PART I. THE POWER OF CE: ILLUSTRATIONS OF THE UNRIVALED SELECTIVITY OF CAPILLARY ELECTROPHORESIS

PART II. QUANTITATIVE ANALYSIS OF POLYPEPTIDES FROM RAT PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

by

DAVID CHRISTIAN DELINSKY

(Under the Direction of Michael G. Bartlett)

ABSTRACT

This dissertation is divided into two parts. **Part I. The Power of CE: Illustrations of the Unrivaled Selectivity of Capillary Electrophoresis** and **Part II. Quantitative Analysis of Polypeptides from Rat Plasma by Liquid Chromatography/Mass Spectrometry.** The purpose is to describe state of the art technologies in bio-analytical chemistry and how they can be applied for pharmaceutical and biomedical analysis. The introduction highlights the history of separation sciences and describes modern biological sample preparation, separation methodology, and detection using mass spectrometry.

Part I, as the title suggests, focuses on capillary electrophoresis and how this extraordinarily selective separation technique can be used to resolve, not only compounds with very similar structure, but individual chiral enantiomers of drugs as well. Chapter 1 describes a method for the quantitative determination of several barbiturates from meconium. The method could be used to assess fetal exposure to barbiturate drugs. Chapter 2 demonstrates a method that is able to selectively monitor two enantiomeric forms of an antiviral drug for the study of possible preferential metabolism of one enantiomer over the other.

Part II presents several related methods for the quantitation of polypeptides of various sizes from blood plasma. In general, peptide quantitative analysis has been difficult without the use of immunoassays. In chapters 3-5, we demonstrate how HPLC/MS methods can be used to quantify polypeptides from plasma in support of pharmacokinetic studies. This could be a useful tool for the biotechnology industry as peptide based drugs are developed and ultimately brought to the marketplace.

INDEX WORDS: Capillary Electrophoresis, Barbiturates, FTC, Antivirals, Chiral Separations, Cyclodextrins, Peptide Quantitation, Polypeptides, LC/MS, Glucagon, Motilin, Galanin

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DEDICATION

This dissertation is dedicated to my father, Richard Thomas Delinsky. He instilled in me the patience and attention to detail that is required for such work to be done. Without him, I would not be the person that I am nor would I have been able to accomplish half of what I already have up to this point in my life.

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INTRODUCTION

History is an important part of understanding anything whether it is the history of the earth, humanity, or separation sciences. To understand where we have been can give us a better understanding of where we may be going. Understanding history can save us from making the same mistakes that others have made in the past. It can also give us ideas for ways to solve problems using older techniques that may work better with current technology. For example, James Jorgenson developed capillary electrophoresis and capillary electro-chromatography in the early 1980's [1-3]. Neither electrophoresis nor chromatography were new techniques, but Jorgenson knew of the work that others had done in the past. By studying the problems with the methods, he was able to develop a new technology that is widely used today. The only way for us to make the advances in chromatography that will lead to tomorrow's methodology is for us to understand the work of our predecessors. Major events in the development of chromatography are listed in Table 1.

A Brief History of Chromatography

Chromatography is nearly 100 years old –Mikhail Tswett's initial description of adsorption column "chromatography" was in the early 1900's. Even though Tswett is considered the father of modern chromatography, its roots are older and stretch back to the middle of the 19th century with Runge's work with the separation of dyes on paper

[4]. Goppelsroeder later pioneered an early version of paper chromatography, but he did not understand the mechanism of the separation and assigned them to be a result of capillary action, which was due to the different viscosities of the various sample components [4].

In the late 1890's, a petroleum chemist by the name David Day suggested that petroleum in the ground could be partially separated by passing through limestone and shale. He believed that this was the reason why oils in different regions (primarily Pennsylvania and Ohio) had differing compositions. Although Day never described his experimental setup, he claimed partial separation of crude Ohio oil by using gentle pressure to pass the crude oil through a bed of limestone then through a bed of finely divided clay resulted in a thinner, lighter colored oil similar to that found in Pennsylvania. Day called his method petroleum filtration [4]. A German chemist, C. Engler, continued Day's work with petrochemicals and was the first to use a flow-through system rather than dividing the column and displacing the separated fraction. Engler was succeeded by Ubbellohde, and in 1909 was the first to suggest that the separation was due to adsorption and not capillary diffusion [4].

At the same time, but unknowing of the American and German discoveries in petroleum separations, a Russian botanist by the name Mikhail Tswett was working on the separation of the two forms of chlorophyll. His work was summarized in several publications between 1903 and 1906. In this method, leaf extract was placed on the top of a petroleum ether saturated column of packed calcium carbonate. Pure ether was added to the top of the column and allowed to run through, thereby resolving the leaf pigments. Tswett called his preparation "a chromatogram, and the corresponding method

the chromatographic method" [4,6]. Contemporary chemists dismissed Tswett's work as careless and irreproducible [6]. Arguments raged between Tswett and various prominent chemists of the time until 1912 when Willstätter was finally able to reproduce Tswett's experiments with chlorophyll. Unfortunately, Willstätter was recognized for his work with chlorophylls by receiving the Nobel Prize in Chemistry in 1915, even though all the work had been done years earlier by Tswett. Even after this, Willstätter refused to accept chromatography as a generally useful technique [4].

For many years, chromatography was not a widely used technique. The time period from about 1910 to 1930 is sometimes referred to as the "dormant period" of chromatography [4]. It wasn't until after Edgar Lederer's initial work with chromatography in late 1930 and 1931 that the world was reawakened to Tswett's method [4]. Lederer used the chromatographic method to semi-preparatively separate xanthophylls [6]. Soon after Lederer published his reports, chromatography swept through Europe and the United States. By the mid-1930's, nearly every laboratory was using the chromatographic technique [4].

World War II obviously slowed scientific work, but it certainly did not stop it. In neutral and isolated countries such as Switzerland, Sweden and the United States, the development of chromatography barely slowed despite the war [4]. Arne Tiselius was a biochemist at Uppsala University, in Sweden. Here Tiselius worked with the theory of adsorption chromatography and pioneered new chromatographic methods in adsorption chromatography, 'frontal analysis' and 'displacement development' [4,6]. In 1940, he also developed the first chromatographic detector, which was used to continuously monitor the effluent from a developing column [4]. In addition to Tiselius's

contributions, he began using the first primitive step gradients in chromatography [4]. In 1948, the Nobel Prize in Chemistry was awarded to Tiselius for both his developments in adsorption chromatography and his 1937 development of moving-boundary electrophoresis [4,6,7].

The war years also yielded the beginnings of thin-layer chromatography (TLC) by Izmailov and Shraiber in 1938 at the Ukranian Institute for Experimental Pharmacy [6,8], but TLC did not rapidly develop until after 1949 when Meinhard and Hall added a binder to hold the stationary phase in place [4,9]. 1939 brought the first ion-exchange chromatography by Taylor and Urey [4]. Within the next few years, one of the most significant achievements in modern chromatography took place.

Beginning in 1932 at Cambridge University, Archer Martin and later with Richard Synge started working on separations that eventually developed into partition chromatography [6]. Martin had begun by exploring the use of "countercurrent separations" for the extraction and separation of vitamin E using separatory funnels. He built elaborate machines to mix and move the two solvents from one chamber to another to aid his separations as he considered 45 individual separations by hand with funnels to be impractical. In 1937, Martin was introduced to Synge and presented with the problem of separating acetylamino acids that were extracted from wool. Synge thought that a machine similar to the one that Martin had used for vitamin E might work for the acetylamino acids. The machine developed did separate the compounds, but the separation took a week, filled the air with chloroform vapors, and had to be constantly monitored. Due to the time required for the separation and the fact that he did not particularly enjoy breathing chloroform, Martin pondered ways to improve the separation

process. His epiphany came in 1940 when he realized that all of the trouble came from having to move two immiscible liquids in opposite directions through each other. He realized that if he could move only one liquid, the separation should still take place and the entire system should be much more simplified. Martin and Synge found that ground silica gel (intended for use as a drying agent) held nearly its own weight in water without being wetted. They packed hydrated silica gel into a glass tube and by passing chloroform through the column were able to separate the acetylamino acids in much less time with less instrumentation and solvents. These findings were published the following year. For this development of partition chromatography, Martin and Synge were awarded the 1952 Nobel Prize in Chemistry [4,6].

In 1943, Martin and Synge were still working together – determining the amino acid sequence of gramicidin-S [6]. They also, while working with Consden and Gordon, developed liquid-paper chromatography for the separation of amino acids [6,10,11]. The same year, Liesegang took paper chromatography one step further by developing the paper in two different directions, giving birth to 2-D paper chromatography [6].

After World War II, Martin continued his work with the introduction of two more chromatographic techniques that turned out to be very important to the scientific community – namely the development of reversed-phase chromatography and gas-liquid partition chromatography. Reversed-phase chromatography was introduced by Howard and Martin in 1950. Although others attempted to reverse the phases in partition chromatography, Howard and Martin were the first to do it successfully. This was done by treating kieselguhr with dichlorodimethylsilane vapor, rendering the packing material unwettable by strongly polar solvents. This hydrophobic phase was then able to retain

nonpolar solvents of a system. It was found that the higher the surface tension between the two solvents, the slower the stationary phase was lost, thereby increasing column stability and lifetime [4,12]. Another step for chromatography came in 1952 with the first true gradient separation by Tiselius's group [4]. Although other chromatographers were using similar techniques by changing solvents, which were in effect step gradients, the difference was that Tiselius used a continuous, gradual change in mobile phase composition [4].

In 1950, Tony James joined Archer Martin's group. The following year at the Oxford Congress for Analytical Chemistry, the two presented a new chromatographic method, gas-liquid chromatography, with which they used to separate natural mixtures of amines. In 1952, they published a detailed report of their experiments, the automatic titrating machine that was the detector, and the theory of gas-liquid chromatography [13]. The theory was actually based on a prediction that Martin and Synge had made in their 1941 introduction of partition chromatography [6,13-15]. After the 1952 landmark publication, gas chromatography (GC) spread very quickly around the world, and new developments came quickly. In the coming years, new detectors for GC were invented. 1958 brought the flame ionization detector (FID), which was developed by McWilliams and Dewer [16]. The FID quickly became the standard detector for GC. 1958 also brought another breakthrough in gas chromatography. Marcel Golay and Greult Dijkstra introduced capillary columns for the use in gas chromatography and revolutionized GC before the technique was even ten years old [6].

1959 brought more advances to gas chromatography with the introduction of several new detectors including the electron capture detector which was developed by

James Lovelock and Seymour Lipsky [17,18]. Also in 1959, R. S. Gohlke published his work on coupling gas chromatography with mass spectrometry [15,19]. This innovation allowed scientists the ability to take advantage of both the highly efficient separating ability of gas chromatography and the specificity, sensitivity, and the ability to gain structural information provided by mass spectrometry.

The 1950's also included advances in other chromatographic areas, even though most were overshadowed by the seemingly pervasive gas chromatography. One was the introduction of gel filtration chromatography in 1956 by Flodin and Porath. Gel filtration became commercially available just two years later [6]. Stahl instituted a standardization of thin-layer chromatography in 1958 [7,20]. Although these advances were undoubtedly important, one would eventually prove to be even bigger than gas chromatography. The Amino Acid Analyzer was introduced in 1958 by Stanford Moore, Darrel Spackman, and William Stein. This instrument was able to automatically analyze the amino acid composition of digested peptides and proteins in less than 24 hours [4,21,22]. It was not the amino acid analysis, but the fact that it was a closed system that used pumps to pressurize the mobile phase and force it through the packed column that made the system so important. The Amino Acid Analyzer is in fact the direct forerunner of modern high performance liquid chromatography or HPLC.

In 1962, Klesper, Corwin, and Turner developed supercritical fluid chromatography (SFC) [23]. The new technique was not commonly used for many years, mainly because of the difficulty associated with maintaining the high temperatures and pressures needed to keep the gas supercritical. Novotny and Lee introduced capillary SFC in 1981, but it was still difficult to maintain the temperature and pressure. It was not

until 1986, with the introduction of the first reliable commercial systems, when interest in SFC began to grow. The 1990's brought more advances in SFC instrumentation, but laboratory interest in the method remains much more in favor of high performance liquid chromatography and gas chromatography [23].

1963 brought more types of detectors to be used with gas chromatography. Meritt *et al*. introduced the first UV detector for GC, but the detector turned out to be less sensitive than others that were already in use [16,24]. The UV detector was revolutionized in 1968 by J. J. Kirkland when he modified a UV detector for a gas chromatograph to be used in a liquid chromatography system [16,25]. The UV detector went on to become the standard detector for nearly all high performance liquid chromatography systems.

Bioaffinity chromatography was first introduced by Axén, Porath, and Ernback in 1967 [4]. They were able to bind enzymes, antibodies, and other biologically active compounds to an insoluble polymer, while maintaining at least partial activity. This polymer could be used as a packing material in chromatographic columns [26,27]. As a result, one could make a separation system that was very specific to a particular compound or a class of compounds, particularly proteins and peptides [28]. Bioaffinity chromatography was welcomed and quickly accepted for use in protein analysis [4].

In 1972, Ronald Majors and Jack Kirkland introduced small particles for LC column packing [5,29,30]. The size of the particles could be controlled to be 10 microns or less with a small size distribution range. These particles allowed higher pressures to be generated and much higher separation efficiency to be reached [6,30]. This and the soon to come improved instrumentation modernized liquid chromatography into the high

performance separation technique that it is today, although the term “high performance liquid chromatography” was coined two years earlier by Csaba Horváth at the 21st Pittsburgh Conference on Analytical Chemistry. Horvath, a pioneer of modern chromatography, gave a talk entitled “High-Performance Liquid Chromatography” [6]. Obviously, the name stuck.

The first mating of liquid chromatography with mass spectrometry also came in 1972 with the first capillary LC inlet by Tal’rose *et al*. [31]. The 1970’s brought a flurry of developments looking to improve LC interfaces with mass spectrometry. This included the first commercially available mass spectrometer inlet for liquid chromatography – the moving belt interface, first available in 1977 from the Finnigan Corporation [31]. The moving belt was a modification of the moving wire detector for LC that was developed and introduced by R. P. Scott and coworkers in 1964 [6].

Modern capillary electrophoresis was introduced in 1981 by James Jorgenson and Krynn Lukacs. This included both capillary zone electrophoresis (CZE) [1,2,32] and capillary electrokinetic chromatography (CEC) [3,33]. CEC effectively combined the “plug flow” characteristics that gives CZE its high separation efficiency with the selectivity of partition chromatography, and allowed the separation of both neutral molecules and ions. This was not the first time that electrophoresis had been demonstrated in a glass capillary (Hjertin did this in 1967 [5,34]), but this was the first time that such high voltages had been used and certain problems such as electroosmosis, Joule heating, and poor sensitivity of the detectors had been addressed. The improved methods provided excellent separation efficiency, with over 400,000 theoretical plates for capillary zone electrophoresis [32] and greater than 30,000 theoretical plates for CEC

[2,35]. A few years later, in 1984, S. Terabe introduced the next advancement in capillary electrophoresis – micellar electrokinetic chromatography [2,3,5,36]. Instead of fixed particles for partitioning, Terabe used micelles freely moving in the capillary and electrolyte solutions to provide a hydrophobic phase in solution for partitioning. Capillary gel electrophoresis was introduced by Cohen, Paulus, and Karger in 1987 [37].

The 1980's brought more advances in the new field of liquid chromatography – mass spectrometry. The first commercial thermospray LC-MS interface was introduced in 1983 [31], but its popularity was short-lived. The electrospray interface was developed by Whitehouse *et al*. in 1985 [31,38]. Bruins, Covey, and Henion introduced pneumatically assisted electrospray in 1987 [39]. This "soft" ionization source became the most widely used means of ionization for LC-MS applications as thermal stability of the analytes was no longer an issue as it was for thermospray. In 1988, an important observation was made with electrospray. Fenn and Henion's groups both concurrently observed multiply charged ions of peptides and proteins when ionized by electrospray [31,40].

