Development of chiral LC-MS methods for small molecules and their applications in the analysis of enantiomeric composition and pharmacokinetic studies

by

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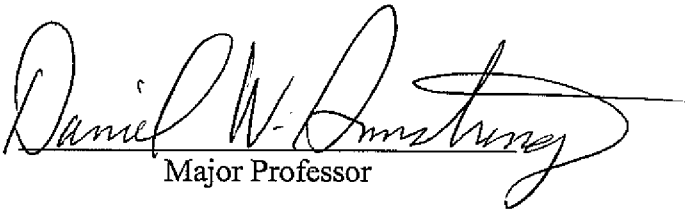
has met the dissertation requirement of Iowa State University



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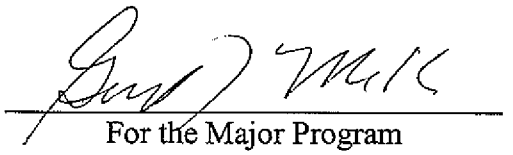

For the Major Program

This dissertation is dedicated to

My parents, for taking a chance on a better life for themselves and their children

My husband, Jason, for being my companion, colleague, and soul mate

and to the loving memory of my Uncle Navin, whose life was an inspiration.

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ABSTRACT

The purpose of this research was to develop sensitive LC-MS methods for enantiomeric separation and detection, and then apply these methods for determination of enantiomeric composition and for the study of pharmacokinetic and pharmacodynamic properties of a chiral nutraceutical.

Our first study, evaluated the use of reverse phase and polar organic mode for chiral LC-API/MS method development. Reverse phase methods containing high water were found to decrease ionization efficiency in electrospray, while polar organic methods offered good compatibility and low limits of detection with ESI. The use of lower flow rates dramatically increased the sensitivity by an order of magnitude. Additionally, for rapid chiral screening, the coupled Chirobiotic column afforded great applicability for LC-MS method development.

Our second study, continued with chiral LC-MS method development in this case for the normal phase mode. Ethoxynonafluorobutane, a fluorocarbon with low flammability and no flashpoint, was used as a substitute solvent for hexane/heptane mobile phases for LC-APCI/MS. Comparable chromatographic resolutions and selectivities were found using ENFB substituted mobile phase systems, although, peak efficiencies were significantly diminished. Limits of detection were either comparable or better for ENFB-MS over heptane-PDA detection. The miscibility of ENFB with a variety of commonly used organic modifiers provided for flexibility in method development. For APCI, lower flow rates did not increase sensitivity as significantly as was previously found for ESI-MS detection.

The chiral analysis of native amino acids was evaluated using both APCI and ESI sources. For free amino acids and small peptides, APCI was found to have better sensitivities over ESI at high flow rates. For larger peptides, however, sensitivity was greatly improved with the use of electrospray. Additionally, sensitivity was enhanced with the use of non-volatile additives. This optimized method was then used to simultaneously separate all 19 native amino acids enantiomerically in less than 20 minutes, making it suitable for complex biological analysis.

The previously developed amino acid method was then used to enantiomerically separate theanine, a free amino acid found in tea leaves. Native theanine was found to have lower limits of detection and better sensitivity over derivatized theanine samples. The native theanine method was then used to determine the enantiomeric composition of six commercially available L-theanine products. Five out of the six samples were found to be a racemic mixture of both D- and L-theanine. Concern over the efficacy of these theanine products led to our final study evaluating the pharmacokinetics and pharmacodynamics of theanine in rats using LC-ESI/MS.

Rats were administered D-, L-, and D,L-theanine both orally and intra-peritoneally. Oral administration data demonstrated that intestinal absorption of L-theanine was greater than that of D-theanine, while i.p. data showed equal plasma uptake of both isomers. This suggested a possible competitive binding effect with respect to gut absorption. Additionally, it was found that regardless of administration method, the presence of the other enantiomer

always decreased overall theanine plasma concentration. This indicated that D – and L-theanine exhibit competitive binding with respect to urinary reabsorption as well. The large quantities of D-theanine detected in the urine suggested that D-theanine was eliminated with minimal metabolism, while L-theanine was preferentially reabsorbed and metabolized to ethylamine. Clearly, the metabolic fate of racemic theanine and its individual enantiomers was quite different, placing into doubt the utility of the commercial theanine products.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction and literature review of the pertinent background information. The following chapters are presented as five complete scientific manuscripts with accompanying tables, figures, and cited references. General conclusions summarize the work.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Background

For many years, liquid chromatography coupled with UV detection has been the dominant technique utilized for enantiomeric separations [1, 2]. UV detection, however, has a number of limitations such as lack of specificity and poor sensitivity for non-UV absorbing species, which have driven scientists to pursue the use of MS detectors as an alternative. An advantage of MS detection over conventional UV detection is that it offers information about the chemical composition of an analyte, thus providing a second dimension of analysis. The high sensitivity of MS detection for a wide variety of analytes also allows for trace quantification. Additionally, MS can be used to detect molecules without chromophores, negating the need for derivatization of samples [3].

Interfacing LC and MS, however, has not always been straight-forward. The high flow rates of LC make maintaining the high vacuum required for MS detection difficult. In addition, conventional gas phase ionization is not suitable for labile, polar, or high molecular weight compounds which are often analyzed by LC methods. A suitable interface must therefore, be able to efficiently evaporate the LC solvent without degrading the analyte and charge many types of analytes including non-volatile and polar compounds [4]. More specifically, a proper interface would accomplish the following: a) transform LC eluent from the condensed phase to the dispersed phase by producing an aerosol; b) remove all mobile phase components such as water, solvents, and buffers; c) ensure efficient transport of dispersed phase analytes to the gas phase towards the MS inlet; and d) ionize solutes during gas phase transfer, for analytes which are unable to form ions in solution [4].

Coupling LC to MS: Creating an Interface

The evolution of LC-MS interfaces is shown in Figure 1. In 1973, Baldwin and McLafferty at Cornell University created the direct introduction interface [5]. In this interface, the sample was placed in a heated capillary ampule at the end of a probe which caused the sample to vaporize. The probe then entered into a vacuum chamber via a vacuum lock and was then pushed into the ion source. From here, vapor pressure efficiently moved the sample vapor into the ionization chamber for MS analysis. Commonly introduced flow rates were below 1 $\mu l\ min^{-1}$. The problem with this interface, however, was that the high vacuum of the ionization chamber caused the capillary eluent to rapidly evaporate leading to the freezing of the solvent eventually plugging the capillary [6].

The first commercially available interface, the moving bed interface, was developed by McFadden in 1976 and marketed by Finnigan [7]. This interface utilized a continuous train of either stainless steel or polyimide ribbon to carry the column effluent to the ion source of the mass spectrometry. During this process, the effluent was heated allowing the solvent to efficiently evaporate and the solute to vaporize. However, less than thirty percent of the solute could be efficiently transferred from the HPLC to the high vacuum housing of the ionization source, most likely a result of poor desorption of the analyte from the belt surface. Additionally, mobile phases with high water content or high flow rates were problematic for effective evaporation [4].

The serendipitous discovery that in the presence of a volatile buffer, ions were formed without any outside ionization source, led to the development of the thermospray (TSP) interface by Vestal in 1983 [8]. In TSP, column eluent flowed through a resistively heated interface towards an evacuated tube. The vaporized sample then exited the interface in a supersonic jet. Analyte ions were then extracted by an electrical potential applied at the mass analyzer inlet, while solvent molecules were purged by a rotary pump. The advantages of TSP were that much higher flow rates (1-2 ml min^{-1}) could be utilized and that a separate ionization source was not required [8]. The sensitivity, however, of the thermospray interfaces were found to be quite poor for larger compounds, such as proteins and peptides [4].

Although dramatic improvements had been made in the design and efficiency of TSP, the popularity of sources in which ionization is carried out at atmospheric pressure began to increase in the late 1980s and early 1990s. By 2000, the LC-MS market was dominated by

atmospheric pressure ionization (API) sources which included electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) [3].

Atmospheric Pressure Ionization Sources

The largest difference between the previously mentioned ionization sources and API is that ionization occurs at atmospheric pressure via either electrical discharges or high voltage electrical fields [9]. Ions that are produced are continuously sampled through a small aperture and pass into the MS due to electrical potential and viscous drag from N_2 sheath gas. Two of the most common API sources, ESI and APCI have very different ionization processes.

ESI is generally considered to be a desorption ionization process; i.e., there are two major steps to ionization: droplet formation and desolvation. A schematic of an ESI probe is shown in Figure 2. For ESI, ionization relies on solution phase chemistry. A potential difference is applied between the ESI needle and the MS inlet which causes the formation of charged droplets at the tip of the needle. This is referred to as the Taylor Cone (Figure 2). The emitted droplets then begin to evaporate forming gas phase ions which are then continuously sampled by the MS inlet to the mass analyzer [10].

There are two generally accepted theories of electrospray ionization, the Dole/Fenn model and the Kebarle model. Both are illustrated in Figure 3. Ions are formed via columbic explosion in the Dole/Fenn model, while ion evaporation is the primary method of forming

ions for the Kebarle model. Both theories stress the requirement that analytes must already exist as ions in solution for ESI [4].

Unlike ESI, ions are formed by gas phase chemistry in APCI. There are three major steps to ionization for APCI: vaporization, ionization of solvent, and charge transfer. A schematic of the APCI probe is shown in Figure 4. For APCI, the analyte and solvent matrix travel through a heated probe, where high temperatures are necessary to desolvate and vaporize the sample. A discharge from a corona needle situated inside the APCI chamber then ionizes the vaporized solvent molecules. The charge is then transferred from the solvent ions to the analyte via gas phase reactions. Therefore, ions need not exist in solution prior to ionization by APCI [11].

Both ESI and APCI provide for facile interfacing with a variety of inlets including LC, flow injection, and capillary electrophoresis (CE). Flow rates up to 1 ml min^{-1} and 2 ml min^{-1} for ESI and APCI, respectively, can be utilized with today's LC-MS equipment [10]. Molecular weights ranging from 10 to 10,000 Da can be detected with fg to pg sensitivity. Additionally, API sources are also compatible with a broad range of analytes from large biomolecules to small non-polar compounds [12].

An analyte compatibility chart for ESI and APCI is shown in Figure 5. Generally, ESI is used for thermally labile, large molecules such as proteins and peptides, while APCI is useful for thermally stable, non-polar or semi-polar, small molecules such as steroids [4]. Additionally, ESI can produce multiply charged ions which appear as low m/z values in the

spectrum allowing for the analysis of compounds outside the molecular weight range of the MS. Unlike APCI where fragmentation can occur, ESI is generally considered a soft ionization source producing the molecular ion with excellent efficiency. Interestingly however, a variety of compounds can be ionized by either probe with differing sensitivities [13].

