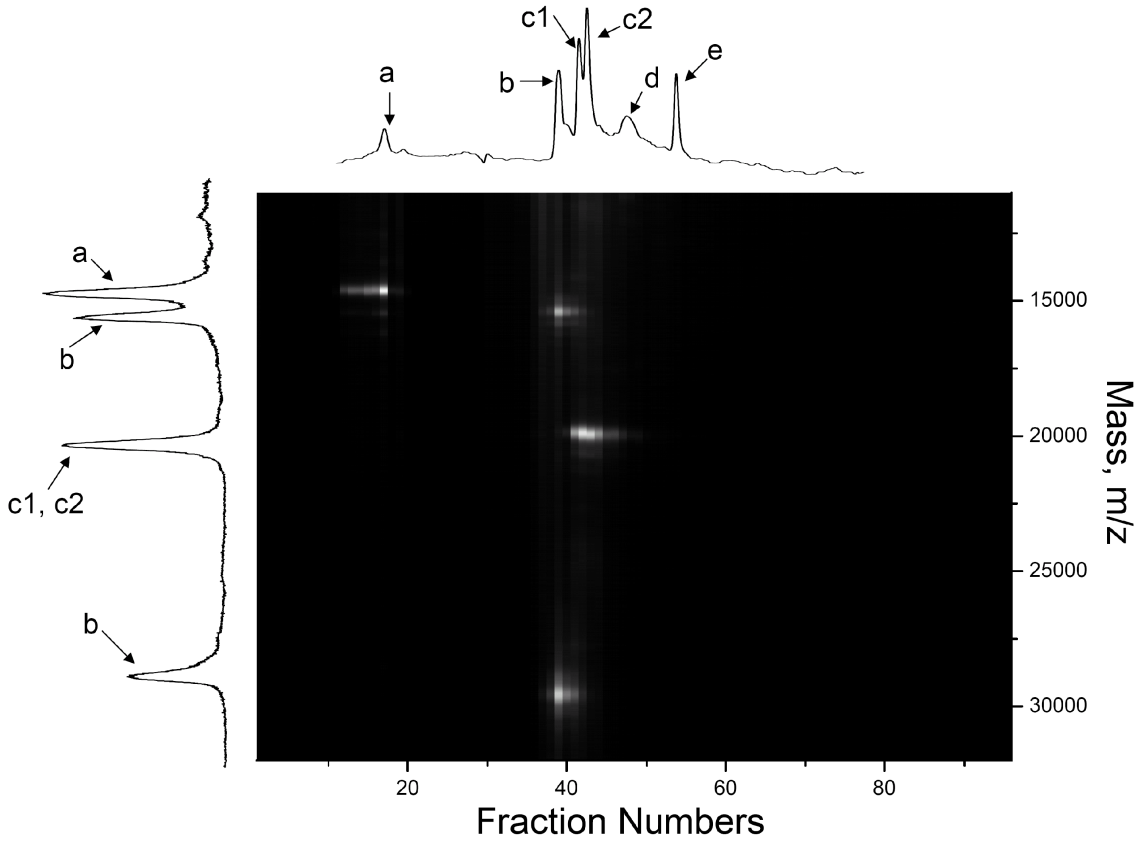


Figure 6.8 Two-dimensional (CIEF-MALDI-MS) separation of *apoA-I*. Reprinted from [68] with permission.

6.4 Concluding remarks

In this chapter, we have combined CIEF and MALDI-TOF-MS for 2-dimensional separations of proteins. We have reconfirmed that Pharmalyte and CHAPS from CIEF severely suppress the MALDI-TOF-MS signal, and developed a simple but effective means to alleviate this effect. We have also demonstrated the potential of this method for practical protein analysis.

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Chapter 7: Conclusions and future directions

7.1 Conclusions

Hyphenated analytical techniques have broad applications in qualitative and quantitative analysis of drug substances and drug products in pharmaceutical industry. The established HSGC-FID method is able to screen 44 ICH Q3C class 2 and 3 residual solvents in drug substances. The LC-MS/MS provides us simple, sensitive and robust analytical methods to determine the concentrations and pharmacokinetic profiles of highly polar aminoglycosides, lipopeptides, and glycopeptides in plasma samples. The studies of TCA concentration on plasma protein precipitation and sample recovery demonstrate a reliable sample preparation procedure for polar compounds. Meanwhile, an efficient LC-MS/MS method is successfully developed and validated for determination of fifteen estrogens and metabolites in human serum. The sample derivatization procedures are optimized, and sample stability is assessed. The method is specific, accurate, precise, sensitive and linear within the calibration range and significantly shortens the separation time and increases sample throughput in epidemiologic research. The hyphenation of CIEF-MALDI-MS could be potentially used in identifying low abundance proteins in proteomics research. Applying a simple interface between CIEF and MALDI target plate with small droplet water alleviates the signal suppression from the MALDI-MS by removing carrier ampholytes and

detergent from the sample spots. Overall, the hyphenated analytical techniques offer mutli-dimensional separation and detection and can accelerate drug discovery and development process in pharmaceutical industry.

7.2 Future directions

As increased sample throughput and fast data processing are desired, laboratory automation needs to be addressed with hyphenated analytical procedures. The future instruments will emphasize simplifying interfaces to combine two or more different techniques for drug discovery. The hyphenated techniques combine new technologies, enabling to analyze more difficult samples and offer us faster and richer information for drug discovery and development [1,206].

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