Table 1. The History of Chromatography

1850-55	Runge – Separation of dyes on paper (for artistic purposes) [4]
1861-1911	Goppelsroeder – Capillary Analysis [4]
1897-1911	Day – Petroleum separations by filtering through limestone and shale [4]
1901	Engler – First flow through system [4]
1903	Tswett – Lecture describing his separation technique based on adsorption [4]
1906	Tswett – First of his publications and giving the term "chromatography" [6]
1930-31	E. Lederer & R. Kuhn – Rebirth of chromatography in Heidelburg, Germany; first preparative use of chromatography [6]
1936	Koschara – Rediscovery of the "flowing chromatogram" [4]

1937	Tiselius – Discovery of moving-boundary electrophoresis [7]
1938	Izmailov & Shraiber – First thin layer chromatography (TLC) [6,8]
1939	Taylor & Urey – First ion-exchange chromatographic separation [4,41]
1940	Tiselius – First chromatographic detector monitored changes in the refractive index of the eluting solvent [4]
1940-41	Martin & Synge – Development of partition chromatography [6,42,43]
1943	Consden, Gordon, Martin & Synge – Reintroduction and development of paper chromatography [6,10,11]
	Liesegang – Introduction of 2 dimensional paper chromatography [6]
1946	First chromatography meeting, the Conference on Chromatography [4]
1948	Nobel Prize in Chemistry awarded to Tiselius for his work in adsorption chromatography and electrophoresis [4]
1949	Meinhard & Hall – Addition of a binder for use in TLC plates [4,9]
1950	Howard & Martin – Development of reversed-phase chromatography [12]
1951-52	James & Martin – First paper describing gas-liquid partition chromatography [6,13-15]
1952	Nobel Prize in Chemistry awarded to Martin and Synge for the development of partition chromatography [6]
	Alm, Williams & Tiselius – First use of true gradient for a separation [4]
1956-58	Flodin & Porath – Introduction of cross-linked dextran gel allowed for the first "gel filtration chromatography" [6]
1958	Moore, Sparkman & Stein – Introduction of the Amino Acid Analyzer (the precursor to HPLC) [4,21,22]
	Golay & Dijkstra – First capillary columns for GC [6]
	Stahl – Standardization of TLC [7,20]
	McWilliams & Dewer – Development of the flame ionization detector [16]
1959	Lovelock & Lipsky – Development of the electron capture detector for GC [17,18]
	Gohlke – GC was first coupled to mass spectrometry (MS) [15,19]
1962	Klesper, Corwin & Turner – Introduction of supercritical fluid chromatography (in packed

	columns) [23]
1963	Meritt *et al.* – UV detector for GC [16,24]
1967	Porath *et al.* – Development of bioaffinity chromatography [26-28]
	Hjerten – First capillary electrophoresis demonstrated [5,34]
1968	Kirkland – Modification of GC UV detector for LC system [16,25]
1972	Majors & Kirkland – Introduction of small particles for column packing in HPLC [5,29,30]
	Tal'rose *et al.* – Capillary LC inlet for the mass spectrometer [31]
1974	Scott *et al.* – Moving-wire detector modified for LC-MS [31,44]
1977	First commercial LC-MS interface – the moving belt [31]
1981	Jorgenson & Lukacs – Introduction of modern capillary zone electrophoresis using high electric fields [1,2,32]
	Jorgenson & Lukacs – Introduction of capillary electrokinetic chromatography [3,33]
	Novotny & Lee – Development of capillary supercritical fluid chromatography [23]
1983	Commercial thermospray LC-MS interface introduced [31]
1984	Terabe *et al.* – Development of micellar electrokinetic chromatography (MEKC) [2,3,5,36]
1985	Whitehouse *et al.* – Electrospray interface for LC-MS [38]
1985-86	Ito *et al.* & Caprioli *et al.* – Development and subsequent commercial availability of frit FAB and continuous-flow FAB interfaces for LC-MS [31,45,46]
1987	Cohen, Paulus & Karger – Introduction of capillary gel electrophoresis [3,37]
	Bruins, Covey & Henion – Pneumatically assisted electrospray (ionspray) for LC-MS [31,39]

Modern Chromatography

More than just a thing of the past, chromatography has had, and continues to have an effect in nearly all fields of human achievement and has played an important role in how we live and play. Various analytical methods have been used for the separation of petroleum products, for the development of pharmaceuticals, and to help us better

understand living organisms and the environment in which we live. Separation sciences have played important roles in forensics, in the development of new consumer products, and even in the sports that we love to watch and participate in. All in all, humanity would not be where it is now without the separation sciences.

Two well known separation techniques are high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). While these two techniques did eventually evolve from the initial chromatography developed by Tswett, they did not come into their current state until the last twenty to thirty years. This was mainly due to the lack of sufficient technology to produce the high pressure pumping systems needed for HPLC and stable high voltage power supplies and sensitive detectors for CE. More is behind these methods than just history. An explanation of more detail how these two methods work will be given below.

High-Performance Liquid Chromatography (HPLC)

As mentioned earlier, the modern HPLC system is a direct descendant of the Amino Acid Analyzer from the late 1950's [4,21,22]. It was not until the late 1960's with the introduction of better high pressure equipment [4] and the development of very small particles (less than 10 microns) for the column packing [5,29,30] that modern HPLC evolved. For the most part, HPLC has replaced traditional liquid chromatography (LC); therefore, the terms will be used interchangeably.

Reversed-phase chromatography is the most commonly used mode of HPLC, especially in the United States. This is due to the large number of differently selective stationary phases that are available for reversed-phase chromatographic separation and

the fact that less organic solvent is used. For these reasons and the fact that part II of this dissertation uses reversed-phase chromatography, discussion of the method will reflect reversed-phase chromatography.

The heart of HPLC is the column. Here, there are the two main parts of the system, the stationary phase (in the column packing) and the mobile phase (which is pumped through the column packing). The stationary phase is generally small particles of derivatized silica (with hydrophobic character for reversed-phase HPLC) while the mobile phase consists of a mixture of water or buffer and a water miscible organic solvent such as acetonitrile or methanol. Separation is achieved by differential retention of an analyte and other compounds from the sample. The difference in retention is a result of partitioning between the stationary phase and the mobile phase. The higher a compound's affinity for the stationary phase over the mobile phase, the longer that compound will be retained. [47]

Most all of the components of an HPLC system can be optimized for the best separation. The column and the mobile phase components should be chosen to favor retention of the intended analyte. From there, the pH of the mobile phase can be adjusted to keep the analyte in either its ionized or unionized form, and a buffer can be selected to help maintain the proper pH. The type of organic solvent and the amount in the mobile phase are chosen for both optimal retention time and good chromatographic peak shape. The percentage of organic solvent can also be altered during the separation process using gradient elution, which helps to minimize the total run time and reduce the analyte peak width. The column temperature and the mobile phase flow rate can also be optimized [47]. Detection in an HPLC system is usually by a UV detector, but it requires solvents

that do not absorb UV light and it also requires the analyte to have a chromophore [47]. In the pharmaceutical industry, this is generally not a problem because most drugs have good chromophores, but in other areas such as monitoring environmental contaminants, there are many compounds of interest that are better detected by methods other than UV detection [48]. Other detection methods for HPLC include fluorescence, refractive index, and mass spectrometry [47,48]. Mass spectrometric detection will be discussed later.

Capillary Electrophoresis (CE)

Capillary zone electrophoresis (no phases for partitioning) works on a different principle than HPLC. Separation in CE is based on an analyte being charged. High voltage is applied across a silica capillary filled with an electrolyte. Cations are attracted towards the cathode and anions are attracted to the anode [1]. Separation is achieved by the charge (i.e. plus one versus plus two) on an analyte and the electrophoretic mobility of an analyte (related to cross-sectional area) [1]. The electrolyte is chosen based on the pH and the ionic strength desired of the solution. The electrolyte pH is modified so that the analyte is charged. The charge of the analyte and the electroosmotic flow (defined below) determines whether the cathode or the anode is positioned at the exit end of the capillary. Detection is achieved by either UV, laser induced fluorescence, electrochemical, or by mass spectrometry [1-3]. In CE systems with a pH greater than 4, there is a phenomenon known as electroosmotic flow. Here, there is a net liquid flow through the capillary towards the cathode while high voltage is applied. This is a result of a net positive charge close to the capillary wall, which is negatively charged. The positive charges migrate towards the cathode and "drag" the rest of the liquid with it [3].

Capillary electrochromatography [33] and capillary micellar electrochromatography [36] utilize the electroosmotic flow as a pump, similar to HPLC. The addition of an organic phase (micelles or stationary phase packed into the capillary) allows analyte partitioning and can result in the separation of both neutral and charged compounds. Chiral selectors such as cyclodextrins can also be added to the electrolyte solution to give the ability to resolve chiral enantiomers [2].

Although the sensitivity of CE tends to be lower than many other chromatographic methods, no other method can match CE in separation efficiency. Theoretical plate values of hundreds of thousands are common with relatively short capillaries and there have been reports of separations with well over one million theoretical plates [33,37]. CE is an incredibly selective separation method able to separate many closely related compounds or, with the addition of chiral selectors, CE can resolve individual enantiomers of chiral compounds. Chapter 1 and Chapter 2 illustrate CE's ability to separate closely related and chiral compounds that are extracted from complex biological matrixes.

Sample Preparation

Generally speaking, biological samples must be prepared in some way no matter what separation technique one might utilize. Gas chromatography may require liquid-liquid extraction or analyte derivatization prior to sample injection or the sample may need to be heated before headspace analysis [15]. Liquid chromatography usually requires some form of extraction whether it is by protein precipitation, liquid-liquid extraction, or solid phase extraction prior to injection [49]. There are online extraction

techniques so that one directly injects plasma into the HPLC system, but whether it is extracted online or uses turbulent flow chromatography [50,51], sample filtration is always a good idea that will help to increase system stability and column lifetime. Capillary electrophoresis generally requires the same sort of sample preparation that is required for liquid chromatography, but final composition of the sample (such as pH, organic solvent content and ionic strength) is important to maintain system stability at the high voltages that are used during the separation process [1-3]. In describing some popular sample preparation steps, focus will be placed on describing solid phase extraction and protein precipitation and what parameters generally have to be adjusted to get optimal analyte extraction.

Solid Phase Extraction (SPE)

Solid phase extraction (SPE) is "a low-efficiency adaptation of HPLC" [47]; it is a chromatographic procedure. SPE involves the extraction of an analyte(s) from a matrix or the removal of interferences from a sample using the same principles that allow retention and separation in liquid chromatography. This is performed using a stationary phase that is in the bed of a cartridge, impregnated in a filter disk, or as a coating on a fiber.

The use of a coated fiber is solid phase microextraction, as the volume of stationary phase tends to be much smaller than the amount that is available in the other SPE devices. Solid phase microextraction is adaptable to both gas chromatography and liquid chromatography. As an advantage of the method, the fiber is usually directly inserted into the injection port thereby limiting sample handling. Another advantage is

that the extraction fiber design makes it easily used to extract an analyte from either a gas or a liquid, and it does not require additional elution solvents. Some of the drawbacks of solid phase microextraction include the cost associated with the fiber. The low surface area limits the amount of analyte that can be extracted. Although the fiber can be directly inserted into a sample, biological samples can severely reduce the lifetime of the fiber. [47]

Solid phase extraction cartridges are the most commonly used device for SPE. They look similar to the barrel of a syringe with packing material retained by frits in the bottom of the barrel. The material is analogous to the packing in separation columns but the particles tend to be larger in size and irregularly shaped. The poorer quality of the packing bed helps to keep the cost of the cartridges down so they remain relatively inexpensive and therefore disposable. Extraction cartridges are available with many different packing materials to help optimize the extraction. [47] Solid phase extraction disks have many of the same properties as cartridges and have as many different phases available. SPE disks allow faster flow of liquids through them, but may lose weakly retained compounds. [47]

General use of SPE cartridges and disks for reversed-phase extractions start with a conditioning step in which an organic solvent is passed through the bedding to “wet” the stationary phase followed by water or buffer to adjust the pH and get the stationary phase ready to retain an analyte. The sample is then added to the cartridge and pulled through, generally at a slower rate than the column is conditioned, but the loading rate does also depend on the packing material used and the strength of the retention mechanism. [47] The sample should also have previously been adjusted to a proper pH without too much

organic solvent. The retained sample may be washed with water or buffer (that does not cause the analyte to be removed) to help remove unwanted matrix components. Before elution, the packing material may be dried to remove excess solvent and help release the analyte(s). The column is eluted with a minimal volume of strong solvent such as an organic solvent. The eluate may either be directly injected into the HPLC system or evaporated to dryness then reconstituted in a more appropriate solvent. [47]

Protein Precipitation

One of many alternatives to solid phase extraction, protein precipitation is favored by some because of its simplicity and speed. The idea behind protein precipitation is to "clean up" a biological sample by altering the sample so that most proteins are no longer soluble and can easily be removed. Although not the only option, there are two popular methods for protein precipitation – precipitation by the addition of an acid or an organic solvent. The addition of a strong acid, such as trichloroacetic acid or perchloric acid, to a biological sample forms protein salts with cationic proteins at low pH, thus precipitating the proteins. Adding a strong base likewise forms salts with the anionic forms of proteins, rendering them insoluble [52]. The addition of an appropriate amount of organic solvent such as acetonitrile, methanol, or ethanol to a biological sample results in a conformational change in dissolved proteins, changing their solubility [52]. Precipitation of blood plasma with acetonitrile requires approximately 1.5 times the volume of the same to completely denature proteins and 2 times the sample volume for ethanol [49]. These volumes are approximate and may need to be optimized to maximize analyte recovery.

Mass Spectrometry

First developed in 1907 by J.J. Thompson in the form of the mass spectrograph, mass spectrometry has been around essentially as long as chromatography [53,54]. Mass spectrometry grew slowly during the first half of the 20th century. With improvements in instrumentation and coupling with separation techniques, mass spectrometry began growing much more rapidly. Today, mass spectrometry is a mature analytical technique, with most advances being small increases in sensitivity here and there and performing tandem mass spectrometry by coupling different types of mass spectrometers. Over the years, mass spectrometry has become the workhorse of the pharmaceutical industry – mainly due to mass spectrometry's combination of sensitivity and selectivity especially when used in conjunction with chromatographic techniques [54,55].

Mass spectrometry can be viewed as similar to chromatography in that it separates ions based on their charge and mass. The mass spectrometer basically consists of three main parts: an ionization source, a mass analyzer, and a detector. The system is run in a high vacuum environment with the vacuum needs dependant on the type of mass analyzer that is used. Different types of mass analyzers include magnetic/electric sectors, TOF (time of flight), quadrupoles, quadrupole ion-traps, and FT-ICRs (fourier transform – ion cyclotron resonance). Ion sources include electron impact ionization, chemical ionization, fast atom bombardment, electrospray ionization, atmospheric pressure chemical ionization, and matrix assisted laser desorption ionization (MALDI), among others. The choice of the ionization technique depends both on the analyte and the type of mass analyzer used. The detectors that are used are usually some type of electron multiplier. [54]

Electrospray is the most commonly used ionization method for LC-MS [55]. Its versatility comes from its ability to ionize many different types of compounds (including nonvolatiles), the fact that it is a "soft" ionization method causing little or no sample fragmentation, and it is capable of ionizing even very large biomolecules. Electrospray ionization starts with solvent nebulization in an electric field, thus charging the small droplets. As solvent evaporates, Coulombic forces increase and the droplets may go through the processes of "Coulombic explosion" and/or charge evaporation (where individual ions are ejected from the droplets). These processes repeat until the solvent is completely evaporated and only gas phase ions remain, which are then directed into the mass analyzer. [54] The exact mechanism for electrospray ionization is not completely understood and is likely a complex mixture of these processes and redox reactions that depend largely on the compound being ionized in addition to other experimental conditions. With larger molecules, especially peptides and proteins, electrospray can put multiple charges on these molecules thereby expanding the mass range of the instrument (because the instrument measures mass to charge ratio rather than mass). [54]

Electrospray has some disadvantages such as its inability to ionize all compounds. Potential analytes must at least have acidic or basic functional groups for ionization [54]. By far, the biggest disadvantage of electrospray is that it is subject to ion suppression. During an event of ion suppression, other compounds are more easily ionized and there are not enough remaining charges to ionize all molecules of the analyte. Trifluoroacetic acid and common matrix components extracted from biological samples are known to suppress ionization of other molecules.

Quadrupole mass spectrometers are most often used with HPLC. This is mainly due to the higher operating pressure that can be tolerated by the quadrupole mass analyzer. Quadrupoles are often called “mass filters” as they do not separate all masses but instead use complex waveforms so that only a single mass to charge ratio is able to pass through the “filter”. The waveform is generated from the magnitude of radiofrequency (RF) signals and direct current (DC) potentials applied to four metal rods (hence the name quadrupole). By changing the magnitude of RF and DC, a different mass to charge ratio is able to pass through the “filter”. This is how the mass spectrometer scans a selected mass to charge range. [54]

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PART I

CHAPTER 1

SIMULTANEOUS CAPILLARY ELECTROPHORESIS DETERMINATION OF BARBITURATES FROM MECONIUM[1]

[1]Delinsky, D.C., Srinivasan, K., Solomon, H.M. and Bartlett, M.G. 2002. *Journal of Liquid Chromatography & Related Technologies.* 25:113-123.
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Abstract

Meconium is the first stool passed by a newborn and as such, represents a record of the fetal environment during the last two trimesters of pregnancy. We have developed the first capillary electrophoresis (CE) method for the analysis of meconium. This method has the potential to help tremendously in the study of fetal drug exposure. Solid-phase extraction (SPE) was used to extract the drugs (pento-, mepho-, pheno-, seco-, and amobarbital) and the internal standard, hexobarbital, from meconium. The extraction efficiency was studied using C_{18}, C_8, Silica (Si), and polymeric cartridges for samples buffered at pHs 2.5, 7.0, and 9.0. The polymeric (Oasis HLB) SPE cartridge at pH 9.0 was selected because it gave clean extractions and high recoveries for most of the studied barbiturates. The CE system consisted of a 75 μm I.D. 77 cm length fused silica capillary and a UV detector set at 254 nm. The run buffer consisted of 150 mM tris buffer at pH 7.8 and the run voltage was 25 kV (at 25 °C). Linear calibration curves show a coefficient of determination of more than 0.99 for all components. The method also showed high between run and within run precision and accuracy. The limit of quantification was 10 μg per gram of meconium. Some common drugs such as aspirin, acetaminophen, and caffeine may be taken in conjunction with barbiturates. The method completely resolved these compounds, along with several other potential interferences, from all the barbiturates in this analysis. Keywords: meconium, barbiturates, pentobarbital, mephobarbital, phenobarbital, secobarbital, amobarbital, hexobarbital, solid-phase extraction, and capillary electrophoresis.