Adapting Chiral LC methods to LC-API/MS

Mobile Phase Compatibility

Most LC solvents are compatible with API sources with few exceptions. For example, hexane can be used sparingly with APCI but not at all with ESI as a result of the explosion hazard. Tetrahydrofuran (THF) can be used with both APCI and ESI but has been known to cause ion suppression [14]. Nevertheless, common LC solvents such as methanol, acetonitrile, and water are easily interfaced with API/MS instrumentation. Mobile phase composition can affect ionization in both ESI and APCI. High water content mobile phases can be problematic for ESI. The desolvation energy and surface tension of water is high making desorption of ions more difficult and thus result in poorer ionization efficiency [14]. For APCI, the solvent composition also affects ionization as the corona discharge first applies charge to the vaporized solvent molecules. The solvent molecules must therefore readily accept charge and then through gas phase reactions transfer charge to the analyte [15].

In addition to solvent composition, additives and buffer types are important considerations in adapting LC methods to LC-MS. Conventional buffers such as phosphate and borate are

generally not compatible with API/MS. Non-volatile buffers such as the aforementioned can contaminate API chambers or block the sample orifice [16]. In addition to volatility, additives should not form strong ion pairs which result in neutralization after desorption in ESI. Since ionization is based on solution phase chemistry for ESI, the pH of a mobile phase system also factors in the ionization of analytes. Additives generally do not impact ionization as much in APCI as in ESI [16].

Chiral Method Development

MS detection for the analysis of chiral compounds offers a variety of advantages over traditional UV detection. For example, enantiomers have the same mass spectrum providing evidence of a successful enantiomeric separation. Additionally for compounds like amino acids, derivatization is unnecessary [17]. To date, few chiral LC-MS methods have been developed and information on which volatile additives work best for various types of compounds is not readily available. Changing conventional LC buffers to LC-MS compatible ones can also negatively affect enantiomeric resolution and/or selectivity [18]. Table I gives some general guidelines for converting existing LC methods to LC-MS compatible methodologies. By far, the biggest challenge when adapting these methods is to enhance analyte ionization without compromising the chromatographic separation.

Only a few studies have described reverse phase or polar organic LC-MS chiral method development [17-20]. Richards et al., demonstrated for the first time the chiral analysis of non-UV-absorbing compounds by polar-organic phase LC-MS. They found that the mass to charge data in addition to isotopic patterns specific to their analytes enabled unequivocal

evidence of enantiomeric resolution, providing specificity unattainable with traditional UV detection [17]. Although, polar organic methods offer excellent sensitivity, reverse phase assays make up the bulk of chiral LC-MS methods [18]. Penmetsa and co-workers utilized reverse-phase for the enantiomeric separation of seven different analytes by LC-MS. The simplicity of the mobile phases (water/acetonitrile) provided for facile interfacing with ESI-MS detection [18]. The broad applicability of these studies, however, is severely limited. The study by Richards et al., focused on only two compounds, while the mobile phases used in the Penmetsa study are not practical for most analytes. Most analytes will require the use of additives for sensitive detection and successful enantiomeric resolution. The chapter following this introduction discusses in detail the obstacles of converting existing methods, as well as offering solutions for reverse phase and polar organic method development for a large variety of compounds of biological, pharmacological, and industrial interest.

In addition to the modes previously discussed, normal phase methods are often utilized in chiral LC. The incompatibility of hexane with API-MS detection, however, makes the conversion of these methods even more difficult. Various normal phase assays have attempted to overcome this issue by diluting the hexane-rich column eluent with MS compatible solvents [21-23]. Unfortunately, this type of post-column addition can adversely affect peak to peak resolution as well as significantly diminish the sensitivity of the assay. The third chapter of this dissertation continues with solutions to method development in this case for normal phase enantiomeric separations using ethoxynonafluorobutane as a substitute for hexane.

Applications for Chiral LC-MS

Enantiomeric Composition

After chiral LC-MS methods have been developed and optimized, it is then possible to utilize these methods to evaluate the enantiomeric composition of various samples. The qualitative determination of enantiomeric composition is of great importance especially with respect to chiral pharmaceuticals. Often the R-enantiomer of a drug has very different pharmacology, metabolism, and/or toxicology from the S-enantiomer. The presence of one enantiomer can also greatly affect the physiological function of the other. Determining enantiomeric composition has become even more necessary after the Food and Drug Administration implemented its policy on stereoisomeric drugs in 1992. The policy states that manufactures must develop assays for determining individual enantiomers early in drug development [24]. These types of assays can be used to determine enantiomeric purity of synthesized drug candidates or chiral consumer products such as flavor additives. As a result LC-MS protocols which offer high sensitivity and specificity for enantiomeric determination have been energetically pursued [25].

One of the first papers utilizing MS detection for enantiomeric determination was published in 1987. The enantiomeric composition was evaluated for amphetamine and methamphetamine samples. Detection limits were found to be similar for the enantiomers of each of the compounds [26]. A similar study was carried out for the determination of enantiomers of 3-tert-butylamino-1,2-propanediol, an intermediate in the synthesis of timolol, a β-blocker. The limits of detection for both enantiomers were found to be ~ 500 ng

ml^{-1}, which was far better than those of UV detection (~50 $\mu g\ ml^{-1}$) [27]. Both of these methods support the finding that enantiomers have identical MS detector response. Although, more quantitative methods of determining enantiomeric excess (ee) by MS exist [28-31], it is possible to use the MS response of the enantiomers as automatic internal standards to one another for determining relative enatiomeric composition. In this dissertation, the enantiomeric composition of commercially available L-theanine samples is evaluated in Chapter 5 utilizing a reverse-phase method developed for amino acids (Chapter 4).

Chiral Analysis in Biological Matrices: Pharmacokinetic/Pharmacodynamic Studies

In addition to enantiomeric composition, the FDA's policy on Stereoisomeric Drugs also states that manufacturers must evaluate the pharmacokinetics of a single enantiomer or mixture of enantiomers in *in vivo* samples prior to initial clinical trials [24]. A growing number of studies have developed methods for enantiomeric determination of pharmaceuticals for *in vivo* analysis. The use of spiked samples is generally the first step for method development in pharmacokinetic/pharmacodynamic studies. Kolbah and Zavitsanos developed an LC-MS technique for the bioanalysis of several chiral drugs and their metabolites. This study evaluated the method using plasma samples spiked with the analyte, prazosin, and provided high specificity with low limits of quantitation [32]. In a follow-up study, Kolbah and Zavitsanos used a similar LC-ESI/MS method for the enantioselective determination of terazosin, an analogue of prazosin, in human plasma after oral administration. The LC-MS findings demonstrated that R-terazosin and S-terazosin had two

different elimination profiles, suggesting an enantioselective mechanism is involved in drug metabolism [22].

A similar study by Pannakker and co-workers validated an assay for the quantitation of enantiomers of Org 4428, a drug candidate in the treatment of major depression, in human plasma. The limits of detection were in the ng ml^{-1} range for each of the individual enantiomers. The assay employed a normal phase extraction prior to MS analysis [33]. This type of extraction is often necessary to separate analytes from the complex matrices of biological samples. This step must often be modified and optimized in order to obtain high extraction efficiency for sensitive analysis. Ceccato et al., developed a solid-phase extraction (SPE) method prior to LC-MS analysis for the enantiomeric determination of tramadol and its main metabolite. Analytes were eluted from the ethyl silica SPE cartridge using methanol with excellent extraction efficiency. This method was then applied successfully in a pharmacokinetic study of enantiomers of tramadol and its metabolites [34]. With the use of innovative 96-well format SPE set-ups, automation of this extraction step has been made possible reducing overall analysis times [35].

In addition to optimizing extraction efficiency, adapting methods to different types of biological matrices is necessary for successful pharmacokinetic analysis. Sample preparation of plasma, urine, and tissue can present very different challenges. Proteins in plasma and tissue can complicate MS analysis of small molecules. Therefore, proteins are usually precipitated with organic solvent before MS analysis. Tissue samples additionally need to be homogenized after collection [36]. Samples such as urine [37] and saliva [38] do not

generally contain many proteins as in plasma and tissue, but can have various other components. The high salt content of urine, for instance, poses an extreme problem for MS analysis by interfering with ionization. Therefore, a desalting step is usually necessary for sensitive determination of enantiomers in urine samples [37].

The final chapter of this dissertation focuses on the pharmacokinetic and pharmacodynamic analysis of theanine in rats. This chapter is the culmination of all the method development and sample preparation derived in the previous chapters.

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Table I. General guidelines for converting chiral LC methods to LC-API/MS methods

1. Use organic acids such as formic and acetic
2. Avoid alkali metal bases which can cause source corrosion
3. Maintain pH similar to previous LC method
4. Keep buffer concentration below 20mM using volatile salts such as ammonium acetate
5. Optimize concentration levels of additives should be maintained from previous LC method

Figure Captions

Figure 1. Timeline of the evolution of LC-MS interfaces.

Figure 2. Schematic of an ESI source. Courtesy of Thermo Finnigan. LCQ Operations Course Manual. Thermo Finnigan Training Institute. West Palm Beach, FL (2002) p. 41.

Figure 3. Illustrations of the two ionization mechanisms theorized for electrospray. Courtesy of Thermo Finnigan. LCQ Operations Course Manual. Thermo Finnigan Training Institute. West Palm Beach, FL (2002) p. 45.

Figure 4. Schematic of an APCI source. Courtesy of Thermo Finnigan. LCQ Operations Course Manual. Thermo Finnigan Training Institute. West Palm Beach, FL (2002) p. 47.

Figure 5. Analyte compatibility of APCI and ESI sources. Courtesy of Thermo Finnigan. LCQ Operations Course Manual. Thermo Finnigan Training Institute. West Palm Beach, FL (2002) p. 38.