Introduction

Meconium is the first stool passed by the newborn baby and is, in essence, a record of the drug history of the mother during the later stages of pregnancy. This biological matrix has not been widely studied but can be a sample of choice when drug abuse during pregnancy is suspected. Most of the work that has been done with meconium involves analyzing for cocaine and its metabolites from the meconium of infants born to mothers that abuse cocaine.[1] Other illicit drugs, xenobiotics, and a few heavy metals have also been studied from meconium.[2-4] The presence of barbiturates in the meconium of neonates born to mothers who have been administered barbiturates has been documented.[5] Thus, development of rugged methods that can quantitate these drugs rapidly and reliably from meconium has assumed importance.

Meconium is a complex biological matrix that contains large amounts of proteins, lipids and pigments. Due to the high level of these endogenous compounds in meconium, recovery of drugs requires significant sample preparation prior to solid-phase extraction. To date, high performance liquid chromatography (HPLC) and gas chromatography (GC) have been the most widely used techniques for determination of drugs out of biological matrices.[1-4] Currently, there are no methods for the analysis of meconium that use capillary electrophoresis (CE). The advantages of CE, such as low sample volumes, high efficiency, and low cost provide powerful alternatives to existing chromatographic methodologies in the area of therapeutic and drug abuse monitoring.[6-11]

Barbiturates are sedative hypnotics that were introduced in 1903. Despite the fact that these compounds have largely been replaced by benzodiazepines as sedative-hypnotics of choice[12], several barbiturates maintain widespread use today.

Phenobarbital is used as a treatment for epilepsy and is considered a very effective drug for this use.[13] Barbiturates are also used in some prescription sedative products.[14] Pentobarbital has been administered for sedation and to relieve stress prior to surgery.[15]

Barbiturates show some adverse reactions at doses from 0.15 to 1.5 mg/kg and are reported to cause bradycardia, hypotension, and syncope. Detrimental effects in all stages of development in the children of addicts have been observed during pregnancy, birth, during breast-feeding, and throughout maturation. Therefore controlled regulation, identification, treatment, and rehabilitation of barbiturate exposure may be warranted. These procedures would require rapid, sensitive, and accurate determination of these drugs.

HPLC[16-21] and GC[18, 22-24] methods have been devised for the analysis of barbiturates from serum, plasma and urine. These methods have found immense clinical significance and have contributed considerably to understanding the pharmacokinetics and pharmacodynamics of barbiturates. The analysis of barbiturates from meconium opens an additional window to study maternal to fetal transfer of drugs during the last two trimesters of pregnancy and would further our understanding of the processes of maternal to fetal drug transport. This paper is also a demonstration of the emerging idea that capillary electrophoresis is a technique which has the capacity to augment, if not replace, other existing chromatographic techniques.

Experimental

Reagents and Chemicals

Pentobarbital, mephobarbital, phenobarbital, amobarbital, secobarbital, and the internal standard, hexobarbital were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Phosphoric acid (85%), sodium dihydrogen phosphate monohydrate and ammonia solutions were obtained from J.T. Baker (Phillipsburg, NJ, USA). C_{18} and C_8 solid-phase extraction columns (100 mg) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). Silica solid-phase extraction cartridges (100 mg) were obtained from Alltech Associates Inc. (Deerfield, IL, USA). Polymer based Oasis^{TM} extraction cartridges were obtained from Waters Corp. (Milford, MA, USA). All SPE cartridges were 1 ml capacity. All solutions were filtered through 0.2 μm nylon membrane filters (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA).

Preparation of Stock and Standard Solutions

Individual stock solutions were prepared in methanol to give a concentration of 1 mg/ml of the analyte. The nominal concentrations of barbiturates studied were 10, 20, 40, 60, 80, 100, 120, 140 μg/g. Appropriate volumes of pentobarbital, mephobarbital, phenobarbital, amobarbital, secobarbital, and the internal standard, hexobarbital were pipetted and volume made up to 1 ml to give the above concentrations. Approximately 0.5 g of meconium was weighed and added to the analyte samples. 2 ml of 25% methanol/acetonitrile was then added to the above and the whole mixture was homogenized. The methanol and acetonitrile added helps in breaking up the meconium facilitating sample handling. This mixture was then centrifuged (3000 rpm for 30 min)

and the supernatant was removed and evaporated under vacuum. The residue was then reconstituted in 30 % (v/v) methanol/buffer and filtered prior to solid-phase extraction. For the extraction studies, sodium dihydrogen phosphate buffer was prepared in double distilled, deionized water and the pH was adjusted to 2.5, 7.0 and 9.0 using 100 mM sodium hydroxide and concentrated phosphoric acid.

Electrophoretic System

All CE experiments were performed using a P/ACE System 5000 (Beckman Inc., Fullerton, CA, USA) equipped with a UV detector. An uncoated fused silica capillary total length 82 cm, effective length 77 cm, 75 μm I.D (Polymicron Technologies, Phoenix, AZ, USA) was used for analysis. The capillary was thermostated at 25 °C and the voltage applied was 25 kV. The typical running current was about 100 μA. A 0.5 cm detection window was created by stripping the polyamide coating of the capillary. The detection was 5 cm from the cathode end of the capillary. The run buffer consisted of an aqueous solution of 150 mM tris buffer pH 7.8 (adjusted with concentrated nitric acid). The analytes were monitored at a wavelength of 254 nm.

New capillaries were conditioned by rinsing with 1 M sodium hydroxide for 5 min followed by 5 min each with 1 M hydrochloric acid, water, and run buffer solutions. The sample introduction was performed using a 5 sec pressure injection (0.5 PSI). Before each analysis, the capillary was rinsed for 2 min first with 0.1 M sodium hydroxide and 2 min with the run buffer.

Assay Procedure

Sample clean up was attempted using C_{18}, C_8, silica and polymeric solid-phase extraction cartridges with extracted samples buffered at three pHs (2.5, 7.0, and 9.0). Prior to SPE, the cartridge was conditioned using 2 ml of methanol and then with 2 ml of the appropriate phosphate buffer (either pH 2.5, 7.0, or 9.0 matched with the pH of the buffer used to reconstitute the sample). The reconstituted sample containing the drug and internal standard in 1 ml of 30% methanol/buffer (pHs 2.5, 7.0 or 9.0) was added to the cartridges and allowed to flow down under low vacuum. The SPE cartridges were not allowed to dry between the pretreatment and sample application steps. The column was then washed with 2 ml of buffer (corresponding to the respective sample pHs) and allowed to dry for 15 min. The analytes were then eluted with 3 ml of methylene chloride. The samples were then evaporated and reconstituted in 1 ml of 30% methanol/water, filtered using a 0.2 μm nylon filter and pressure injected into the CE instrument for 5 seconds. Absolute recoveries were calculated by comparing the drug peak height from spiked meconium samples to unextracted stock solutions that had been injected directly into the electrophoretic system.

Results and Discussion

The analytes eluted after the EOF (analogous to the solvent front in HPLC) in tris buffer at a pH of 7.8 with migration times from 12-19 min. Figure 1.1A shows an electropherogram of blank meconium. Figure 1.1B shows the electropherogram of the barbiturates and internal standard spiked into meconium. Fig. 1.2 shows the structures of

(A) hexobarbital, (B) phenobarbital, (C) pentobarbital, (D) amobarbital, (E) mephobarbital, and (F) secobarbital.

To increase sensitivity by reducing band broadening thereby achieving sharper peaks, the sample was prepared in a lower conductivity solvent (methanol/water) than the electrolyte solution. When a voltage of 25 kV is applied across the capillary, a greater field develops across the sample plug. This causes the ions to move faster. When the ions reach the buffer they slow down due to the reduced field to which they are subjected, this results in analyte stacking within a narrow zone of the capillary.[25,26]

SPE was attempted on four different cartridges (polymeric, C_{18}, C_8, and Silica) with samples at three different pHs 2.5, 7.0, and 9.0. The recovery of the barbiturates studied is reported in Table 1.1. The polymeric (Oasis HLB) SPE cartridge at pH 9.0 was selected because it gave clean extracts and good recovery for most components.

Common drugs such as aspirin, caffeine, and acetaminophen must not interfere with the separation of the barbiturates as they may also be taken by the mother prior to giving birth. While caffeine and acetaminophen elute in the EOF (electrosmotic flow, difference in migration time [t_Δ] of 2.8 min before the first barbiturate), aspirin has a t_Δ of 5.1 min after the last migrating barbiturate (phenobarbital). Anticonvulsants such as phenytoin may also be present; phenytoin migrates past the detector before the internal standard hexobarbital (first in the migration order) maintaining baseline resolution with a t_Δ of 0.43 min. The difference in migration times allows baseline resolution of the drugs. Atropine, an anticholinergic, also elutes in the EOF.

The calibration curve showed good linearity over the range from 10 to 140 μg/g for all the barbiturates. The coefficient of determination was greater than 0.99 (n=3).

Representative linear regression equations obtained were $y = 0.01604x + 0.01755$ (secobarbital), where y and x were drug to internal standard peak area ratios and concentration, respectively. The within-run (n=5) and between-run (n=15) precision and accuracy as expressed by % error and % RSD are shown in Table 1.2 and Table 1.3, respectively. The limit of quantitation for this method is 10 μg/g, and the limit of detection is 5 μg/g.

Conclusions

The HPCE assay described herein is sensitive and suitable for simultaneous determination of barbiturates from meconium. The solid phase extraction method provides excellent sample clean up with no endogenous interferences and good recovery. This method also shows excellent within-run and between-run linearity, precision and accuracy in the range of 10-140 μg/g. The method is sensitive and sturdy and would be a good alternative to existing HPLC or GC methods.

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Figure 1.1

Typical electropherogram of (A) blank meconium and (B) meconium spiked with pentobarbital (14.38 min), secobarbital (14.87 min), amobarbital (15.33 min), mephobarbital (16.42 min), phenobarbital (19.97 min), and the internal standard hexobarbital (13.07 min) on a 77 cm, 75 μm fused silica capillary. The run buffer contained 150 mM tris buffer (pH 7.8) with detection at 254 nm. The capillary was thermostated at 25°C and the run voltage was 25 kV.

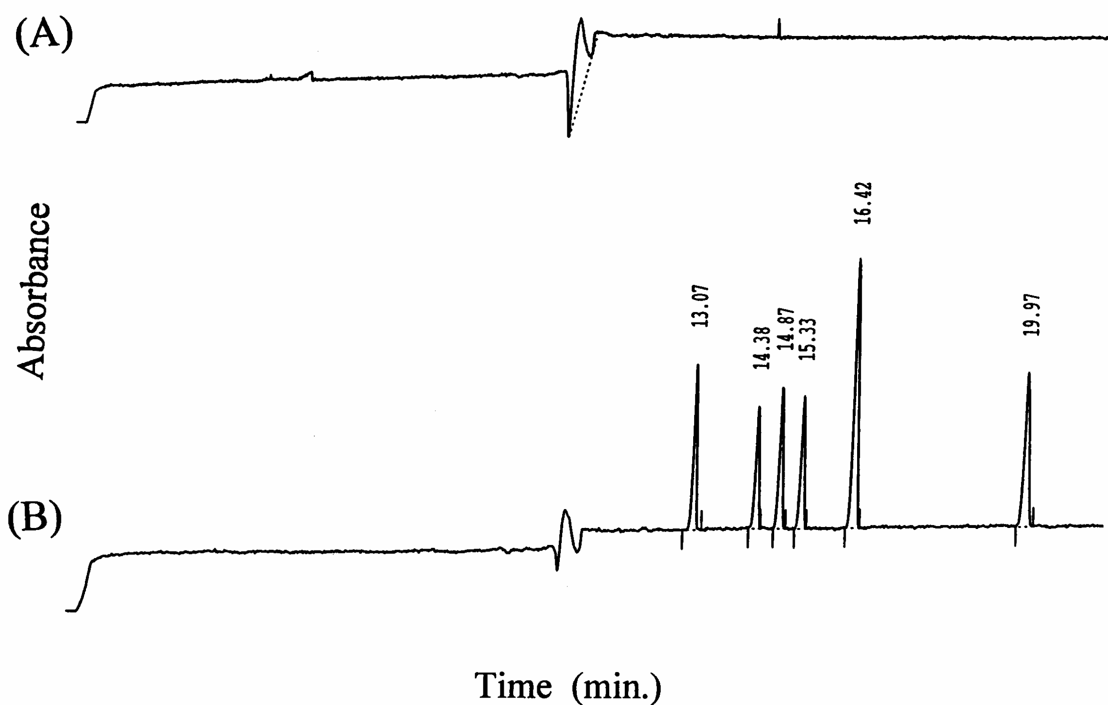

Figure 1.2

Structures of (A) hexobarbital, (B) phenobarbital, (C) pentobarbital, (D) amobarbital, (E) mephobarbital, and (F) secobarbital.

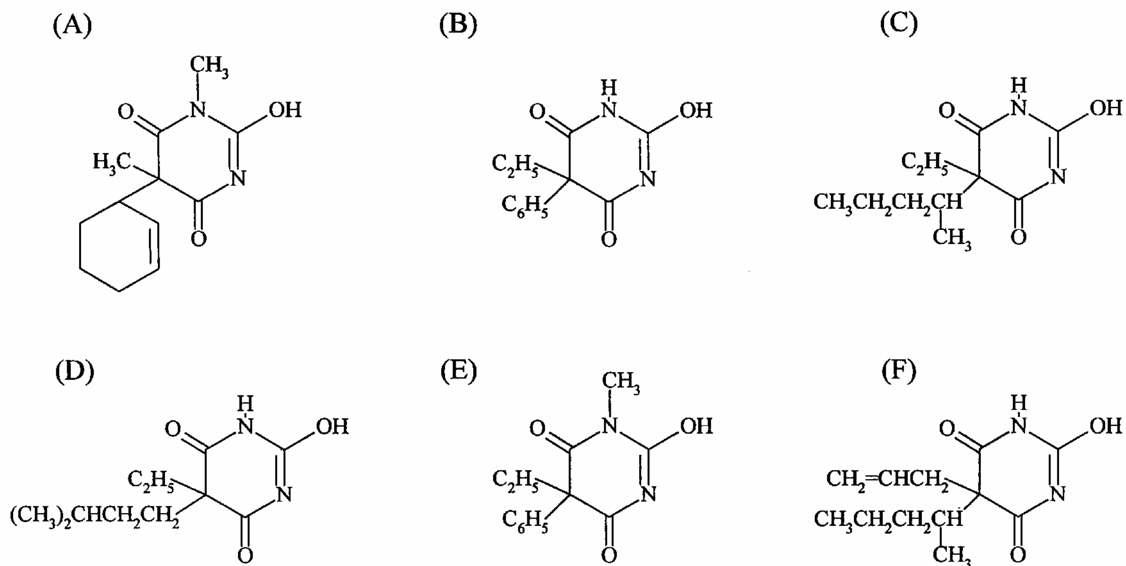

Table 1.1

Extraction Efficiency (%)

SPE	Sample pH	Hex	Pent	Sec	Amo	Meph	Pheno
Polymer	9.0	72.6	99.3	103.6	99.2	82.2	22.9
Polymer	7.0	78.2	76.9	75.5	73.6	73.7	28.2
Polymer	2.5	88.3	82.1	74.5	68.8	70.7	21.6
C8	9.0	67.0	89.9	104.1	90.0	64.2	246.6
C8	7.0	91.1	100.7	100.9	100.8	96.2	33.9
C8	2.5	85.5	97.8	100.9	99.2	89.3	36.2
C18	9.0	73.2	100.1	100.2	109.6	73.4	19.6
C18	7.0	85.5	94.0	99.1	96.8	92.9	35.1
C18	2.5	92.2	97.8	99.1	100.0	98.5	32.6
Silica	9.0	16.2	22.4	25.5	19.2	16.6	52.8
Silica	7.0	0.0	0.0	0.0	0.0	0.0	0.0
Silica	2.5	0.0	0.0	0.0	0.0	0.0	0.0

Table 1.2

Within-Run Data, n=5 (Run 3)

	Conc. Found				
Spike Conc. (μg/g)	Pent	Sec	Amo	Meph	Pheno
19.93	21.69	20.21	19.81	18.61	19.22
20.13	19.09	20.50	19.01	17.85	23.05
20.00	18.96	19.42	18.91	19.34	20.95
19.94	20.89	20.85	19.36	18.28	20.83
20.20	20.50	20.95	19.01	18.94	19.15
120.70	128.21	125.94	126.16	122.20	111.38
120.31	125.53	122.48	124.40	123.30	110.80
120.89	125.40	123.98	125.97	123.56	109.13
120.14	122.45	119.26	118.39	119.83	134.81
119.26	127.99	127.46	129.04	127.97	121.27
Avg.	% Error				
20.04	5.11	2.91	4.06	7.14	6.51
120.26	4.70	3.26	4.36	2.70	7.85
Avg.	% RSD				
20.04	4.75	2.24	1.52	2.32	5.64
120.26	1.39	1.91	2.18	1.55	7.19

Table 1.3

Between-Run Data, n=15

	Avg. Spike Conc. (μg/g)	Conc. Found				
		Pent	Sec	Amo	Meph	Pheno
Run 1	19.93	18.59	19.83	21.05	20.75	19.22
Run 2	19.86	17.85	20.01	19.20	18.50	19.79
Run 3	20.04	20.39	20.39	19.22	18.60	20.64
Run 1	119.96	124.53	123.31	122.61	119.85	126.41
Run 2	120.14	120.65	119.12	121.33	122.74	115.96
Run 3	120.26	125.91	123.82	124.79	123.37	117.48
	Nominal Conc.	% Error				
	20	7.80	3.84	5.21	6.06	6.99
	120	3.27	3.20	2.66	1.75	7.05
	Nominal Conc.	% RSD				
	20	5.13	3.58	3.27	3.01	6.19
	120	1.34	2.28	1.54	1.18	6.12

CHAPTER 2

CHIRAL CAPILLARY ELECTROPHORETIC DETERMINATION OF 2',3'-DIDEOXY-5-FLUORO-3'-THIACYTIDINE IN RAT PLASMA[1]

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[1]Delinsky, D.C., Abu-Raddad, E.J. and Bartlett, M.G. Accepted by *Journal of Liquid. Chromatography & Related Technologies.*
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Abstract

Chiral drugs have the potential for differential pharmacokinetics and metabolism of individual enantiomers. Analytical methods are needed for the separation and quantitation of the enantiomers. Here, we present a method for the separation and quantitation of two enantiomers of 2’,3’-dideoxy-5-fluoro-3’-thiacytidine (FTC) from rat blood plasma using organic protein precipitation with liquid-liquid extraction and capillary electrophoresis (CE). Lamivudine (3TC) was used as an internal standard. The CE system consisted of a 75μm I.D., 37cm length fused silica capillary and a UV detector monitoring a wavelength of 280nm. The run buffer was aqueous containing 90mM hydroxypropyl-β-cyclodextrin in 50mM phosphate at pH 2.5. The system was maintained at 25°C, and the separation voltage was 25kV with a runtime of 15 min. The method was linear over the range from 0.5 to 100μg/ml. The method had baseline resolution of the enantiomers and showed high precision and accuracy both within and between runs at three different concentrations including the lower limit of quantitation (0.5μg/ml).

Introduction

Currently, much research is ongoing for the discovery of new antiviral compounds. Of these, 2’,3’-dideoxy-5-fluoro-3’-thiacytidine (FTC) is a relatively new chiral antiviral, currently in phase III clinical trials, with potent activity against the hepatitis B virus (HBV) and human immunodeficiency virus (HIV).[1-4] For many antiviral drugs, tests have shown that the S(-) enantiomers generally have greater activity than the corresponding R(+) enantiomers.[2,4-9]

Biological systems have a tendency to be chiral, an example being that most higher organisms preferentially utilizing L-amino acids for protein synthesis. Due to this chiral environment, differential metabolism or other bio-interactions may occur between different enantiomers of a single chiral compound. This may include different potency between enantiomers, chiral inversion, and enantiomer-enantiomer interactions.[5-9] If an achiral assay is used, data may be skewed if the dispositions of the individual enantiomers are different. In order to protect against misleading information, chiral assays should by used whenever possible.[10] Chiral assays continue to be developed using common analytical techniques including high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE).

CE is particularly adept for chiral separations due to its very high separation efficiency. Chiral CE most often employs the use of modified cyclodextrins.[11,12] Other methods for chiral CE include the use of Crown ethers, polysaccharides, macrocyclic antibiotics, and electrokinetic chromatography with optically active micelles and/or proteins.[13-15] Cyclodextrins are used for chiral separation in order to take advantage of very slight differences in the inclusion rate constants between the individual enantiomers and the cyclodextrin. Changing the number of subunits or the modification to the cyclodextrin can greatly alter the size and shape of the pocket for analyte inclusion.[15]

Experimental

Reagents and Chemicals

Racivir and the individual enantiomers of Racivir ((+)FTC and (-)FTC) were supplied by Pharmasset Inc. (Tucker, GA, USA). The internal standard, 3TC, was

provided by Dr. Raymond Schinazi (Emory University, Atlanta, GA, USA). Hydroxypropyl-β-cyclodextrin was obtained from Cerestar (Hammond, IN, USA). HPLC grade acetonitrile, phosphoric acid, and granular ammonium sulfate were purchased from J.T. Baker, Inc. (Philipsburg, NJ, USA). Monobasic sodium phosphate was acquired from EM Science (Gibbstown, NJ, USA). Blank rat plasma was purchased from Harlan Laboratories (Indianapolis, IN, USA).

Preparation of Stock and Standard Solutions

Stock solutions of (+)FTC and (-)FTC and the internal standard, 3TC, were prepared in deionized water. Appropriate volumes of stock solutions (between 10 and 20μl) were added to microcentrifuge tubes with enough blank rat plasma to bring the total volume up to 100μl. Concentrations of the two enantiomers were 100, 75, 25, 10, 2.5, and 0.5μg/ml for the calibration curves and 90, 5, and 0.5μg/ml for validation points.

Sample Preparation

All samples were prepared by organic protein precipitation followed by liquid-liquid extraction. To 100μl plasma, 50μl 3TC (60μg/ml) was added and mixed thoroughly. 600μl cold acetonitrile was then added while gently mixing. The mixture was vigorously mixed for 30 sec. Excess ammonium sulfate was added and vigorously mixed for an additional 45 sec and centrifuged for 3 min at 9000 x g. The mixture separated into two phases with an organic layer on top of the saturated ammonium sulfate aqueous layer. The organic layer was removed and placed in a clean tube and evaporated to

dryness under vacuum. The dried samples were reconstituted in 75µl methanol/water (30% v/v).

Electrophoretic System

All CE experiments were performed using a P/ACE System 5000 (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a UV detector. An uncoated fused silica capillary with a total length of 42cm, an effective length of 37cm, and a 75µm I.D (Polymicron Technologies, Phoenix, AZ, USA) was used for analysis. The capillary was thermostated at 25°C and the voltage applied was 25kV. A 0.5cm detection window was created by stripping the polyamide coating off the capillary. The detection window was 5cm from the cathode end of the capillary. The run buffer consisted of an aqueous solution of 50mM phosphate buffer at pH 2.5 (adjusted with concentrated phosphoric acid) and 150mM hydroxypropyl-β-cyclodextrin. The analytes were monitored at a wavelength of 280nm.

Sample introduction was performed using a 5 sec pressure injection (0.5 PSI). The separation voltage was ramped to 25kV over 30 sec. Before each analysis, the capillary was rinsed for 2 min first with 0.1 M sodium hydroxide and 2 min with the run buffer. New capillaries were conditioned by rinsing with 1 M sodium hydroxide for 5 min followed by 5 min each with 1 M hydrochloric acid, water, and run buffer solutions.

Results and Discussion

Baseline resolution was achieved for the two enantiomers of FTC with the use of 90mM hydroxypropyl-β-cyclodextrin in phosphate buffer. Figure 2.1A shows an

electropherogram of extracted blank plasma. Figure 2.1B shows a typical electropherogram of the two enantiomers of FTC and the internal standard spiked into blank plasma. Figure 2.2 shows the structures of (-)FTC, (+)FTC, and the internal standard, 3TC.

Analyte stacking was used to increase sensitivity by reducing band broadening thereby sharpening peaks. In order to do this, the sample was prepared in a lower conductivity solvent (methanol/water) than the run buffer. When high voltage is applied across the capillary, a greater electric field develops across the sample plug than the rest of the capillary filled with buffer. The higher electric field causes sample ions to move faster until they move out of the sample plug. This results in analyte stacking within a narrow zone of the capillary.[16,17]

Several cyclodextrins of various sizes and modifications were tested against FTC using a long capillary and long separation time. Hydoxypropyl-β-cyclodextrin gave the best resolution of the two enantiomers of FTC. The capillary length was then optimized to give baseline resolution with the shortest runtime. The system was able to recognize the difference between FTC and 3TC, resulting in a very large difference in migration times. 3TC had no or very little affinity for the cyclodextrin and therefore had a short migration time (ca 4.5 min). FTC, on the other hand, was able to form inclusion complexes with the cyclodextrin. The affinity for the cyclodextrin resulted in broadening the analyte peaks, which is due to the added partitioning in and out of the cyclodextrin. When the compound enters a cyclodextrin molecule, it slows considerably. The slight difference between the affinities of the enantiomers provided a difference in migration times between the two enantiomers of FTC.

The calibration curve showed good linearity over the range from 0.5 to 100μg/ml for both enantiomers. The method was validated over the calibration range using replicate extractions at three concentrations: 90μg/ml, 5μg/ml, and 0.5μg/ml (the lower limit of quantitation, LLOQ). The coefficients of determination were 0.999 or better (n=3). The within run precision and accuracy (n=6), expressed as percent relative standard deviation (%RSD) and % error, were less than 2.9% and 5.6%, respectively for concentrations above the LLOQ. At the LLOQ, within run precision and accuracy was 13.0% RSD and 11.3% error or better. Detailed within run validation data is listed in Table 2.1. Between run precision and accuracy (n=18) were less than 3.2% RSD and 4.8% error at concentrations above the LLOQ and were 15.5% RSD and 11.2% error or better at the LLOQ. Detailed between run precision and accuracy data is listed in Table 2.2.

Conclusions

The HPCE assay as described is sensitive and selective enough for the separation and quantitation of (+)FTC and (-)FTC from plasma. The organic precipitation and liquid-liquid extraction provides good sample cleanup with no endogenous interferences. This method has very good within-run and between-run precision and accuracy over the range of 0.5 to 100μg/ml. This method is reliable for the separation and quantitation of FTC enantiomers.

Acknowledgements

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Figure 2.1

Typical electropherogram of (A) blank plasma and (B) plasma spiked with (+)-FTC (12.14 min), (-)-FTC (12.65 min), and the internal standard 3TC (4.57 min) on a 37cm, 75µm fused silica capillary. The run buffer contained 90mM hydroxypropyl-β-cyclodextrin in 50mM phosphate buffer (pH 2.5) with detection at 280nm. The capillary was thermostated at 25°C and the run voltage was 25kV.

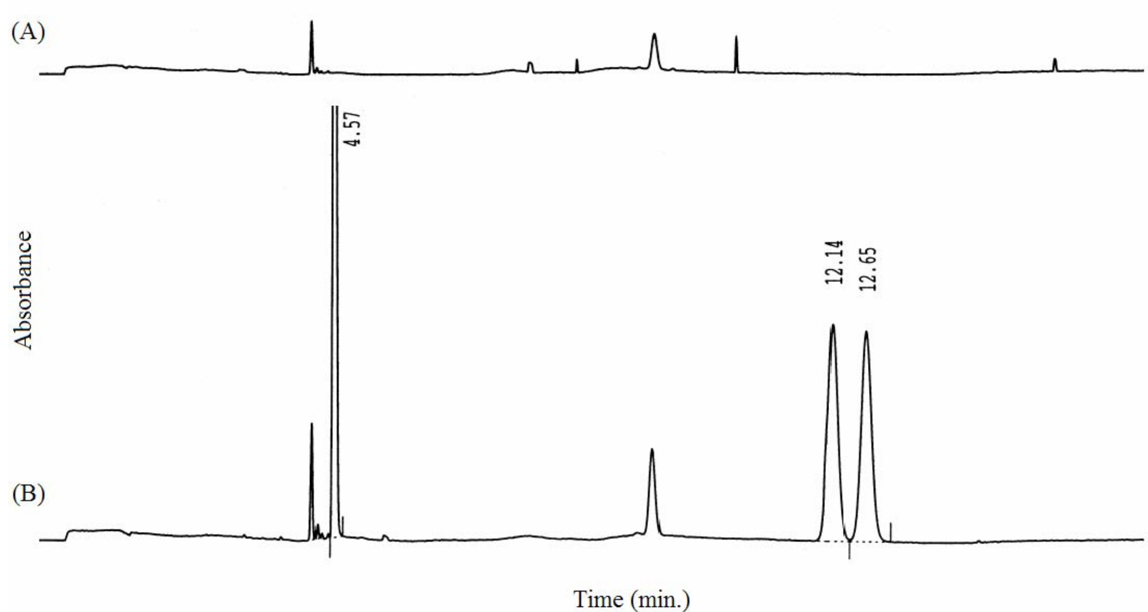
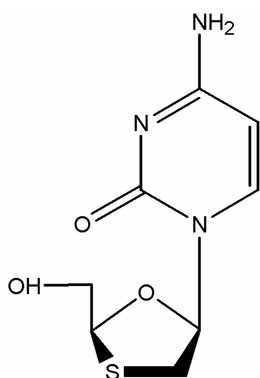
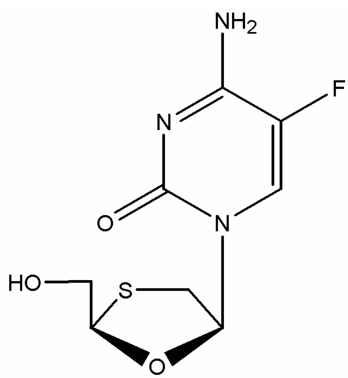


Figure 2.2

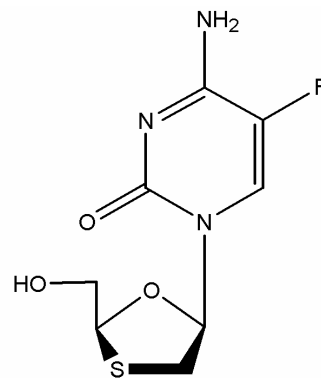
Structures of 3TC (the internal standard), (-)-FTC, and (+)-FTC



3TC



(-)-FTC



(+)-FTC

Table 2.1

Within-Run Precision and Accuracy Data, n=6

Spike Conc. (μg/ml)	Average Conc. Found		% error		% RSD	
	(+)-FTC	(-)-FTC	(+)-FTC	(-)-FTC	(+)-FTC	(-)-FTC
0.5	0.510	0.545	8.9	11.3	13.0	11.1
5	5.28	5.26	5.5	5.3	2.4	2.8
90	91.0	91.3	1.1	1.5	0.5	0.6

Table 2.2

Between-Run Precision and Accuracy Data, n=18

Spike Conc. (μg/ml)	Average Conc. Found		% error		% RSD	
	(+)-FTC	(-)-FTC	(+)-FTC	(-)-FTC	(+)-FTC	(-)-FTC
0.5	0.503	0.527	9.9	11.2	15.5	13.6
5	5.23	5.21	4.7	4.4	2.7	3.1
90	91.6	92.1	2.0	2.6	1.6	1.8

PART II

CHAPTER 3

QUANTITATION OF THE LARGE POLYPEPTIDE GLUCAGON BY PROTEIN PRECIPITATION AND LC/MS[1]

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[1]Delinsky, D.C., Hill, K.T., White, C.A. and Bartlett, M.G. Submitted to *Journal of Mass Spectrometry*, 07/2003.

Abstract

We present a method for the quantitation of glucagon from rat plasma by protein precipitation and LC/MS. No internal standard was used as a labeled standard was not available and similar peptides did not show comparable extraction characteristics to glucagon. The LC system included a Keystone C_{18}, 300 Å pore size column; a linear gradient was used with a mobile phase consisting of water and acetonitrile, each containing 0.2% acetic acid and 0.02% trifluoroacetic acid. Glucagon was detected with the mass spectrometer in positive ion mode monitoring the 4+ charge state at *m/z* 871.7. The method has an approximate limit of detection of 1 ng/ml. The lower limit of quantitation (LLOQ) is 25 ng/ml, which could be reduced with an appropriate internal standard. External calibration was used and calibration curves were found to be linear over the range from 25 to 1000 ng/ml. The method showed a high degree of precision and accuracy both within and between runs at four validation points, including the LLOQ.

Introduction

The quantitative analysis of polypeptides has traditionally been performed by immunoassay based technologies and liquid chromatography (LC) with UV detection.[1-6] The development of peptide based drugs has produced some very potent therapies because they more closely model the system they treat.[7] Due to their potency; cheaper, sensitive, and more specific assays are needed to assay these drugs. While LC-UV methods have been around for a long time, low sensitivity is a major disadvantage when used to assay low levels of compounds from a biological sample. While immunoassays

have generally very good sensitivity, one main disadvantage is the possible cross-reactivity with other compounds with similar structures, which can lead to erroneous results.[1,8,9] Another weakness associated with immunoassays is the cost and time required for method development, especially when analyte specific antibodies are not readily available. With the impending release of biotechnology derived peptide based drugs, it has become increasingly important to develop very specific, sensitive methods in a timely and cost efficient manner for the support of drug development and clinical trials.