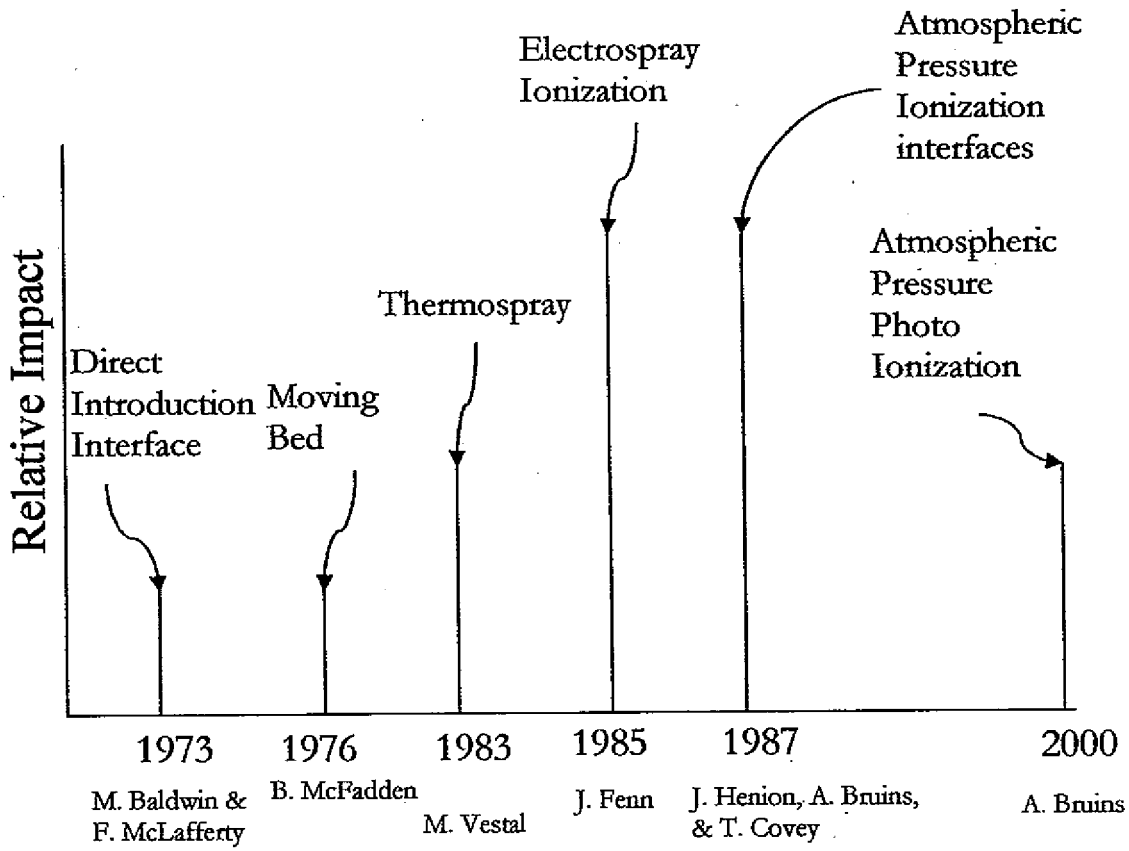
Relative Impact
Direct Introduction Interface
Moving Bed
Thermospray
Electrospray Ionization
Atmospheric Pressure Ionization interfaces
Atmospheric Pressure Photo Ionization
1973
1976
1983
1985
1987
2000
M. Baldwin & F. McLafferty
B. McFadden
M. Vestal
J. Fenn
J. Henion, A. Bruins, & T. Covey
A. Bruins

Figure 1.

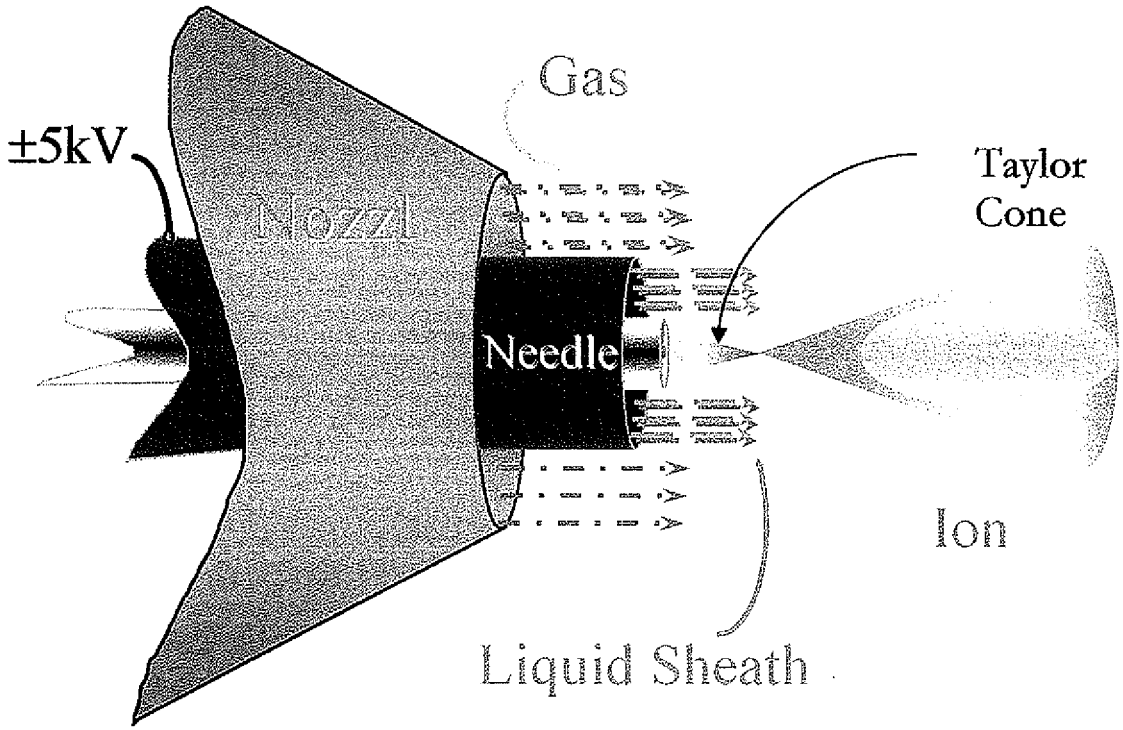

Figure 2.

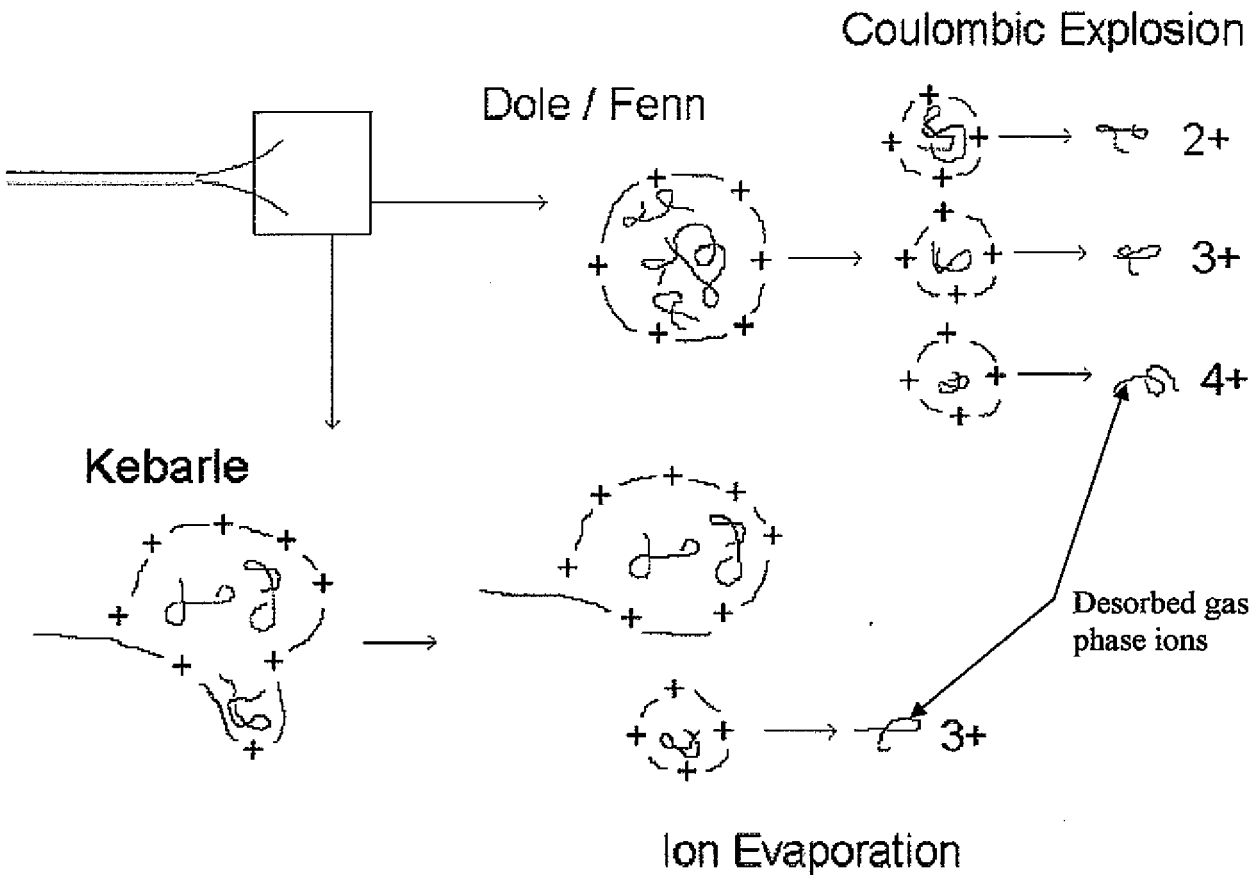
Coulombic Explosion
Dole / Fenn
2+
3+
4+
Kebarle
Desorbed gas
phase ions
3+
Ion Evaporation

Figure 3.