Due to its high specificity, sensitivity, and relatively short method development time, there has been an increasing interest in using LC/MS for the quantitation of peptides from complex biological matrices.[7,8,10-18] Despite the increase in developed methods for small peptides, there are relatively few methods that involve the quantitation of polypeptides greater than 3000 Da.[7,8,11-16] Here, we present the determination of glucagon from rat plasma as an example of using LC/MS to quantify a large polypeptide. Glucagon is a large polypeptide consisting of 29 amino acid residues with a molecular weight of 3483 Da. As a therapeutic agent, glucagon is used to treat hypoglycemia, mainly in diabetics.[19] Glucagon acts by stimulating the liver to produce glucose through gluconeogenesis.[19,20]

Experimental

Materials

Human glucagon was obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

Glacial acetic acid was purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA). Blank rat plasma was purchased from Harlan Laboratories (Indianapolis, IN, USA).

LC/MS assay

An Agilent 1100 series LC system (Palo Alto, CA, USA) consisting of a solvent degassing module, quaternary gradient pump, autosampler, and thermostated column compartment was coupled to a Micromass Quattro II mass spectrometer fitted with a Z-spray ion source (Manchester, England, UK). Glucagon was separated on a Biobasic C_{18} (2.0 x 50mm) with a 5μm particle size and a 300 Å pore size with a Biobasic C_{18} cartridge guard column (Keystone Scientific, Bellefonte, PA, USA). The column was maintained at 60°C. The mobile phase consisted of (A) water and (B) acetonitrile each containing 0.2% acetic acid and 0.02% trifluoroacetic acid (TFA). The gradient used for separation was 22 to 38% B over eight minutes followed by a 95% B column wash step prior to column re-equilibration (see Table 3.1). The flow rate was 0.2 ml/min, and the total run time was 25 minutes. The LC flow was directed into the mass spectrometer source without splitting. Prior to the injection of a sample set, at least 10 blank extractions were run in order to stabilize the retention time of the analyte.

The mass spectrometer was operated in positive ion mode with a capillary voltage of 4.0 kV and a cone voltage of 43 V. The source block was heated to 120°C, the desolvation gas was heated to 400°C at a flow rate of approximately 300 L/hr, and the nebulizing gas was set at approximately 30 L/hr. Selected ion monitoring (SIM) was used to monitor for glucagon in its 4+ charge state at *m/z* (mass to charge ratio) 871.7. Glucagon did not produce a stable product ion upon collisionally induced dissociation.

The instruments were controlled and data was processed by Micromass Masslynx v. 3.1 software.

Preparation of Stock and Standard Solutions

A stock solution of glucagon was made by dissolving the lyophilized powder in blank rat plasma to give a concentration of 100 μg/ml and was stored at -20°C until use. Serial dilutions were made with blank plasma to give the following concentrations: 30, 24, 18, 12, 9, 6, 3, 2.25, 1.5, and 0.75 μg/ml. All standards were made fresh each day. 5 μl of each of these standards were added to 145 μl aliquots of rat plasma. This yielded final concentrations of 1000, 800, 600, 400, 300, 200, 100, 75, 50, and 25 ng/ml. Calibration and validation samples were then processed as described below.

Sample Preparation

The initial sample volume of 150 μl plasma was placed in 1.5 ml polypropylene centrifuge tubes. The sample was precipitated with 300 μl acetonitrile (ACN), which was added over 30 sec with an infusion pump while vortexing. The sample was then centrifuged at 16,000xg for five minutes. The supernatant was transferred to clean tubes and evaporated to dryness under vacuum. Dried samples were then reconstituted in 50 μl of a mixture of water, ACN, acetic acid, and TFA (80:20:0.2:0.02). Samples were then centrifuged at 60,000xg for 10 minutes in order to remove insoluble particulates. 40 μl of the reconstituted sample was injected into the LC system for analysis.

Method Validation

Calibration curves for glucagon were generated by spiking glucagon into blank rat plasma. The calibration curves were constructed over the range from 25 to 1000 ng/ml using weighted (1/y) least-squares linear regression analysis of peak areas. Precision and accuracy was determined using five replicates of each of four concentrations; 25, 75, 300, and 1000 ng/ml. Precision is indicated by the percent relative standard deviation within a concentration set, and accuracy is expressed as the average percent error of the calculated concentration to the nominal concentration of each validation sample. Within-run precision and accuracy is calculated from all of the replicates of each of the four concentrations run in one day. Between-run precision and accuracy is calculated from all replicates of the same concentrations on three separate days.

Animal Treatment

Animal studies were approved by the Animal Use and Care Committee at the University of Georgia. Rats were anesthetized using ketamine: acepromazine: xylazine (50:3.3:3.4 mg/kg) and a catheter was placed in the right jugular vein. Glucagon was dissolved in a buffer containing 0.01 M phosphate and 0.15 M sodium and chloride. Glucagon was intravenously administered as a single dose of 0.5 mg/kg to male Sprague-Dawley rats. Each rat weighed approximately 300 grams. Blood was collected by jugular canula (c.a. 500 μl) and placed in heparinized 1.5 ml centrifuge tubes. Collection time points were 0, 1.5, 3, 5, 7.5, 10, 12.5, 15, and 20 minutes after administration. The blood samples were centrifuged at 9,500xg for five minutes. 150 μl plasma aliquots were placed in clean tubes and immediately processed as described above.

Results and Discussion

LC/MS Assay

The chromatographic gradient was optimized both for initial conditions as well as the rate of change of the amount of organic in the system. This is to gain separation from endogenous interferences while maintaining a minimal analyte peak width. A small amount of trifluoroacetic acid (TFA) was used to improve chromatographic peak shape while keeping analyte signal suppression from the TFA to a minimum. Acetic acid was added to reduce the pH of the mobile phase to aid in ionization. The resulting chromatography gave baseline separation of glucagon from other compounds with the same m/z (See Figure 3.1).

Flow injection analysis was performed in order to optimize the source conditions for the mass spectrometer. This was done by adding an infusion of glucagon (10 μg/ml) at a rate of 50 μl/min to a flow from the LC system at 150 μl/min (total of 200 μl/min at the approximate composition of organic at the time of analyte elution). At the pH of the mobile phase, glucagon mainly ionized to four different charge states, 2+ to 5+ (Figure 3.2). By altering the capillary voltage, we were able to discriminate for and maximize the 4+ charge state. Monitoring the 4+ charge state allowed for both the largest possible signal as well as the least amount of observed endogenous interferences. Using collision-induced dissociation, it was not possible to generate an abundant product ion for use in a multiple reaction monitoring (MRM) transition.

We also attempted to use an internal standard in the assay. Due to the cost associated with obtaining a stable isotope labeled glucagon, we were forced to try non-labeled compounds. Several polypeptides of similar size to glucagon were tested. Extraction of the possible internal standards did not correlate well with glucagon. As a

result, calibration curves generated by plotting known glucagon concentration vs. the peak area ratio of glucagon to internal standard had lower correlation coefficients (R^2) than curves generated using glucagon peak area alone.

Variability in the retention time of the analyte was found to be a problem for the first several sample injections of a set. A general increase of one minute in the retention time over the first six to eight samples injected was observed. This was different from general column equilibration issues that usually result in a gradual decrease in the retention time. The phenomenon observed may be attributed to an accumulation of compounds from plasma which had a strong affinity for the analytical column. These compounds were not completely removed by the high organic wash and slowly bled from the column. The compounds on the column may have provided an additional interaction with glucagon resulting in the observed increase in the retention time. In support of this idea, it was found that allowing the system to run for several hours without injection reduced the retention time to its original time.

Preparation of Stock and Standard Solutions

All stock and standard solutions were prepared in blank plasma rather than water. It was found that when these solutions were made in water, the extraction appeared to have more variation. We also observed that sensitivity was poor and the calibration curves were not linear. By dissolving the lyophilized glucagon in plasma and making all dilutions in plasma, we were able to achieve better sensitivity and improve the linearity of our calibration curves. We attributed this phenomenon to analyte adhesion to the sample tubes. The addition of plasma possibly adds additional peptides that compete for

tube wall binding sites. Stability experiments showed the rapid disappearance of glucagon from sample tubes in the absence of plasma. With plasma in the tubes, glucagon disappeared at a much slower rate. This allowed enough time for all samples to be prepared and processed. The slow loss of glucagon mandated fresh standard solutions to be made daily. The 1 mg/ml stock solution was kept frozen to maintain its stability. General response from samples made with this stock solution was similar on a day to day basis, indicating no appreciable loss of glucagon in the stock solution. Borosilicate glass tubes as well as deactivated glass tubes did not have improved analyte adhesion characteristics.

Sample Preparation

The most important step in the sample preparation was the slow addition of acetonitrile during protein precipitation. Quickly precipitating plasma samples produced clumped precipitate that could possibly trap the polypeptide analyte. When samples were quickly precipitated, LC/MS responses had a high degree of variability. Slower precipitation while vortexing produced a finer protein precipitate with much improved response variability.

We also found that glucagon binds very strongly to membranes that are common in filters used to remove particulates prior to LC analysis. Filtration resulted in the near 100% loss of glucagon from extracted plasma samples. In order to remove insoluble particulates prior to injection, all samples were centrifuged at 60,000xg.

Due to problems associated with analyte binding, there was concern whether glucagon would be stable in the autosampler long enough to complete analysis. Several

samples of glucagon (100 ng/ml) were allowed to remain in the autosampler and were injected every few hours. There was no observable decrease in analyte response and hence it was found that glucagon was stable in the reconstitution solution and autosampler vials for at least 20 hours. This was more than the time needed for the analysis of a full set of samples.

Method Validation

The method was validated from 25 to 1000 ng/ml glucagon in plasma using a starting volume of 150 µl. Accuracy and precision were determined from four validation points at 25, 75, 300, and 1000 ng/ml. Each point had five replicates.

Calibration curves were generated by plotting concentration vs. peak area with 1/y weighting. Each calibration curve had an R^2 of at least 0.99. The 1/y weighting allowed more emphasis to be placed on the lower concentrations for linear regression analysis. This resulted in reduced error at the low end of the calibration range. Calibration and validation samples were randomized prior to analysis to eliminate bias based on injection order.

The lower limit of quantitation (LLOQ) was set at 25 ng/ml. Figure 3.1 shows the separation of glucagon at the LLOQ. The observed peak is much greater than the 10:1 signal to noise ratio commonly used to define the LLOQ. Due to the lack of an internal standard and variation associated with protein precipitation, precision and accuracy was too poor to validate significantly lower concentrations.

Accuracy and precision are represented as percent error and percent relative standard deviation (% RSD), respectively. Within run accuracy and precision at the

LLOQ was determined to be 4.4% error and 6.2% RSD. The within run precision and accuracy was found to be less than 8.9% error and less than 8.3% RSD at all other points. Between run accuracy and precision at the LLOQ was found to be 8.3% error and 10.4% RSD, respectively. All other validation points were found to be less than 8.2% error and less than 10.0% RSD for between run accuracy and precision. Detailed accuracy and precision data can be found in Table 3.2.

Recovery could not be calculated for the extraction. This is because stock solutions of glucagon had to be dissolved in plasma to prevent analyte loss due to tube wall binding. As a result of analyte loss, all water solutions had a much lower instrument response than the same concentrations extracted from plasma.

Method Application

This method was used to analyze plasma samples from rats treated with a single IV bolus dose of glucagon (0.5 mg/kg). Due to the short half life of glucagon, blood was drawn within 1.5 minutes of dosing. No loss of glucagon was observed for at least 5 minutes in whole blood. This was enough time for samples to be individually processed after collection. A concentration vs. time plot of glucagon is shown in Figure 3.3. Pharmacokinetic analysis shows first order elimination of glucagon with a calculated elimination phase half-life of 2.3 minutes. This corresponds well with literature values of 3.3 minutes.[20]

Conclusions

We have demonstrated a new LC/MS method for the quantitation of the large polypeptide glucagon from rat plasma. Validated over the range 25 to 1000 ng/ml, we have shown that quantitative analysis of large polypeptides from biological matrices is possible by LC/MS even if no stable MS/MS transitions exist. Without the use of an internal standard, we were able to reach a sensitivity of 25 ng/ml. Estimated from the peak height, with an appropriate internal standard, the lower limit of quantitation may be as low as 5 ng/ml. The method has been shown to be specific, sensitive, accurate, and suitable for the determination of pharmacokinetic parameters.

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Table 3.1

Gradient used for separation, (A) water and (B) acetonitrile, each containing 0.2% acetic acid and 0.02% trifluoroacetic acid.

Time	% A	% B
0	78	22
8	62	38
9	5	95
13	5	95
15	78	22
25	78	22

Table 3.2

Accuracy (average % error) and precision (% standard deviation) at four concentrations for within run (n=5) and between run (n=15) validation.

Within run

conc (ng/ml)	avg % error	% stdev
25	4.4	6.2
75	6.4	6.9
300	8.8	8.2
1000	6.1	8.1

Between run

conc (ng/ml)	avg % error	% stdev
25	8.2	10.4
75	7.9	7.4
300	8.1	9.9
1000	6.4	7.0

Figure 3.1

Typical ion chromatograms of m/z 871 for glucagon (11.74 min) at the LLOQ (25 ng/ml) and blank extracted plasma.

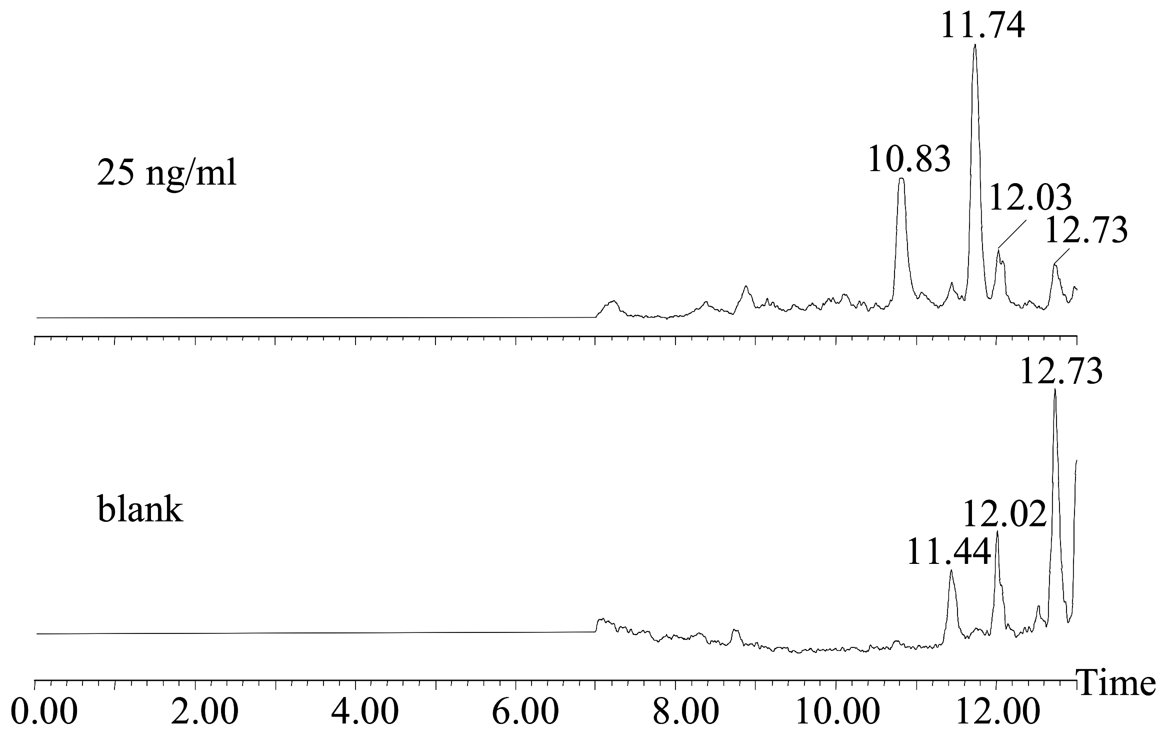


Figure 3.2

Spectrum of glucagon showing its multiple charge states.