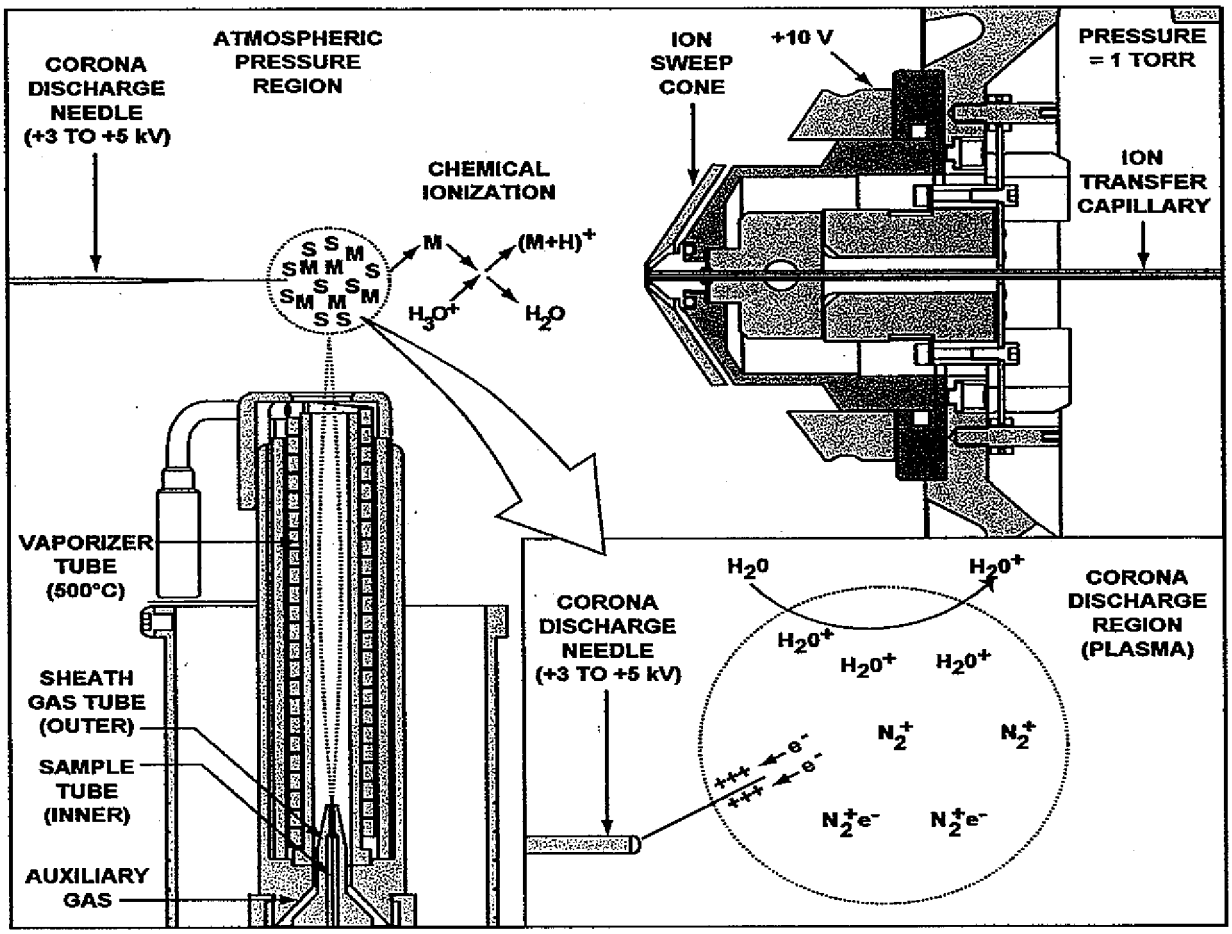
CORONA DISCHARGE NEEDLE (+3 TO +5 kV)
ATMOSPHERIC PRESSURE REGION
CHEMICAL IONIZATION
M
(M+H)+
H3O+
H2O
ION SWEEP CONE
+10 V
PRESSURE = 1 TORR
ION TRANSFER CAPILLARY
VAPORIZER TUBE (500°C)
SHEATH GAS TUBE (OUTER)
SAMPLE TUBE (INNER)
AUXILIARY GAS
CORONA DISCHARGE NEEDLE (+3 TO +5 kV)
H2O
H2O+
N2+
e-
N2+e-
CORONA DISCHARGE REGION (PLASMA)

Figure 4.

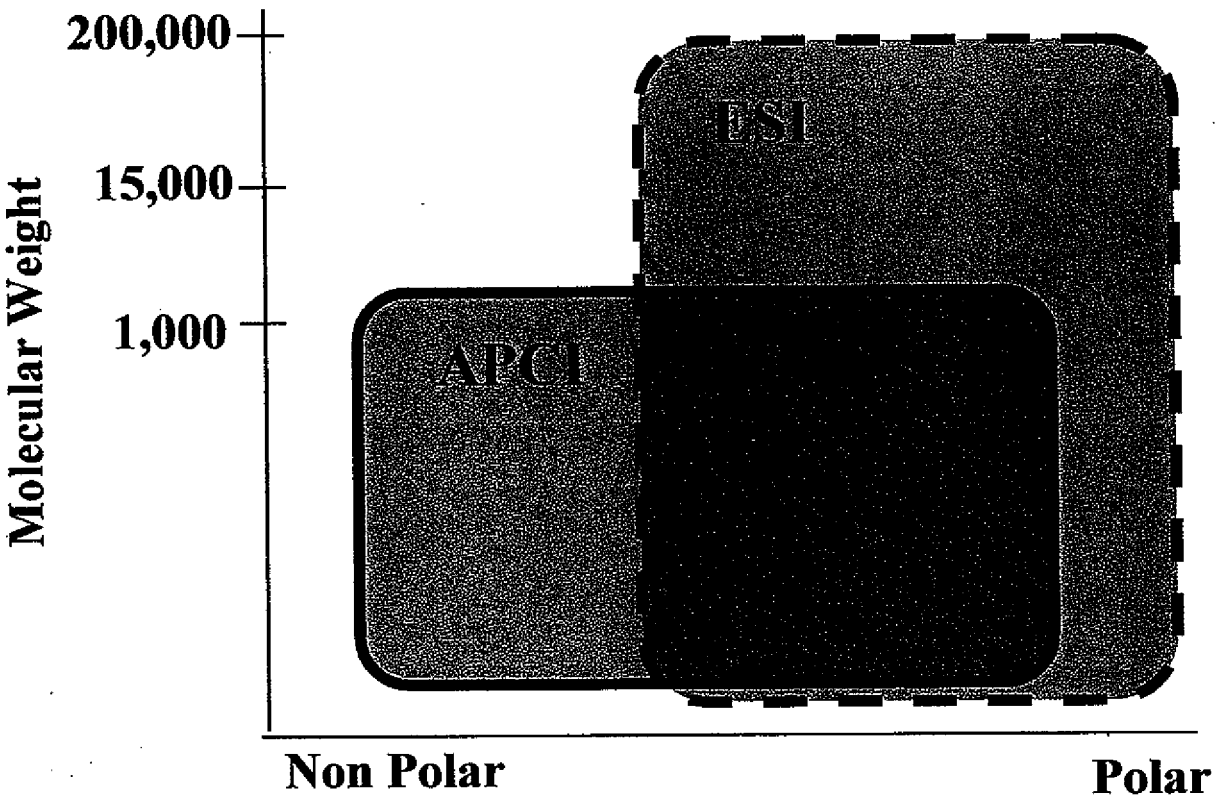
200,000
15,000
1,000
Molecular Weight
ESI
APCI
Non Polar
Polar

Figure 5.

CHAPTER 2. TRANSFORMING CHIRAL LC METHODOLOGIES INTO MORE SENSITIVE LC-ESI-MASS SPECTROMETRY WITHOUT LOSING ENANTIOSELECTIVITY

A paper published in the *Journal of Chromatography A*[1]

Meera J. Desai and Daniel W. Armstrong

ABSTRACT

LC-ESI/MS conditions were optimized for the individual chiral separation of 19 compounds of pharmaceutical interest using the macrocyclic glycopeptide based chiral stationary phases in both polar organic and reverse phase modes. The influence of mobile phase composition and MS additive type on sensitivity was investigated for all classes of compounds tested. Compounds with amine or amide groups were efficiently separated, ionized, and detected with the addition of 0.1 % (w/w) ammonium trifluoroacetate to the solvent system in either the reverse phase or polar organic modes. Macrocyclic glycopeptide coupled column technology was initially used to screen all chiral compounds analyzed. Baseline resolution of enantiomers was then achieved with relatively short retention times and high efficiencies on Chirobiotic T, Chirobiotic V or Chirobiotic R narrow bore chiral stationary phases. The polar organic mode offered better limits of detection (as low as 100 pg/ml) and sensitivity over reverse phase methods. An optimum flow rate range of 200 to 400 µl/min was necessary for sensitive chiral LC/ESI-MS analysis.

INTRODUCTION

Chirality has long been an important criterion for drug discovery and analysis. As a direct result of the advances made in the LC separation of enantiomers in the 1980's, the Food and Drug Administration developed a new policy for the characterization and testing of enantiomeric compounds [1]. HPLC has become the dominant technique employed for the analysis (and sometimes preparation) of chiral molecules in the pharmaceutical industry [2,3]. Consequently, a variety of chiral stationary phases (CSPs) are commercially available for the enantiomeric separation of stereogenic compounds, although only a few dominate the market. Recently, HPLC coupled to atmospheric pressure mass spectrometry (API-MS) has become a popular method for the analysis of pharmaceutical compounds due to its sensitivity, speed, and specificity. However, most existing enantiomeric separation methods were developed using UV detection and they cannot be directly used with LC-MS due to various mobile phase and additive incompatibilities. Simply changing the mobile phase and additives of known enantioselective LC methods to ones that are API-MS compatible often results in diminished or lost enantiomeric separations. When converting existing chiral LC methods to chiral LC-MS methods, the goal is to achieve the highest sensitivity and gain the increased information of MS without losing enantiomeric resolution and/or selectivity.

Many chiral LC methods require the use of the normal phase mode for the enantiomeric separation. When coupled with mass spectrometric ionization sources, such as electrospray ionization (ESI) these techniques, however, are highly incompatible [4]. Normal phase solvents such as hexane do not support the formation of ions which is well known to be

critical for ESI [5]. In addition, high hexane composition introduces a possible explosion hazard in the presence of the high voltage of the electrospray needle for ESI [4]. In order to overcome these difficulties, there is no other choice but to employ extensive post-column addition of MS compatible solvent systems [6,7], which can severely affect resolution and sensitivity. This type of massive post-column dilution is only acceptable when one is not sample limited and has very good separations. However, the compatibility of the reverse phase mode (RPM) and the polar organic mode (POM) with LC-MS interfaces and detection (without the need for post-column dilution) makes them attractive direct approaches for the LC-MS of chiral compounds.

In order to achieve optimal ESI-MS sensitivity, there are restrictions not only on solvent type, but also on solvent additives. Commonly used LC additives, such as phosphate buffers, are incompatible with MS as they can contaminate ionization sources and decrease sensitivity [8]. Unfortunately when doing enantiomeric separations, simply changing the additive type to one that is MS compatible can decrease or eliminate enantiomeric resolution and/or selectivity. Many other chromatographic parameters (such as flow-rate) also can impact MS detection [9-11].

The macrocyclic glycopeptide based chiral stationary phases, teicoplanin [12-15], vancomycin [16-18], and ristocetin A [19,20], have been used successfully in the enantiomeric separation of a variety of chiral compounds. The multi-modal capability (normal phase, reverse phase, or polar organic modes) of these CSPs enables facile interfacing with MS ionization sources [15, 21-23]. The usefulness of these macrocyclic

stationary phases results from their broad selectivity and in the complementary nature of these columns, making them ideal candidates for chiral LC-MS method development [14, 23].

LC chiral method development often employs the technique of directly coupling columns in series to resolve and screen a variety of chiral compounds [24-26]. Kristensen and co-workers used a combination of achiral and chiral columns to resolve methadone enantiomers in serum [24]. Johnson and Wainer coupled two chiral columns to improve the resolution of chiral ketones and diastereomeric alcohols [25]. More recently, Wang et al. reported the coupling of the macrocyclic glycopeptide CSPs as a fast column screening approach for HPLC [26]. All three macrocyclic glycopeptide columns, Chirobiotic R, Chirobiotic T, and Chirobiotic V were coupled together with zero dead volume fittings forming a single chiral screening column. The applicability of this technique for HPLC coupled to atmospheric pressure chemical ionization (APCI) mass spectrometry was demonstrated by Bakhtiar and co-workers [21, 22].

In this study, the optimal conditions for doing chiral LC-ESI/MS were determined and the potential of adapting macrocyclic glycopeptides as a broadly applicable, LC-MS compatible class of CSPs was considered. The glycopeptide coupled column system was used to screen a variety of compounds of pharmaceutical interest by LC-ESI/MS. The enantiomeric separations were then optimized on Chirobiotic T (teicoplanin), Chirobiotic V (vancomycin), or Chirobiotic R (ristocetin A) chiral stationary phases in either reverse phase or polar organic phase mode. MS compatible mobile phases were evaluated for each class of chiral

compound tested. The influence of flow-rate on MS detector sensitivity, as well as on chromatographic parameters such as resolution and selectivity were also investigated.

EXPERIMENTAL

Reagents and Samples

Ammonium trifluoroacetate (NH_4TFA), ammonium acetate (NH_4OAc), and trifluoroacetic acid (TFA) were purchased from Aldrich (Milwaukee, WI, USA). All racemic compounds were obtained from Sigma (St. Louis, MO, USA), except phensuximide, coumafuryl, chloroquine, trimipramine, and metoprolol which were donated by Astec (Whippany, NJ, USA). HPLC grade methanol (MeOH) and water were acquired from Fisher (Pittsburgh, PA, USA). Formic acid and 100 % pure ethyl alcohol (EtOH) were purchased from J.T. Baker (Phillipsburg, NJ, USA) and Apper Alcohol (Shelbyville, KY, USA), respectively. All compounds were dissolved in either 100 % methanol or 50:50 (methanol: water) and diluted to 10 μg/ml prior to injection.

Apparatus and Instrument Conditions

Experiments were performed on a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with an ESI ion source. The MS was operated in positive ion mode using selected ion monitoring (SIM) mode of detection at the appropriate $[M+H]^+$ for each compound. Nitrogen (Praxair,

Danbury, CT, USA) was used as both sheath and auxiliary gases. Ultra-high purity helium (Linweld, Lincoln, NE, USA) was used as the dampening gas in the ion trap. Sheath and auxiliary gases ranged between 35-40 and 10-40 arbs (arbitrary units), respectively. MS parameters were optimized to the following: source voltage = +4.50 kV, capillary voltage = 10.0 V, tube lens offset = 30.0 V, and capillary temperature = 200 degrees Celsius.

Separations were carried out at room temperature on 250 x 4.6 mm ID or 250 x 2.0 mm ID Chirobiotic R, Chirobiotic V, or Chirobiotic T chiral columns from Astec (Whippany, NJ, USA). All three columns, Chirobiotic R, Chirobiotic V, and Chirobiotic T (100 x 4.6 mm ID) were also coupled together with zero dead volume fittings for screening of chiral compounds. The CSPs were coupled together in order of increasing polarity, ristocetin A, followed by vancomycin, followed by teicoplanin (RVT). Reverse phase systems contained either ethanol: water or methanol: water with an MS compatible reagent such as ammonium acetate, formic acid, TFA or NH_4TFA. Polar organic systems contained a mixture of 0.1 % (w/w) NH_4TFA in methanol and 100 % methanol, at varying compositions. Mobile phase flow-rates varied from 200 to 800 µl/min.

RESULTS AND DISCUSSION

Using MS Compatible Mobile Phases

Unlike the normal phase mode, the ability of RPM and POM to seamlessly interface with MS doesn't place very many limitations on the assay. However, when these methods (which were

developed using UV detection) are converted to ones that are MS compatible, a number of factors, including chromatographic selectivity and efficiency, additive volatility, and ion formation or suppression, must be considered. In this study, 19 chiral compounds of pharmaceutical interest, such as beta-blockers, antidepressants, and antimalarial drugs, were individually separated using volatile MS additives. Figure 1 shows the structures and monoisotopic molecular masses for all the compounds tested.

For reverse phase solvent systems, formic acid and TFA were used for protonation of the analytes, in addition to salts such as ammonium acetate and ammonium trifluoroacetate. The traditional mobile phase composition for the polar organic mode usually consists of methanol and/or acetonitrile and small percentages of glacial acetic acid and triethylamine (TEA). Although, acetic acid and TEA are volatile additives the combination of the acidic and basic additives can cause the neutralization of analyte ions [5]. For all the compounds tested in the polar organic mode, the use of 0.1 % NH_4TFA instead of a combination of acetic acid and TEA allowed the enantiomeric separation and proper ionization of the analytes for MS detection.

In general, the chromatographic resolution and selectivity were not significantly affected by changing the nature of the LC mobile phase to MS compatible additives described herein as long *as the optimized concentration levels of these additives were maintained.* However, the choice of volatile additive had a significant impact on signal intensity. For chloroquine enantiomers, for example, the use of ammonium trifluoroacetate provided a signal intensity that was one order of magnitude higher than that found when the same concentration (%

w/w) of ammonium acetate was used. In addition, it was found that compounds with amine or amide functional groups could be effectively ionized with ammonium trifluoroacetate in both the RPM and POM. However, coumafuryl, a compound which does not contain any of those functional groups, could not be ionized at all with the addition of NH_4TFA. Ionization and separation of coumafuryl could only be achieved using a small percentage (0.001 %) of TFA in the reverse phase system.

Limits of Detection for ESI-MS: Reverse Phase vs. Polar Organic

The limits of detection for reverse phase and polar organic phase LC-ESI/MS methods were investigated. Compounds were detected by SIM at their corresponding $[M+H]^+$ values. Concentrations of 0.0001, 0.001, 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, and 10.0 μg/ml were injected of each compound. As can be seen in Table I, detection limits as low as 100 pg/ml and high sensitivities (sensitivity as defined by IUPAC is the slope of the dose/response curve [27]) were achieved for many analytes such as the β-adrenergic blockers in the polar organic mode. Compounds, such as the amino acids separated in the reverse phase mode, had the worst limits of detection and the lowest sensitivities for ESI-MS detection of all compounds tested. The significant differences in detection limit and sensitivity may be attributed to the significant presence of water in reverse phase analysis. Since ESI is a desorption ionization process, the two most important considerations for MS detection are the creation of ions and the desolvation of the analyte. As it is well known that although water supports the formation of ions, its surface tension and solvation energy make it more difficult to desolvate than organic solvents such as methanol or ethanol [5], contributing

greatly to the lower ionization efficiency of reverse phase mode separations, compared to polar organic separations when using ESI-MS detection. The sensitivity of MS detection of amino acids in the reverse phase mode, however, is increased tremendously by switching ionization sources from ESI to APCI [23].

Table I also presents the linearity and r-squared values of the calibration curves for selected compounds. The calibration curves were linear over two orders of magnitude. The limits of detection and linearity of both polar organic and reverse phase methods demonstrate their applicability for mass limited sample analysis. Typical examples of mass limited analysis of chiral samples include those found in biological matrices as well as pharmacokinetic and pharmacodynamic studies.

Flow-rate and Sensitivity for ESI

Mass spectrometers are generally considered mass flow-dependent detectors; that is, detector response is proportional to the total number of molecules being detected per unit of time [28]. As a result, flow-rate is an important parameter in the optimization of any chiral or non-chiral method. The effect of flow rate on sensitivity was evaluated for leucine enantiomers. To our knowledge, specific data on exactly how much the reduction of flow-rate affects MS detection sensitivity has not been published. Using a 4.6 mm ID Chirobiotic T column flow rate was varied from 400 µl/min to 800 µl/min. Figure 2 shows the dose response curves for D- and L-leucine at the two different flow rates. The sensitivity for leucine at 400 µl/min was nearly an order of magnitude higher than that found at 800 µl/min. This observed behavior

supports the known theory that ion sampling and gas phase ionization in ESI play a predominant role in determining sensitive detector response. Thus, ESI-MS detectors seem to be concentration-sensitive [28].

Flow-rate and Chromatographic Parameters

In this study, we determined that the use of narrow bore columns allowed for facile LC-ESI/MS interfacing without compromising enantioselectivity or chromatographic resolution. The optimum flow-rate using these columns was then investigated for the separation of clenbuterol enantiomers. Figure 3 shows the separation of clenbuterol enantiomers on Chirobiotic T at flow rates varying from 100 µl/min to 600 µl/min. Flow-rates greater than 600 µl/min could not be evaluated due to high column back pressure. At the highest flow rates, decreased peak efficiencies were observed (N< 2000 plates). While resolution improved with decreasing flow rate, selectivity remained relatively constant ($\alpha \sim 1.2$). Interestingly, a flow rate of 300 µl/min resulted in the best overall resolution, 3.08, and peak efficiencies (N> 5000 plates). This observation could possibly be attributed to attaining an optimum linear velocity for the narrow bore column in conjunction with the ESI source resulting in the best chromatographic and MS response.

The smaller column diameter also resulted in an increase in detector sensitivity over conventional columns (data not shown) which can be attributed to the increased sample concentration at the detector. This supported the findings of Abian and co-workers, which stated that samples separated with narrow bore columns were 5 times more concentrated than

samples run on conventional columns having the same length [28]. As a result of the enhanced detector response, the amount of sample necessary for detection can be decreased. The use of the narrow bore columns also allowed for a significant decrease in solvent consumption over columns run at typical 4.6 mm ID flow rates.