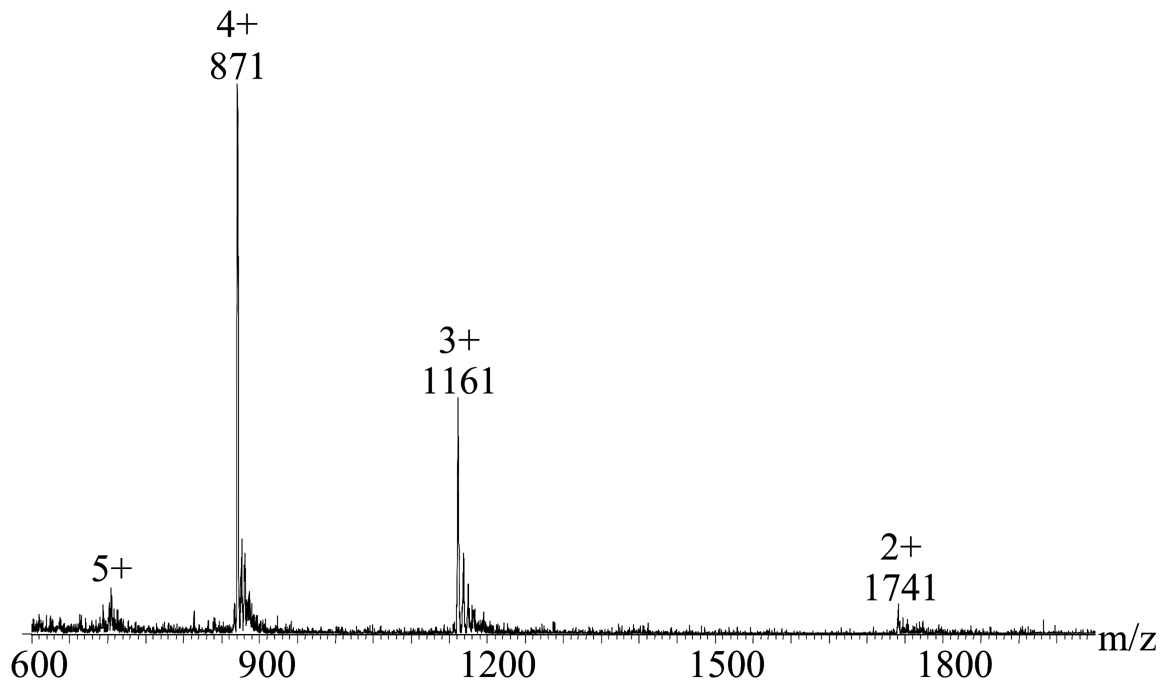
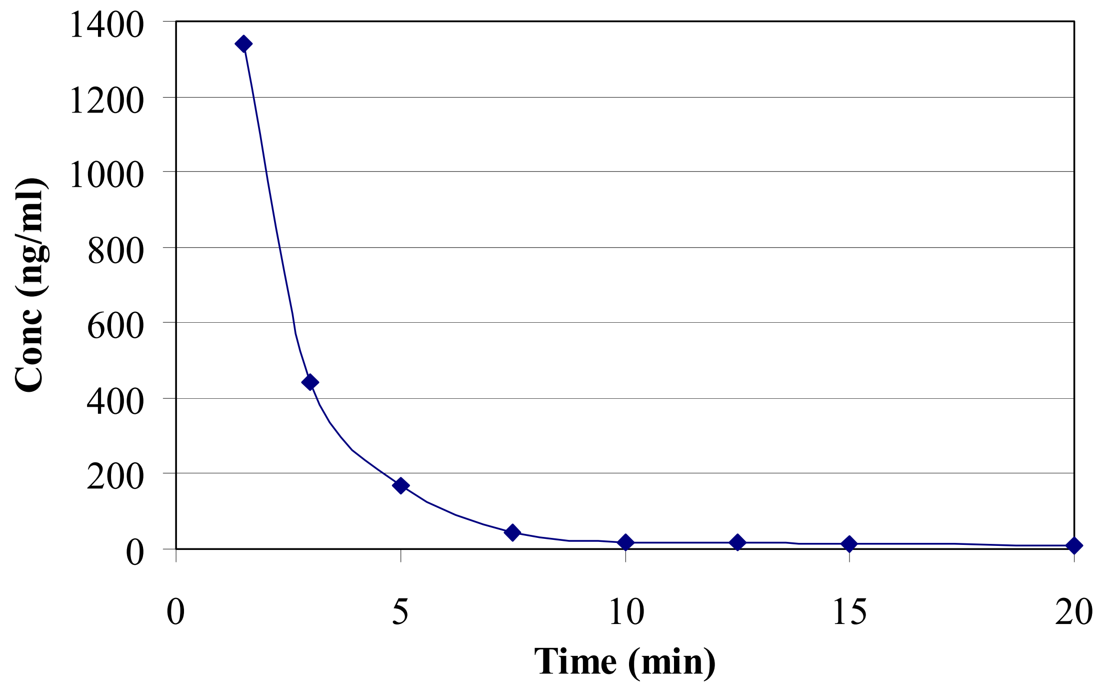


Figure 3.3

Concentration vs time plot for glucagon administered to a male Sprague-Dawley rat with a dose of 0.5 mg/kg. The half-life was determined to be 2.3 minutes.



CHAPTER 4

QUANTITATIVE DETERMINATION OF THE POLYPEPTIDE MOTILIN IN RAT PLASMA BY EXTERNALLY CALIBRATED LIQUID CHROMATOGRAPHY/ELECTROSPRAY IONIZATION MASS SPECTROMETRY[1]

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[1]Delinsky, D.C., Hill, K.T., White, C.A. and Bartlett, M.G. Submitted to *Rapid Communications in Mass Spectrometry*, 07/2003.

Abstract

We present a method for the quantitation of motilin from rat plasma by protein precipitation and LC/MS. Using external calibration, the method was linear over the concentration range from 10 to 1000 ng/ml with an initial sample volume of 150 µl. The LC system included a Keystone C_{18}, 300 Å pore size column. A linear gradient was used with a mobile phase consisting of water and acetonitrile, each containing 0.2% acetic acid and 0.02% trifluoroacetic acid. Motilin was detected with the mass spectrometer in positive ion mode monitoring the 4+ charge state at *m/z* 675.5. The approximated limit of detection was less than 1 ng/ml and the lower limit of quantitation (LLOQ) was 10 ng/ml. The method showed a high degree of precision and accuracy both within and between runs at five validation points, including the LLOQ.

Introduction

In the past, quantitative analysis of polypeptides was mainly limited to liquid chromatography (LC) with UV or fluorescence detection for high concentrations and immunoassays for lower concentrations including physiological levels.[1-6] Recently, other methods have been employed for the quantitation of polypeptides including capillary electrophoresis with UV, laser induced fluorescence, and even mass spectrometry for detection.[6-9]

For many years, polypeptides have been used to treat diseases. Insulin, for example, is used to treat diabetic patients by helping to control blood glucose levels. More recently, it has been reported that bombesin has anti-inflammatory properties.[10] This polypeptide has also been used to help in the healing of chronic gastric ulcers.[10]

Currently, polypeptides are also being used to study system regulation, signal progression, and how they relate to system failure.[10-12] Various polypeptides have been related to many forms of cancer and some could be used as biomarkers for cancers as well as other diseases.[10,11,13-16] For example, bombesin and related peptides have been shown to be specific markers of cancers of the lung, breast, prostate, ovary, pancreas, colon, and brain, among others.[10,14] Another example is human atrial and brain natriuretic peptides; these polypeptides may be useful as diagnostic and prognostic indicators for patients with acute myocardial infarction or congestive heart failure.[17]

Until recently, nearly all quantitative analysis of polypeptides has been done by immunoassays. This technique has several drawbacks including false positive results due to cross reactivity. Cross reactivity is when another compound, not the analyte, is indistinguishable by the detection technique from the analyte of interest.[1,18,19] This may be from a related compound or one with the same recognition characteristics as the analyte. Radioactive labels may be required for some assays and these increase the expense of the test as well as the cost for disposal. Immunoassays also require antibodies specific to the analyte of interest. For new compounds, these antibodies are often unavailable and must be custom made. This is both extremely time consuming and costly.

Newer technologies include the development of biotechnologically derived peptides and polypeptides for the treatment of infections and diseases. These include synthetic peptide antibiotics and other peptide based drugs.[10,13,20-25] In support of new peptide drugs, sensitive and more specific assays which can be developed more cheaply are needed. One alternative to immunoassays is LC with UV detection, but this

technique is limited by its sensitivity. Fluorescence and laser induced fluorescence detection offers improved sensitivity but depends on native fluorescence or complicated derivatization of the analyte.[9] Liquid chromatography offers a strong advantage in that it can be used to separate even closely related compounds prior to detection. To get the sensitivity of traditional immunoassays, one could fraction collect the LC eluent followed by immunoassay of the individual fractions, but this method would, again, be unable to distinguish different compounds if they coelute.[3]

Due to its high specificity, sensitivity, and relatively short method development time, there has been an increasing interest in using LC/MS for the quantitation of peptides from complex biological matrices.[18,21-27] Mass spectrometers are generally easily coupled to LC instrumentation and detection of individual masses allows greater selectivity and therefore greater confidence in the true identity of observed signals. The development of LC/MS methods involves much less time and financial resources than the development and production of new antibodies for immunoassays. We present the determination of motilin from rat plasma as an example of using LC/MS to quantify a polypeptide. Motilin is a polypeptide consisting of 22 amino acid residues with a molecular weight of 2699 Da. Motilin is a gastrointestinal peptide mainly present in the upper small intestine.[28] It increases gastric motility, stimulates pepsin secretion, and is thought to inhibit the release of luteinizing hormone.[28,29]

Experimental

Materials

Porcine motilin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Glacial acetic acid was purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA). Blank rat plasma was purchased from Harlan Laboratories (Indianapolis, IN, USA).

LC/MS assay

An Agilent 1100 series LC system (Palo Alto, CA, USA) consisting of a solvent degassing module, quaternary gradient pump, autosampler, and thermostated column compartment was coupled to a Micromass Quattro II mass spectrometer fitted with a Z-spray ion source (Manchester, England, UK). Motilin was separated on a Biobasic C_{18} analytical column (2.0 x 50mm) with a 5µm particle size and a 300 Å pore size with a Biobasic C_{18} cartridge guard column (Keystone Scientific, Bellefonte, PA, USA). The column was maintained at 60°C. The mobile phase consisted of (A) water and (B) acetonitrile each containing 0.2% acetic acid and 0.02% trifluoroacetic acid (TFA). The gradient used for separation was 19 to 35% B over four minutes followed by a 95% B column wash step prior to column re-equilibration (see Table 4.1). The flow rate was 0.2 ml/min, and the total run time was 20 minutes. The LC flow was directed into the mass spectrometer source without splitting. Prior to the injection of a sample set, at least 10 blank extractions were run in order to stabilize the retention time of the analyte.

The mass spectrometer was operated in positive ion mode with a capillary voltage of 4.0 kV and a cone voltage of 20 V. The source block was heated to 100°C, the desolvation gas was heated to 350°C at a flow rate of approximately 250 L/hr, and the nebulizing gas was set at approximately 25 L/hr. Selected ion monitoring (SIM) was used to monitor for motilin in its 4+ charge state at *m/z* (mass to charge ratio) 675.5. The instruments were controlled and data was processed by Micromass Masslynx v. 3.1 software.

Preparation of Stock and Standard Solutions

A stock solution of motilin was made by dissolving the lyophilized powder in blank rat plasma to give a concentration of 100 μg/ml and was stored at -20°C until use. Serial dilutions were made with blank plasma to give the following concentrations: 30, 24, 18, 12, 9, 6, 3, 2.25, 1.5, 0.75, and 0.30 μg/ml. All standards were made fresh each day. 5 μl of each of these standards were added to 145 μl aliquots of rat plasma. This yielded final concentrations of 1000, 800, 600, 400, 300, 200, 100, 75, 50, 25, and 10 ng/ml. Calibration and validation samples were then processed as described below.

Sample Preparation

The initial sample volume of 150 μl plasma was placed in 1.5 ml polypropylene centrifuge tubes. The sample was precipitated with 300 μl acetonitrile (ACN), which was added over 20 sec with an infusion pump while vortexing. The sample was then centrifuged at 16,000xg for five minutes. The supernatant was transferred to clean tubes and evaporated to dryness under vacuum. Dried samples were then reconstituted in 50 μl

of a mixture of water, ACN, acetic acid, and TFA (85:15:0.2:0.02). Samples were then centrifuged at 60,000xg for 10 minutes in order to remove insoluble particulates. 40 µl of the reconstituted sample was injected into the LC system for analysis.

Method Validation

Calibration curves for motilin were generated by spiking the peptide into blank rat plasma. The calibration curves were constructed over the range from 10 to 1000 ng/ml using weighted (1/y) least-squares linear regression analysis of peak areas. Precision and accuracy was determined using five replicates of each of five concentrations; 10, 25, 75, 300, and 1000 ng/ml. Precision is indicated by the percent relative standard deviation within a concentration set, and accuracy is expressed as the average percent error of the calculated concentration to the nominal concentration of each validation sample. Within-run precision and accuracy is calculated from all of the replicates of each of the five concentrations run in one day. Between-run precision and accuracy is calculated from all replicates of the same concentrations on three separate days.

Animal Treatment

Animal studies were approved by the Animal Use and Care Committee at the University of Georgia. Rats were anesthetized using ketamine: acepromazine: xylazine (50:3.3:3.4 mg/kg) and a catheter was placed in the right jugular vein. Motilin was dissolved in a buffer containing 0.01 M phosphate and 0.15 M sodium and chloride. Motilin was intravenously administered as a single dose of 0.5 mg/kg to male Sprague-Dawley rats. Each rat weighed between 250 and 300 grams. Blood was collected by

jugular canula (c.a. 500 µl) and placed in heparinized 1.5 ml centrifuge tubes. Collection time points were 0.5, 1.0, 1.5, 2, 3, 4, 5, 7.5, 9, 12, 15, 17.5, and 20 minutes after administration. The blood samples were centrifuged at 9,500xg for five minutes. 150 µl plasma aliquots were placed in clean tubes and immediately processed as described above.

Results and Discussion

LC/MS Assay

The chromatography combined with the selectivity of the mass spectrometer resulted in chromatograms with very little visible endogenous compounds, see Figure 4.1. The organic gradient used was optimized for both reduced run time as well as to maintain a minimal peak width. Although trifluoroacetic acid (TFA) is a known signal suppressor in mass spectrometers, a small amount was necessary to maintain good chromatographic peak shape. The low percentage of TFA was found to cause only a minimal amount of signal suppression. Acetic acid was also added to the mobile phase primarily to reduce the pH of the solution and thus aid in ionization.

The source conditions for the mass spectrometer were optimized by flow injection analysis. For this experiment, an infusion of motilin (10 µg/ml) was added at a rate of 50 µl/min to a 150 µl/min flow rate from the LC system. This resulted in a total flow rate of 200 µl/min entering the source of the mass spectrometer. The total flow had an organic and pH composition estimated to be the same as at the time of elution from the column.

At the pH of the mobile phase and under the optimized source conditions, motilin mainly ionized to its 3+ and 4+ charge states with the 2+ and 5+ charge states still visible

(Figure 4.2). We were able to maximize the 4+ charge state by altering the capillary and cone voltages. The 4+ charge state was chosen as this channel had the smallest amount of endogenous interferences. Upon collisionally induced dissociation, motilin did produce an abundant product ion with an m/z of 614.0. Despite the multiple reaction monitoring (MRM) transition, single ion reaction (SIR) was used to monitor the nonfragmented 4+ ion because it gave better sensitivity than the MRM transition.

The method used external calibration because no suitable internal standard was found. Since no commercially available stable isotope labeled motilin was available and custom synthesis was too expensive, we tried to use similar peptides. Several peptides including β-endorphin, the individual chains of insulin, and galanin, were tested for use as an internal standard. In every case, the peptide did not correlate well with motilin after extraction and/or did not easily fit chromatographically with motilin. This resulted in calibration curves with better correlation coefficients (R^2) when no internal standard was used.

During the first several injections of a sample set, a gradual shift in the retention time was observed. This was an increase in the retention time of about one minute over the first eight injections of extracted plasma (blank or spiked). This maybe attributed to an accumulation of peptides and proteins which have a strong affinity for the analytical column. The presence of these compounds may have provided an additional partition interaction with motilin which allowed for an increase in the retention. Although these compounds were not removed by an organic rinse, they were found to eventually elute from the column. After allowing the system to run for several hours, even without a high

concentration of organic solvent, the retention time was found to return to its original time.

Preparation of Stock and Standard Solutions

All standard and stock solutions were prepared in blank plasma rather than water. More variation in motilin peak area was observed when initial solutions were made in water. It was also found that sensitivity was better and calibration curves were more linear when motilin was dissolved in plasma prior to spiking samples. We expect that this is a result of the analyte adhering to the sample vials. The addition of plasma to the sample vials likely adds additional peptides and proteins that compete with motilin for wall binding sites. It was found that borosilicate glass and deactivated glass tubes did not have improved analyte adhesion characteristics.

Stability experiments were conducted and it was found that in the absence of plasma, motilin concentrations decreased rapidly. Upon the addition of plasma, motilin concentrations decreased at a much slower rate. This allowed enough time to prepare and process all samples before motilin concentrations diminished significantly. Because there was still a slow loss of motilin over time, fresh standard solutions were made just prior to each sample set. The stock solution (1 mg/ml) was kept frozen at -20°C to maintain stability. No significant loss of motilin was observed in the stock solution as peak areas from samples were similar on a day to day basis.

Sample Preparation

Slowly adding the acetonitrile during protein precipitation was found to be a very important step in sample preparation for minimizing variability in the analyte response. Rapid precipitation of proteins resulted in a clumped precipitate that may trap polypeptide analytes. It was found that LC/MS peak areas had a large amount of variation when spiked plasma samples were precipitated in this manner. Upon slower precipitation while vortexing, a finer protein precipitate was produced and a much improved response variability was achieved.

It was also observed that motilin strongly binds to commonly used filtration membranes that are used to remove particulates prior to injection into the LC system. When extracted spiked plasma samples were filtered, close to 100% of motilin in the sample was lost. All samples were centrifuged at 60,000xg prior to injection in order to remove most insoluble particulates.

As a result of motilin binding to both sample vials as well as filtration membranes, there was concern over the possibility that motilin may not be stable in the autosampler. Several extracted samples with motilin were mixed and redistributed into separate autosampler tubes. The samples remained in the autosampler, and one was injected every few hours. No decrease in motilin peak area was observed for at least 20 hours. This was ample time for the analysis of a full set of samples (up to 48).