Coupled Column for Chiral Screening

The macrocyclic glycopeptide coupled column (RVT) was originally developed for 4.6 mm ID columns coupled to UV detection [26]. As previously mentioned, the applicability of the RVT technology has already been demonstrated for LC coupled to APCI-MS [21, 22] with conventional columns. In our study, the RVT coupled column technology was used to screen the 19 racemic compounds using LC-ESI/MS. The separations were then optimized using narrow bore (2.0 mm ID) glycopeptide columns. These molecules were analyzed in either the reverse phase mode or the polar organic mode. The results of the coupled column screening and the optimized chiral separation conditions for each compound are listed in Table II.

According to Wang et al, if a split peak is observed on the glycopeptide coupled column, a baseline separation can be expected on at least one of the three columns, Chirobiotic R, Chirobiotic V, or Chirobiotic T [26]. Resolutions as low 0.12 on the RVT coupled column were able to produce baseline resolutions when conditions were optimized on at least one of the macrocyclic glycopeptide columns. All separations were optimized with run times less than 25 minutes on the 2.0 mm ID Chirobiotic columns. Figure 4 illustrates examples of

compounds screened on the RVT column then optimized on the Chirobiotic T or Chirobiotic V columns. Figure 5 shows the RVT screen and the baseline reverse phase separation of phensuximide enantiomers on the Chirobiotic R column. The coupled column screening technique can also be applied to compounds with more than one chiral center such as labetolol (see Figure 6).

CONCLUSIONS

In this study, existing chiral LC methods were adapted to make LC-ESI/MS compatible ones. Some general rules of thumb when converting these methods to MS amenable methodologies are as follows: a) Polar organic mobile phases are most compatible and easily adaptable to chiral LC-ESI/MS analysis. b) Normal phase methods are incompatible with direct LC coupling to ESI-MS. They can be used if post-column dilutions of a large excess of ESI-MS compatible solvents is acceptable in terms of sensitivity and band broadening. c) When possible avoid high water content reverse phase methods when using ESI-MS detection as it tends to decrease the ionization efficiency. However, switching to APCI for reversed phase separations produces much greater sensitivity. d) Ammonium trifluoroacetate enhances ionization for molecules with amine or amide functionalities. e) Optimized concentrations levels of additives should be maintained when converting existing chiral LC methods to LC-MS compatible methodologies.

In addition, the applicability of the macrocyclic glycopeptide coupled column was demonstrated for the rapid LC-ESI/MS screening of a variety of chiral compounds of pharmaceutical interest. Slight split peaks on the RVT coupled column provided for baseline separations on at least one of the three narrow bore Chirobiotic columns. Optimum flow rates for ESI/MS using these columns ranged between 200 and 400 µl/min. Clearly, LC-ESI/MS can be used as a valuable tool for chiral drug discovery and development.

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FIGURE CAPTIONS

Figure 1. Structures and monoisotopic molecular masses of enantiomeric compounds separated by reverse phase and polar organic phase modes. Chiral centers are indicated with asterisks (*).

Figure 2. Influence of flow-rate on the sensitivity of detection for LC-ESI/MS. Dose/response curves for D- and L-leucine are shown for 0.8 ml/min and 0.4 ml/min. The slopes of the calibration curves at 0.4 ml/min and 0.8 ml/min were approximately 6×10^6 and 9×10^5, respectively.

Figure 3. Effect of flow-rate on the separation of clenbuterol enantiomers. LC-ESI/MS in SIM mode was used at *m/z* 278.0. An optimum flow rate of 300 µl/min provided for the best resolution and enantioselectivity. The separation conditions for clenbuterol are reported in Table II. Rs: resolution, α: selectivity.

Figure 4. Examples of chiral compounds screened on the RVT column then optimized on Chirobiotic T or Chirobiotic V in polar organic phase mode. A) Separation of terbutaline enantiomers, SIM at *m/z* 226.0; B) Separation of mianserin enantiomers, SIM at *m/z* 265.0. Optimized conditions are reported in Table II.

Figure 5. Reverse phase separation of phensuximide enantiomers screened on RVT column then optimized on Chirobiotic R. LC-ESI/MS in SIM mode was used at *m/z* 190.0. Separation conditions are reported in Table II.

Figure 6. Coupled column screening technique applied to labetolol, a compound with two chiral centers. Separation was optimized on Chirobiotic V under the following conditions: 34:66 (0.1% NH_4TFA in methanol:100 % methanol); flow rate: 0.3 ml/min, SIM at *m/z* 329.0.

Table I. Limits of Detection for Selected Compounds

Compound	SIM (m/z)	Column	Linearity	r^2	LOD*
leucine	132	T	y = 5E+06x + 623361	0.9976	50 ng/ml
atenolol	267	T	y = 3E+08x + 6E+07	0.9985	100 pg/ml
promethazine	284	V	y = 9E+07x + 2E+07	0.9978	1 ng/ml
fluoxetine	310	V	y = 4E+07x + 5E+06	0.9976	1 ng/ml

* based on a signal to noise ratio = 3; T: Chirobiotic T, V: Chirobiotic V

Table II. Results of Chiral Method Development using the Macrocyclic Coupled Column System

Compound Number	Compound Name	Classification	Coupled Column		Optimized Column (R/V/T)	Optimized Mobile Phase Conditions	Optimized Column	
			Rs	α			Rs	α
Reverse Phase Mode								
1	isoleucine	amino acid	0.40	1.11	T	50:50 (100% EtOH: 100% H_2O) 0.4ml/min	1.73	1.26
2	leucine	amino acid	0.55	1.07	T	50:50 (100% EtOH: 100% H_2O) 0.4ml/min	3.45	1.39
3	methionine	amino acid	0.37	1.06	T	80:20 (1.0 % NH_4TFA in MeOH: 100 % H_2O) 0.4 ml/min	5.24	1.57
4	phensuximide	anticonvulsant	0.34	1.04	R	66:34 (0.1% NH_4OAc in H_2O: 100 % MeOH) 0.4 ml/min	1.74	1.12
5	coumafuryl	rodenticide	1.07	1.10	V	85:15 (0.001 % TFA in H_2O: 100 % MeOH) 0.4ml/min	1.53	1.43
6	chloroquine	antimalarial	0.17	1.01	V	10:90 (0.1 % NH4TFA in MeOH: 0.1 % formic acid in H2O) 0.3 ml/min	1.92	2.53
Polar Organic Mode								
7	trimipramine	antidepressant	0.12	1.01	V	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	1.50	1.11
8	fluoxetine	antidepressant	0.36	1.03	V	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	1.50	1.12
9	mianserin	antihistamine	0.67	1.08	V	30:70(0.1% NH_4TFA in MeOH: MeOH) 0.4 ml/min	1.78	1.77
10	promethazine	antihistamine	0.67	1.05	V	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.2 ml/min	1.58	1.20
11	clenbuterol	B-adrenergic agonist	0.43	1.03	T	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	3.08	1.21
12	terbutaline	B-adrenergic agonist	2.52	1.18	T	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	2.67	1.36
13	atenolol	B-adrenergic blocker	0.44	1.03	T	50:50(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.4ml/min	1.52	1.13
14	pindolol	B-adrenergic blocker	0.38	1.02	T	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	1.67	1.12
15	alprenolol	B-adrenergic blocker	1.62	1.11	T	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	1.71	1.13
16	propranolol	B-adrenergic blocker	0.55	1.03	T	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	1.58	1.13
17	metoprolol	B-adrenergic blocker	1.39	1.09	V	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	1.45	1.12
18	nicardipine	calcium channel blocker	0.71	1.07	V	10:90(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.4 ml/min	1.43	1.68
19	bupivacaine	local anesthetic	0.44	1.03	V	34:66(0.1% NH_4TFA in MeOH: 100 % MeOH) 0.3 ml/min	1.26	1.13

$Rs = 2(t_2 - t_1) / (w_1 + w_2)$; where t_2 and t_1 are the retention times and w_2 and w_1 are the baseline peak widths of the second and first peak, respectively. $\alpha = (t_2 - t_0) / (t_1 - t_0)$

where T: Chirobiotic T, V: Chirobiotic V, & R: Chirobiotic R

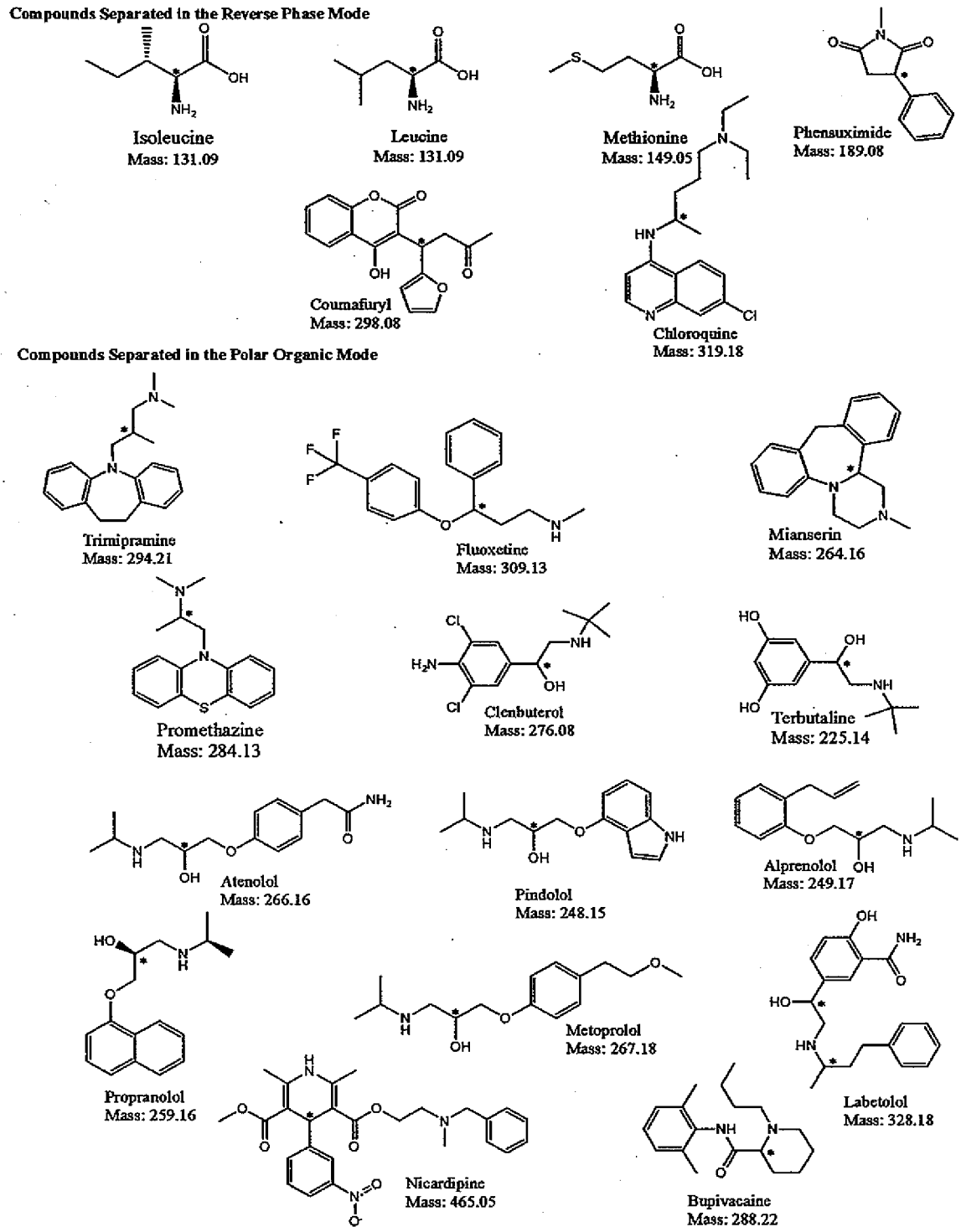
Compounds Separated in the Reverse Phase Mode
Isoleucine
Mass: 131.09
Leucine
Mass: 131.09
Methionine
Mass: 149.05
Phensuximide
Mass: 189.08
Coumafuryl
Mass: 298.08
Chloroquine
Mass: 319.18
Compounds Separated in the Polar Organic Mode
Trimipramine
Mass: 294.21
Fluoxetine
Mass: 309.13
Mianserin
Mass: 264.16
Promethazine
Mass: 284.13
Clenbuterol
Mass: 276.08
Terbutaline
Mass: 225.14
Atenolol
Mass: 266.16
Pindolol
Mass: 248.15
Alprenolol
Mass: 249.17
Propranolol
Mass: 259.16
Metoprolol
Mass: 267.18
Labetolol
Mass: 328.18
Nicardipine
Mass: 465.05
Bupivacaine
Mass: 288.22

Figure 1.

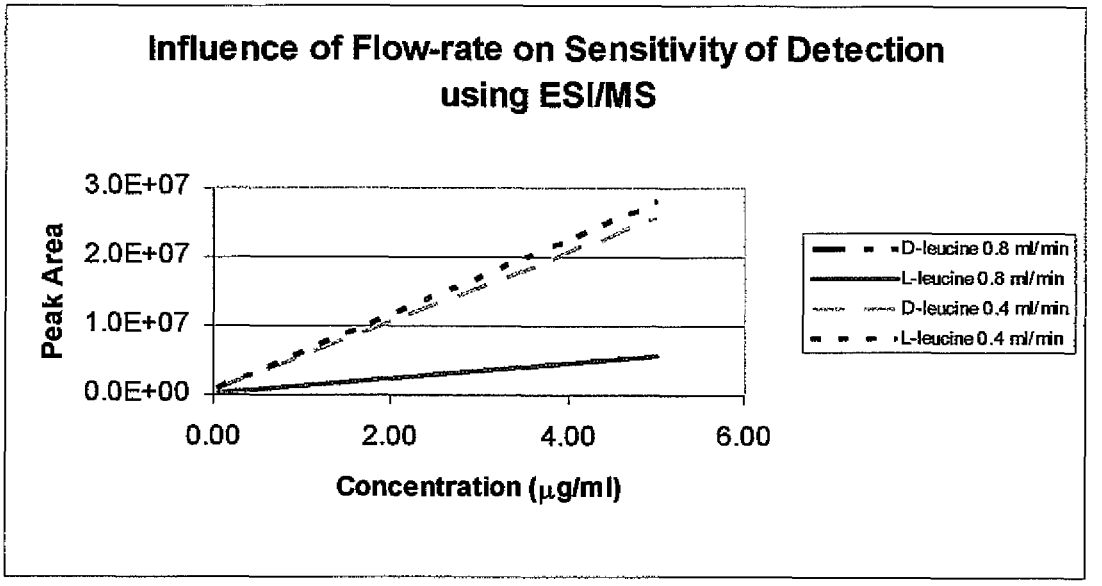
Influence of Flow-rate on Sensitivity of Detection using ESI/MS
Peak Area
3.0E+07
2.0E+07
1.0E+07
0.0E+00
0.00
2.00
4.00
6.00
Concentration (μg/ml)
D-leucine 0.8 ml/min
L-leucine 0.8 ml/min
D-leucine 0.4 ml/min
L-leucine 0.4 ml/min

Figure 2.

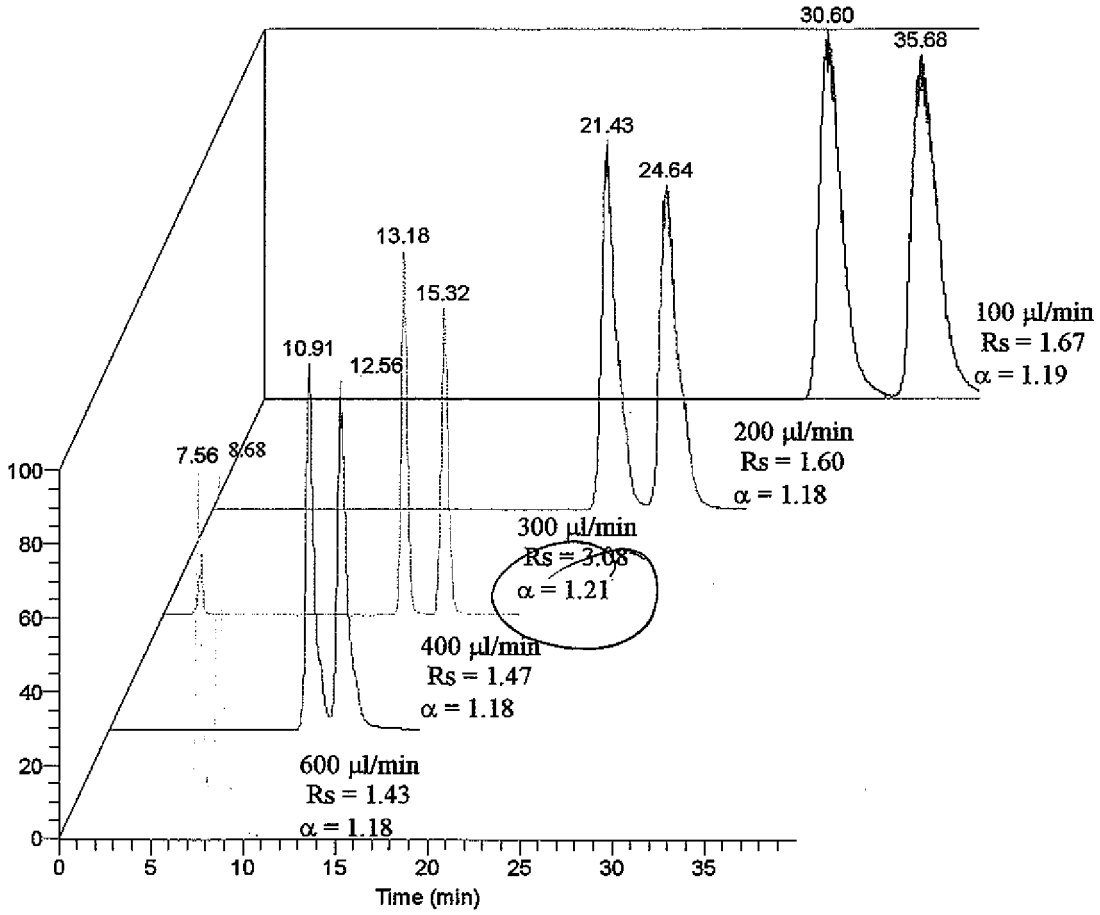
30.60
35.68
21.43
24.64
13.18
15.32
10.91
12.56
7.56
8.68
100 µl/min
Rs = 1.67
α = 1.19
200 µl/min
Rs = 1.60
α = 1.18
300 µl/min
Rs = 3.08
α = 1.21
400 µl/min
Rs = 1.47
α = 1.18
600 µl/min
Rs = 1.43
α = 1.18
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time (min)

Figure 3.

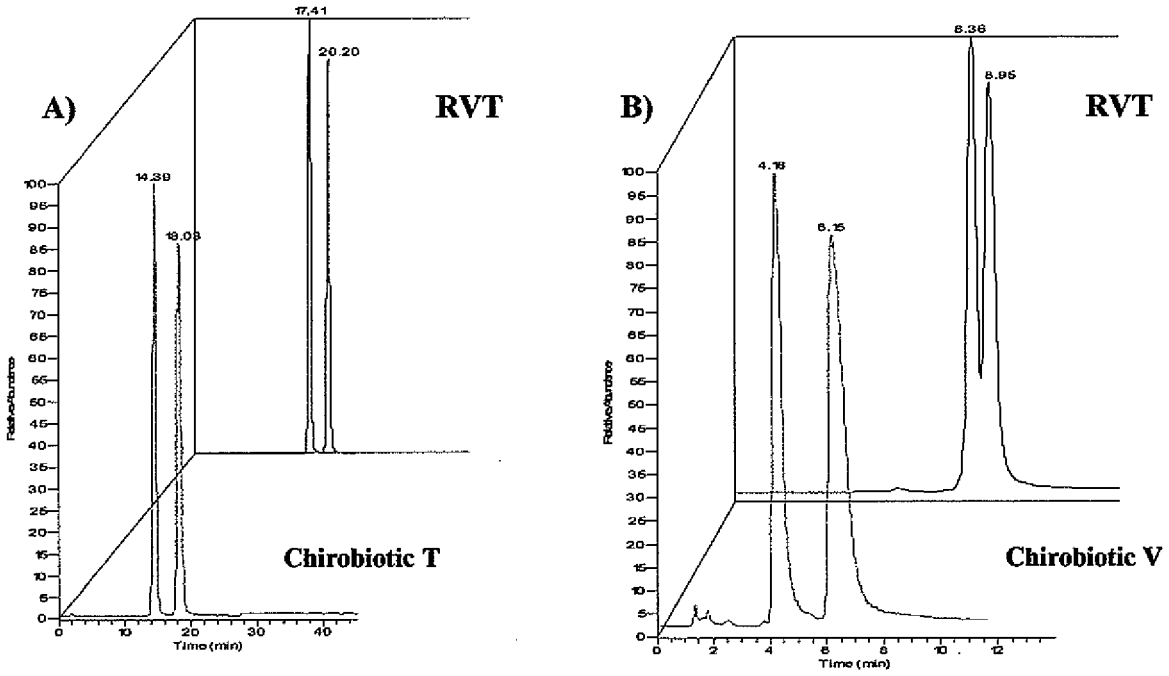
A)
RVT
17.41
20.20
14.39
18.03
Chirobiotic T
Time (min)
B)
RVT
8.36
8.95
4.18
6.15
Chirobiotic V
Time (min)

Figure 4.