Method Validation

This method was validated over the range from 10 to 1000 ng/ml motilin in plasma using an initial volume of 150 μl. Accuracy and precision were determined using

five validation points at 10, 25, 75, 300, and 1000 ng/ml. Five replicates of each point were used.

Calibration curves were generated by plotting the known motilin concentration vs. its corresponding peak area using a 1/y weighting scheme. All calibration curves had a minimum R^2 of at least 0.99. 1/y weighting allowed additional emphasis to be placed on lower concentrations during linear regression analysis. The weighting scheme allowed for more accurate calculations at the low end of the calibration curve. All samples in a set were randomized prior to analysis to help eliminate bias based on injection order.

The lower limit of quantitation (LLOQ) was set at 10 ng/ml. The separation of motilin at the LLOQ is shown in Figure 4.1. The peak for motilin is greater than the 10:1 signal to noise ratio often used to define an LLOQ. Due to the variation associated with protein precipitation and, more importantly, due to the lack of an internal standard, confidence could not be placed in concentrations less than 10 ng/ml. Thus, we did not attempt to determine the precision and accuracy of lower concentrations.

Accuracy and precision are represented as percent error and percent relative standard deviation (% RSD), respectively. Within run accuracy and precision at the LLOQ was determined to be 10.8% error and 9.8% RSD. The within run precision and accuracy was found to be less than 11.6% error and less than 5.6% RSD for all other points. Between run accuracy and precision at the LLOQ was 13.5% error and 16.4% RSD, respectively. All other validation points were found to have an error of less than 8.8% and less than 10.2% RSD for between run accuracy and precision. Detailed accuracy and precision data can be found in Table 4.2.

It was not possible to calculate recovery for the extraction. This is a result of the motilin stock solutions being dissolved in plasma rather than in a clean solvent. It was observed that solutions of motilin dissolved in water as well as motilin extracted from water had much lower peak areas than other samples of the same concentration that were extracted from plasma. This is understood to be a result of more motilin binding to the walls of sample tubes when it is not initially dissolved in plasma.

Method Application

To demonstrate the utility of this method to determine pharmacokinetic parameters, we determined the half-life of motilin in rats. We analyzed plasma from rats that were given a single IV bolus dose of motilin (0.5 mg/kg). Due to the expected short half-life of motilin, blood was drawn within 30 seconds of dosing. Prior to animal dosing, we determined that motilin was stable in fresh whole blood for at least 30 minutes. This was enough time to collect and process all samples. A concentration vs. time plot of motilin is shown in Figure 4.3. Pharmacokinetic analysis shows first order elimination of motilin and a half-life of 6.2±1.9 minutes. This is similar to the elimination half-life of other polypeptides.

Conclusions

We have described and proven a new LC/MS method for the quantitation of the polypeptide motilin from rat plasma. Without the use of an internal standard, we were able to validate this method from 10 to 1000 ng/ml. With an appropriate internal standard, we are confident that an LLOQ of as low as 1 ng/ml could be reached. Our

method has proven to be specific to motilin, sensitive, accurate, and well suited for the determination of pharmacokinetic parameters.

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Table 4.1

Gradient used for separation, (A) water and (B) acetonitrile, each containing 0.2% acetic acid and 0.02% trifluoroacetic acid. The total run time was 20 minutes.

Time	% A	% B
0	81	19
4	65	35
5	5	95
9	5	95
11	81	19
20	81	19

Table 4.2

Accuracy (average % error) and precision (% standard deviation) at five concentrations of motilin extracted from plasma for within run (n=5) and between run (n=15) validation.

Within run

conc (ng/ml)	avg % error	% stdev
10	10.8	9.8
25	6.3	6.5
75	11.6	2.7
300	5.6	6.1
1000	4.6	7.5

Between run

conc (ng/ml)	avg % error	% stdev
10	13.5	16.4
25	7.8	10.1
75	8.7	9.5
300	4.9	4.6
1000	4.2	5.7

Figure 4.1

Typical ion chromatograms of m/z 675.8 for motilin (8.26 min) at the LLOQ (10 ng/ml), (a) and blank extracted plasma, (b)

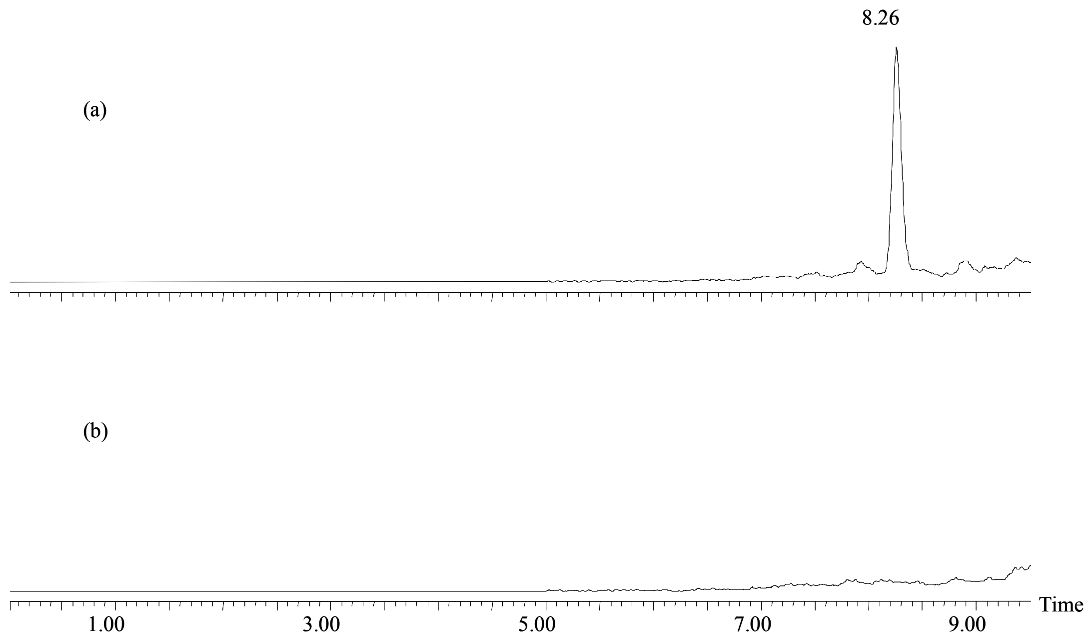


Figure 4.2

Spectrum of motilin showing its multiple charge states

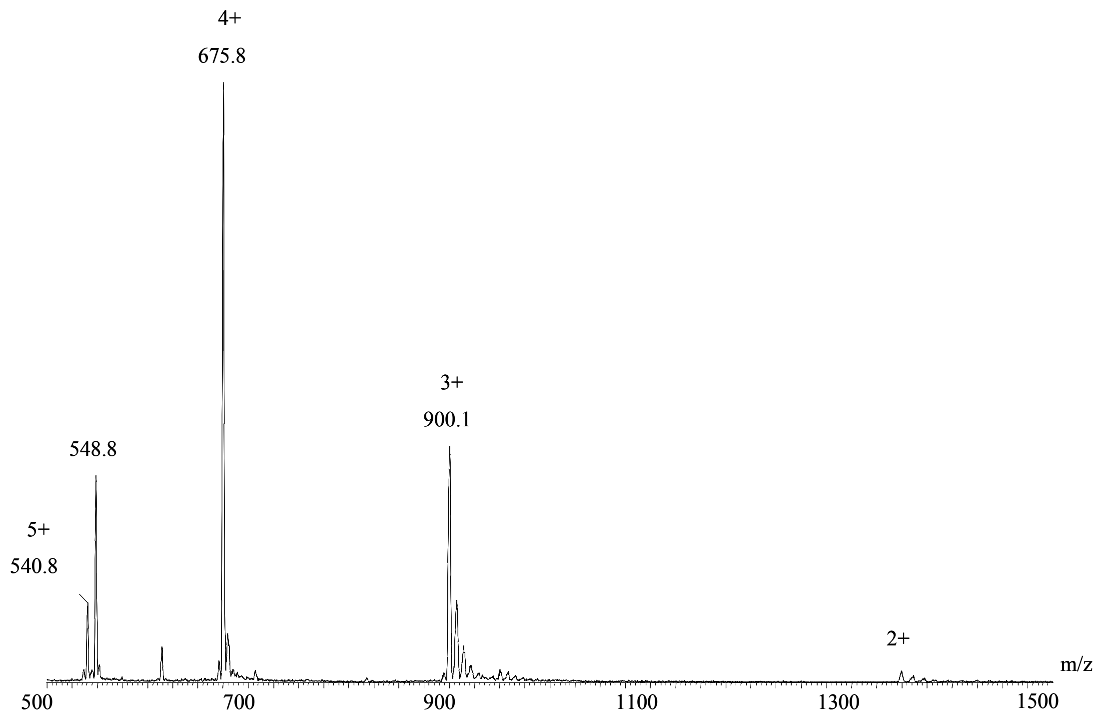
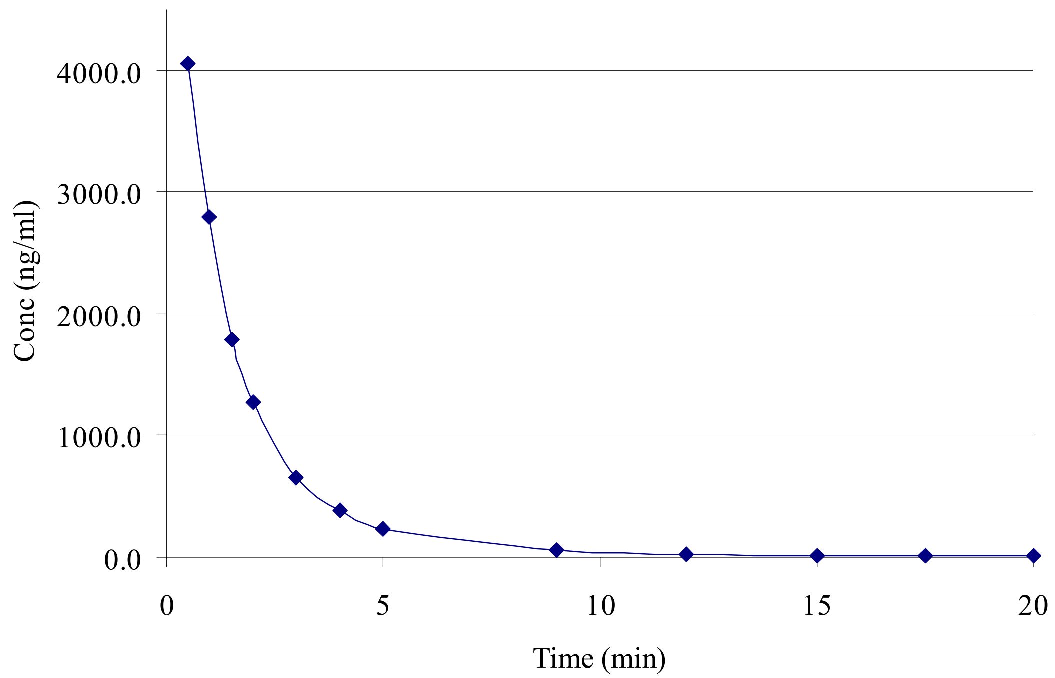


Figure 4.3

Concentration vs time plot for motilin administered to a male Sprague-Dawley rat with a dose of 0.5 mg/kg. The half-life was determined to be 6.2 minutes.



CHAPTER 5

QUANTITATION OF THE POLYPEPTIDE GALANIN BY PROTEIN PRECIPITATION AND EXTERNAL CALIBRATION LC/MS[1]

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[1]Delinsky, D.C., Hill, K.T., White, C.A. and Bartlett, M.G. Submitted to *Journal of Chromatography B*, 07/2003.

Abstract

We present a method for the quantitation of galanin from rat plasma by protein precipitation and LC/MS. The method was externally calibrated, as no suitable internal standard was available. The LC system included a Keystone Biobasic C_{18}, 300 Å pore size column maintained at 60°C. A linear gradient was used with a mobile phase consisting of water and acetonitrile, each containing 0.2% acetic acid and 0.02% trifluoroacetic acid. Galanin was detected with the mass spectrometer in positive ion mode by selected ion monitoring of the 3+ charge state at *m/z* 1053.4. The method has an approximate limit of detection of less than 1 ng/ml. The lower limit of quantitation (LLOQ) was 10 ng/ml and calibration curves were found to be linear over the range from 10 to 1000 ng/ml. The method was both accurate and precise within and between runs using five validation points, including the LLOQ. The method was then demonstrated by a limited pharmacokinetic study of galanin in rats.

Introduction

Immunoassays have commonly been used for the detection and quantitation of polypeptides since the 1960's when an immunoassay was worked out for determining insulin from human plasma [1]. These methods have been used because of their high sensitivity as well as the lack of better techniques. Immunoassays use specific antibodies that recognize and bind to certain structures of an intended analyte. One drawback to this is the very real possibility of cross-reactivity with other compounds (endogenous or other drugs) that can lead to erroneous test results. Even the use of monoclonal antibodies only

reduces rather than eliminates cross-reactivity [2]. Quantitative techniques that further improve the confidence in a method's results are needed.

Another severe drawback to immunoassays is the long period of time and the large amount of money that is necessary for the development of new methods. If antibodies are not available for the analyte in question, it may take months to years before enough antibodies could be raised against a particular antigen.

Current advancements in the biotechnology industry may result in many new peptide based drugs for the treatment of various infections and diseases [3-7]. As is common in the pharmaceutical industry, only a small percentage of lead compounds are ever approved for retail sale. This means that there may be hundreds of potential compounds that fail toxicity and efficacy screenings. For these tests, sensitive quantitative methods are needed for each compound. Due to the high cost and time associated with the development of new immunoassays, assays that are developed more quickly and cheaply would be highly desirable to the biotechnology industry. Less severe problems associated with immunoassays include the fact that some immunoassays use radiolabels, which increase the cost of the assay and complicates sample handling and disposal.

Liquid chromatography (LC) coupled with mass spectrometry (MS) has gained ever increasing interest in the quantitation of peptides from biological matrices [4-8], as this technique can potentially meet these needs for a more reliable, inexpensive, and speedy alternative to immunoassays. LC can effectively separate the analyte from other compounds. MS has the ability to separate and detect ionized molecules by their mass and charge. The combination of these two techniques can produce an analytical method

that is both specific (minimizing the chance for false results) and sensitive to a polypeptide analyte.

Just as there are drawbacks with any analytical method, there are obstacles associated with the analysis of polypeptides by LC/MS. The first being difficulties associated with the extraction of polypeptides from a biological matrix. Biological matrices are very complex and usually contain a large amount of proteins and peptides. As a result of many peptide-protein interactions, it is not necessarily easy to remove a specific polypeptide from such a mixture. Peptides in general can be quite hydrophobic and can thereby bind to glass or plastic containers, especially when dissolved in only water. Another problem with the analysis of polypeptides is that LC methods that are used to separate peptides often use ion pairing agents, such as trifluoroacetic acid [9]. These largely ionic compounds are often found to decrease ionization efficiency in the sources of mass spectrometers.

Despite the increased interest in quantitation of peptides by LC/MS, there are relatively few published methods to quantify polypeptides larger than 3000 Da [4-8,10]. To illustrate the ability of LC/MS methods to quantify large polypeptides, we present a method for the quantitation of the polypeptide galanin from rat plasma using protein precipitation sample preparation followed by LC separation and MS detection. Galanin is a 30 amino acid polypeptide with a molecular weight of 3157 Da [11]. As a peptide neurotransmitter [11], galanin is a critical part of the cholinergic system [12], and as such, it is involved in insulin release, spinal reflex, growth, depression, learning, and memory, among others [11-14]. As related to memory, galanin is associated with Alzheimer's disease [12-14].

Experimental

Materials

Synthetic human galanin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Glacial acetic acid was purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA). Blank rat plasma was purchased from Harlan Laboratories (Indianapolis, IN, USA).

LC/MS assay

An Agilent 1100 series LC system (Palo Alto, CA, USA) consisting of a solvent degassing module, quaternary gradient pump, autosampler, and thermostated column compartment was coupled to a Micromass Quattro II mass spectrometer fitted with a Z-spray ion source (Manchester, England, UK). Galanin was separated on a Biobasic C_{18} (2.0 x 50mm) with a 5µm particle size and a 300 Å pore size with a Biobasic C_{18} cartridge guard column (Keystone Scientific, Bellefonte, PA, USA). The column was maintained at 60°C. The mobile phase consisted of (A) water and (B) acetonitrile each with 0.2% acetic acid and 0.02% trifluoroacetic acid (TFA). The organic gradient used for separation was linear from 19 to 35% B over eight minutes. Prior to column re-equilibration, the column was washed with 95% B (see Table 5.1). The total run time was 24 minutes at a flow rate of 0.2 ml/min. The LC flow was directed into the mass spectrometer source without splitting. Prior to the injection of a sample set, at least 10 blank extractions were run in order to stabilize the retention time of the analyte.

The mass spectrometer was operated in positive ion mode with a capillary voltage of 4.0 kV and a cone voltage of 43 V. The source block was heated to 100°C, the desolvation gas was heated to 350°C at a flow rate of approximately 250 L/hr, and the nebulizing gas was set at its maximum flow rate. Selected ion monitoring (SIM) was used to monitor for galanin in its 3+ charge state at *m/z* (mass to charge ratio) 1053.4. Galanin did not produce an abundant product ion upon collisionally induced dissociation. The instruments were controlled and data was processed by Micromass Masslynx v. 3.1 software.

Preparation of Stock and Standard Solutions

A stock solution of galanin was made by dissolving the lyophilized powder in blank rat plasma to give a concentration of 100 µg/ml and was stored at -20°C until use. Serial dilutions were made with blank plasma to give the following concentrations: 30, 24, 18, 12, 9, 6, 3, 2.25, 1.5, 0.75, and 0.30 µg/ml. All standards were made fresh each day. 5 µl of each of these standards were added to 145 µl aliquots of rat plasma. This yielded final concentrations of 1000, 800, 600, 400, 300, 200, 100, 75, 50, 25, and 10 ng/ml and a total volume of 150 µl. Both calibration and validation samples were then processed as described below.

Sample Preparation

The initial sample volume of 150 µl plasma was placed in 1.5 ml polypropylene centrifuge tubes. Acetonitrile was added to the plasma over 20 seconds with an infusion pump while vortexing to precipitate the plasma proteins. The sample was then

centrifuged at 16,000xg for five minutes. The supernatant was transferred to clean tubes and evaporated to dryness under vacuum. Dried samples were then reconstituted in 50 μl of a mixture of water, ACN, acetic acid, and TFA (85:15:0.2:0.02). Reconstituted samples were then centrifuged at 60,000xg for 10 minutes in order to remove insoluble particulates. 40 μl of the reconstituted sample was injected into the LC system for analysis.

Method Validation

Calibration curves for galanin were generated as spiked plasma samples. The calibration curves were constructed over the range from 10 to 1000 ng/ml using weighted (1/y) least-squares linear regression analysis of galanin peak areas. The precision and accuracy of the method was determined using five replicates of each of five concentrations of galanin; 10, 25, 75, 300, and 1000 ng/ml. Precision is indicated by the percent relative standard deviation within a single concentration in a validation set, and accuracy is expressed as the average percent error of the calculated concentration to the nominal concentration of each validation sample. Within-run precision and accuracy is calculated from all of the replicates of each of the five concentrations run in one set. Between-run precision and accuracy is calculated from all replicates of the same concentrations over three separately prepared sets.

Animal Treatment

Animal studies were approved by the Animal Use and Care Committee at the University of Georgia. Rats were anesthetized using ketamine: acepromazine: xylazine

(50:3.3:3.4 mg/kg) and a catheter was placed in the right jugular vein. Galanin was dissolved in a buffer containing 0.01 M phosphate and 0.15 M sodium and chloride. The solution was then intravenously administered as a single dose of 0.5 mg/kg to male Sprague-Dawley rats. Each rat weighed approximately 250 to 300 grams. Blood was collected by jugular canula (c.a. 500 µl) and placed in heparinized 1.5 ml centrifuge tubes. Collection time points were 0, 0.5, 1, 2, 3, 4, 5, 7, 9, and 12 minutes after administration. Freshly collected samples were stored on ice no longer than 30 minutes before processing. The blood samples were centrifuged at 9,500xg for five minutes to separate the plasma from the red blood cells. 150 µl plasma aliquots were placed in clean tubes and immediately processed as described above.

Results and Discussion

LC/MS Assay

The chromatographic gradient was optimized to provide good separation between galanin and endogenous compounds in the ion chromatograms. Initial organic composition was optimized with the gradient to produce a minimal analyte peak width. Some TFA (trifluoroacetic acid) was necessary to obtain a satisfactory peak shape. No significant ion suppression was observed with the low levels of TFA used. The addition of acetic acid was to lower the pH of the mobile phase for chromatographic separation as well as to aid analyte ionization in the source of the mass spectrometer. The resulting chromatography provided baseline resolution of galanin from endogenous compounds, see Figure 5.1.

The source conditions for the mass spectrometer were optimized by flow injection of a solution of galanin (10 μg/ml). For optimization, the mobile phase composition needed to be as close as possible to conditions of analyte elution. To do this, a make-up flow of 150 μl/min from the LC system was added to the flow from the infusion pump (50 μl/min). This resulted in a total flow rate of 200 μl/min entering the source of the mass spectrometer. The total flow had an organic and pH composition estimated to be the same as at the time of elution from the column. By altering the capillary and cone voltages, we were able to selectively maximize the 3+ charge state of galanin. This particular charge state was chosen because it had the largest signal when optimized as compared to optimizing for other charge states. The optimized source conditions produced the spectrum seen in Figure 5.2. Using collision-induced dissociation, it was not possible to generate an abundant product ion for use in a multiple reaction monitoring (MRM) transition.

There was no commercially available stable isotope labeled galanin, so other peptides were tested as possible internal standards. Several peptides were tested including angiotensins I, II, and III, β-endorphin, motilin, and glucagon. In every case, galanin and the possible internal standard did not extract in the same way. Galanin always produced a better calibration curve with less variability in replicate samples when no internal standard was used. For this reason, we did not use an internal standard. If stable isotope labeled galanin was used as an internal standard, the LLOQ could likely be reduced to less than 5 ng/ml.

During the first several injections of a sample set, variability in the retention time of galanin was observed. The time increased by approximately one minute over the first

eight injections of extracted plasma. We attribute this to an accumulation of peptides and proteins on the analytical column. They appear to have a strong affinity for the column as the organic wash does not remove them, although they do eventually run off of the column. We believe that these compounds provide an additional partitioning interaction with galanin resulting in an increased retention time. Allowing mobile phase alone to run through the column for several hours returned the elution back to its original retention time.

Preparation of Stock and Standard Solutions

All stock and standard solutions were prepared in blank plasma. This was done because plasma samples spiked with water standards were found to have more variability in resulting peak areas. When stocks were made in plasma, improvement was seen in response variability, sensitivity, and the linearity of calibration curves. This is likely a result of galanin adhering to sample vials when dissolved in water. The addition of plasma may add additional peptides and proteins that compete or saturate vial wall binding sites. It was found through stability experiments that galanin concentrations of standard solutions dropped very quickly when they were in water alone. By dissolving galanin in plasma, standard solution stability was greatly improved. Standard solutions of galanin dissolved in plasma still lost approximately 10% of the peptide within 20 minutes, but this allowed enough time to prepare and process all samples before galanin concentrations diminished to a great extent. Yet because of the eventual loss of galanin in the standard solutions, fresh standard solutions were made daily, just prior to use. The stock solution (1 mg/ml) was kept frozen at -20°C to maintain stability. No significant

loss of galanin was observed in the stock solution as indicated by similar peak areas from the same concentration samples prepared on different days. Neither borosilicate glass nor deactivated glass tubes had improved analyte adhesion characteristics.

Sample Preparation

One of the most important steps for minimizing variability in the sample preparation was found in the precipitation of plasma proteins. It was found that the addition of acetonitrile for precipitation must be done slowly. Rapid precipitation yielded more variability in the responses and reduced sensitivity. This may be a result of clumping of the precipitate, possibly trapping some of the analyte. Slower precipitation while vortexing produced a much finer precipitate and it also improved the sensitivity as well as the variability in galanin peak areas.

It was also found that extracted galanin samples could not be filtered. Galanin bound so strongly to the filtration membranes that when samples were filtered, nearly 100% of the galanin was lost. In order to remove insoluble particulates prior to LC injection, all extracted samples were centrifuged at 60,000xg.

Due to galanin's tendency to adhere to sample vials and filtration membranes, there was concern over the possibility of galanin not being stable in the autosampler over the entire time needed for analysis for a sample set. In order to test this, eight galanin samples were extracted. After they were reconstituted and centrifuged, they were mixed and then redistributed into eight autosampler tubes and placed in the autosampler. The samples were mixed to remove any variability between samples as a result of preparation. The samples remained in the autosampler, and one was injected every three hours. No

decrease in galanin peak areas was observed. The standard deviation of the peak areas was 5.9%, indicating that the samples were stable for at least 21 hours. This was more than the time needed for the analysis of a whole sample set.

Method Validation

With an initial sample volume of 150 µl plasma, this method for the quantitation of galanin was validated over the range from 10 to 1000 ng/ml. Accuracy and precision of the method were determined over three sample sets using five validation points at 10, 25, 75, 300, and 1000 ng/ml with five replicates of each point on each day. Calibration points were at 10, 25, 100, 250, and 1000 ng/ml. Calibrations curves were generated by plotting these known galanin concentrations vs. the corresponding peak area with a 1/y weighting scheme. 1/y weighting puts additional emphasis on lower concentrations during linear regression. This weighting scheme was chosen over 1/x, $1/y^2$, and $1/x^2$ weightings because it gave the lowest sum of the percent residuals for the calibration curve. Percent residuals are calculated by dividing the residual for each point (from the linear regression) by the corresponding peak area and multiplying by 100%. This allowed the optimization of the calibration curve to give the most accurate calculated concentration both at the top and at the bottom of the calibration range. All calibration curves had a minimum R^2 value of 0.99. In order to eliminate any bias based on injection order, all samples in a set were randomized prior to injection.

The LLOQ (lower limit of quantitation) for the method was set at 10 ng/ml. A typical chromatogram for galanin at the LLOQ is shown in Figure 5.1. Due to variation associated with protein precipitation, differing levels of endogenous compounds appear

from sample to sample, as seen if Figure 5.1, peaks at 6.2, 7.5, 8.1 and 9.1 minutes. Even so, the extraction of galanin (8.4 minutes) remains consistent. With an appropriately labeled galanin internal standard, one may be able to decrease the LLOQ.

Accuracy and precision are represented as percent error and percent relative standard deviation (% RSD), respectively. Within run accuracy and precision at the LLOQ was determined to be 11.4% error and 10.3% RSD. The within run precision and accuracy was found to be less than 11.6% error and less than 6.5% RSD for all other points. Between run accuracy and precision at the LLOQ was 17.4% error and 10.9% RSD, respectively. All other validation points were found to have an error of less than 8.0% and less than 6.8% RSD for between run accuracy and precision. Detailed accuracy and precision data can be found in Table 5.2.

Recovery could not be calculated for the extraction because all galanin stock and standard solutions were made in plasma rather than water. Again, this was to slow analyte loss due to sample tube wall binding. As a result of galanin loss, water solutions were found to have much lower peak areas than equal concentration samples extracted from plasma.

Method Application

We demonstrated the utility of this method by determining the half-life of galanin in rats. We analyzed plasma from rats that were given a single IV bolus dose of galanin (0.5 mg/kg). Due to the expected short half-life of galanin, blood was drawn within the first 30 seconds after dosing. After collection, all samples were processed by the described method within 30 minutes. A concentration vs. time plot of galanin is shown

in Figure 5.3. Pharmacokinetic analysis shows that human galanin has an elimination half-life of 1.0 minutes in rats. The calculated elimination half-life is reasonable considering the half-life of galanin in humans is approximately 3.5 to 4.0 minutes [15,16].

Conclusion

We have described a new LC/MS method for the quantitation of the polypeptide galanin from rat plasma. We were able to validate this method from 10 to 1000 ng/ml without the use of an internal standard. The LLOQ may be reduced, with an appropriate internal standard, to a level as low as 1 ng/ml. The method is sensitive, accurate, and specific to galanin. The method has proven to be useful and well suited for the determination of pharmacokinetic parameters.

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Time	% A	% B
0	81	19
8	65	35
9	5	95
13	5	95
15	81	19
24	81	19

Table 5.2

Accuracy (average % error) and precision (% standard deviation) at five concentrations of galanin extracted from plasma for within run (n=5) and between run (n=15) validation.

Within run		
Conc (ng/ml)	avg % error	% stdev
10	11.4	10.3
25	5.8	6.4
75	2.5	2.9
300	11.5	3.4
1000	5.1	6.2
Between run		
conc (ng/ml)	avg % error	% stdev
10	17.4	10.9
25	5.7	6.7
75	3.9	3.1
300	7.9	3.7
1000	6.0	6.4

Figure 5.1

Typical ion chromatograms of m/z 1053.4 for galanin (8.4 min) at the LLOQ (10 ng/ml), (a) and blank extracted plasma, (b)

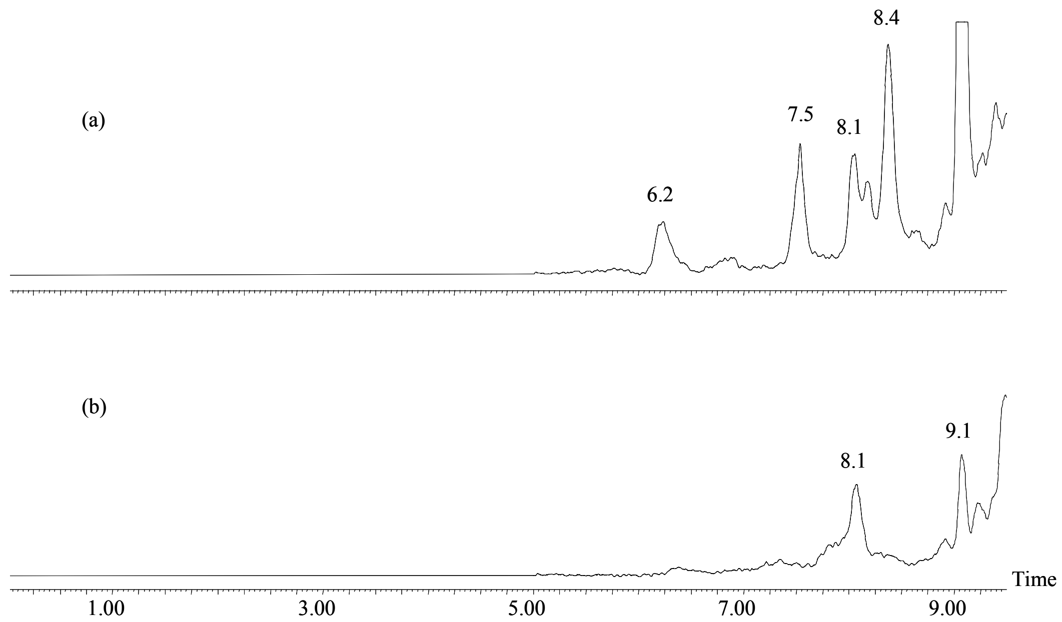

Figure 5.2

Spectrum of galanin showing its multiple charge states

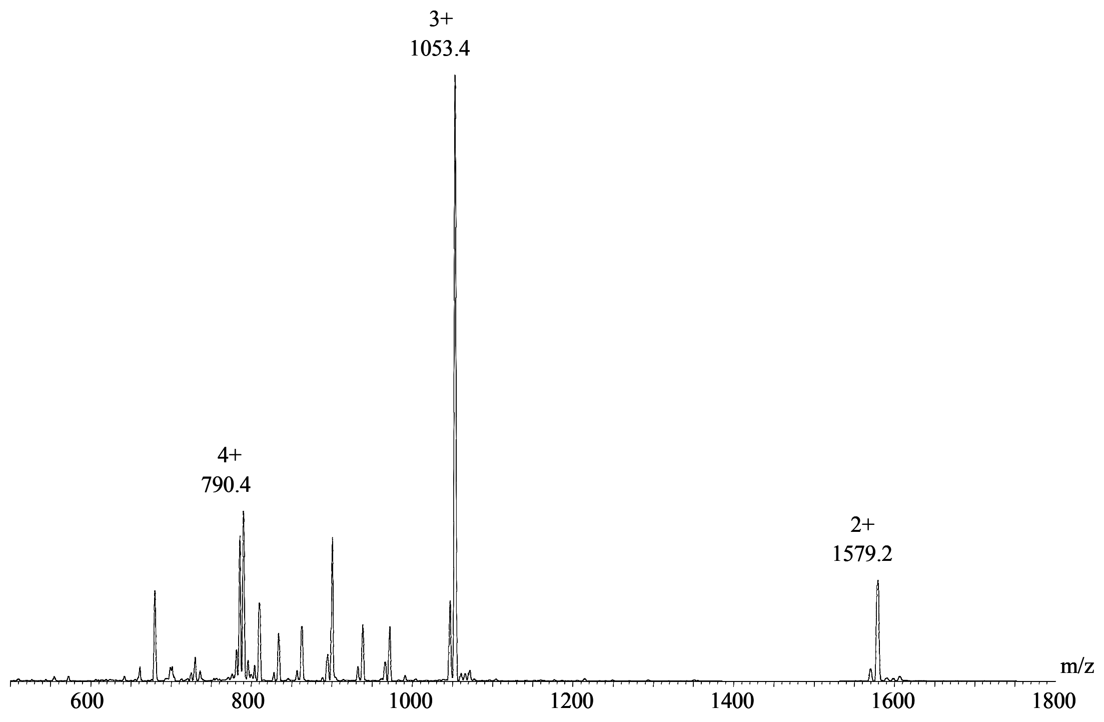

Figure 5.3

Concentration vs time plot for galanin administered to a male Sprague-Dawley rat with a dose of 0.5 mg/kg. The half-life was determined to be 1.0 minutes.