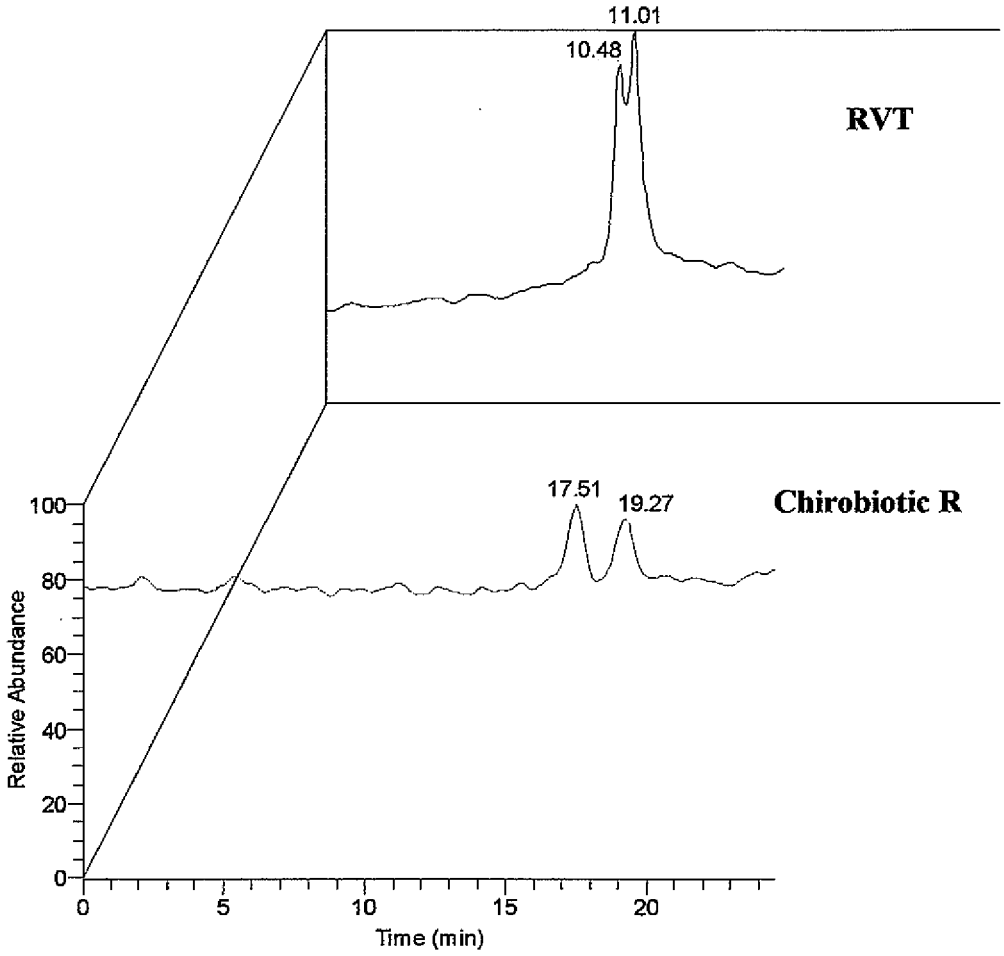
11.01
10.48
RVT
17.51
19.27
Chirobiotic R
100
80
60
40
20
0
Relative Abundance
0
5
10
15
20
Time (min)

Figure 5.

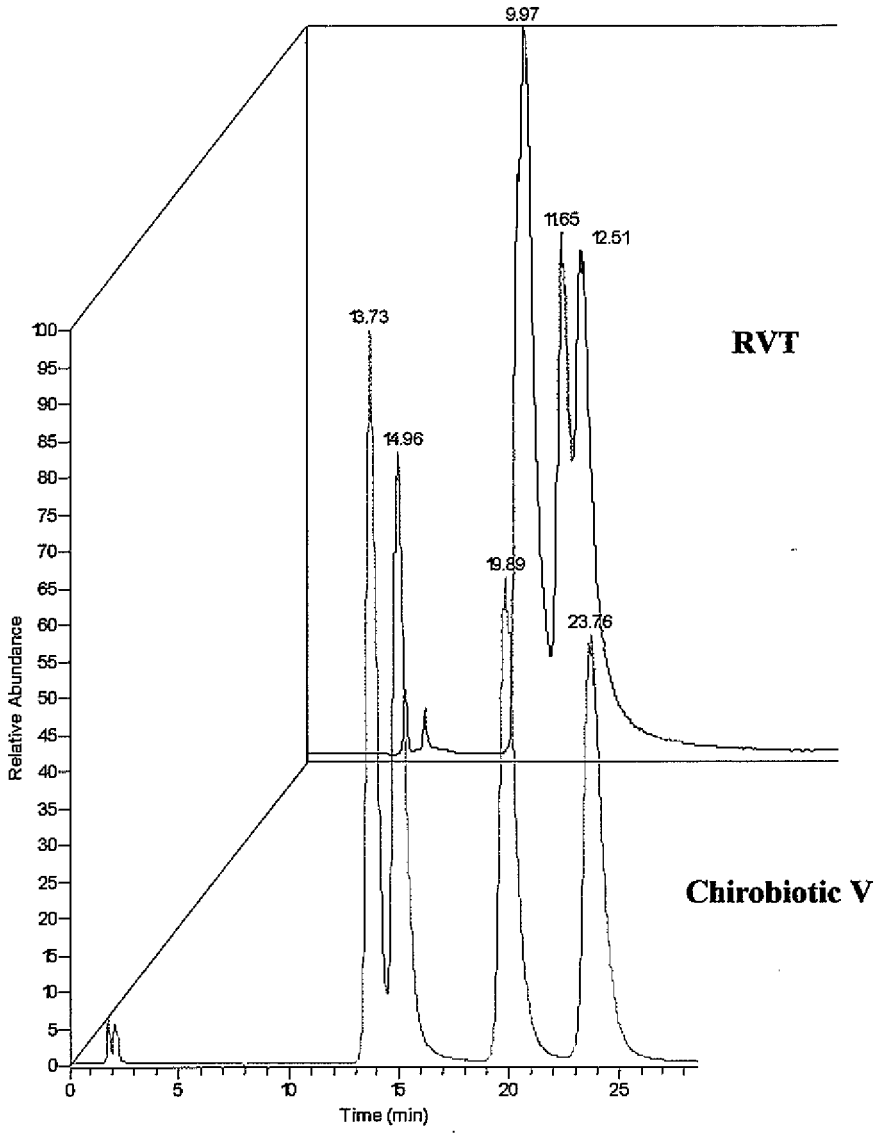
9.97
11.65
12.51
13.73
14.96
19.89
23.76
RVT
Chirobiotic V
Relative Abundance
Time (min)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100

Figure 6.

CHAPTER 3. NORMAL-PHASE CHIRAL LIQUID CHROMATOGRAPHY/ATMOSPHERIC PRESSURE CHEMICAL IONIZATION MASS SPECTROMETRY USING ETHOXYNONAFLUOROBUTANE AS A SUBSTITUTE FOR HEXANE/HEPTANE

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ABSTRACT

The applicability of ethoxynonafluorobutane (ENFB) as a viable substitute for hexane/heptane in normal phase mode for chiral separations using atmospheric pressure chemical ionization/mass spectrometry (APCI-MS) was studied. The compatibility of hexane/heptane with MS detection is questionable as a result of flammability and low flashpoint. ENFB was found to provide comparable separations to heptane mobile phases with little difference in resolution and selectivity. Peak efficiency, however, was compromised as a result of using ENFB. The overall sensitivity and limits of detection were either equivalent or better for ENFB with APCI-MS detection over those of heptane with PDA detection for the compounds analyzed. The miscibility of ENFB with a variety of more polar organic solvents that are used as mobile phase modifiers allowed for flexibility in method development. For the compounds analyzed, ethanol offered the best compromise of chromatographic parameters such as resolution, selectivity, and efficiency. Ethanol and methanol provided the best sensitivity with APCI-MS detection. The flow rate did not appear to impact sensitivity significantly for APCI-MS.

INTRODUCTION

Liquid chromatography coupled to mass spectrometry (LC-MS) has become an increasingly useful tool for analyzing small molecules of biological as well as pharmaceutical and industrial interest. The chiral nature of many of these compounds contributes to their bioactivity and/or their various pharmaceutical/industrial uses. As a result, the Food and Drug Administration has developed policies for analyzing the enantiomers of chiral compounds [1]. The vast majority of existing chiral separation techniques utilize HPLC-UV for chiral separation, detection, and characterization [2,3]. However, the limitations of UV detection, including poor sensitivity for non-UV absorbing compounds and lack of specificity, have motivated scientists to pursue MS detection as an alternative for chiral analysis.

Normal phase LC is used for many enantioselective separations. Only a few studies have demonstrated the use of normal phase conditions for non-chiral LC-MS [4,5]. These studies were able to utilize MS-compatible aqueous solvents under normal phase conditions with silica gel columns, which were more polar than the mobile phase components. Unfortunately most chiral methods rely on bonded or coated stationary phases and conventional normal phase systems containing hexane or heptane to achieve enantioselective separations. These mobile phases, however, are highly incompatible with MS ionization sources such as electrospray ionization (ESI) as they can pose an explosion hazard [6]. In addition, hexane-type solvents do not readily support the formation of ions for other atmospheric pressure ionization sources such as atmospheric pressure chemical ionization (APCI) [7]. In order to

overcome these limitations, a number of studies have used post-column addition of MS-compatible polar organic or aqueous solvents [8-10]. Post-column additions can, however, greatly reduce the sensitivity of an assay and are therefore problematic when sample limited. In addition, massive post-column dilution can also affect chromatographic resolution.

Recently, Kagan proposed the use of ethoxynonafluorobutane, an environmentally friendly, fluorinated solvent, as an alternative to *n*-hexane for the non-chiral normal phase LC of various groups of compounds, such as steroids and benzodiazapines [11]. Separations with ENFB were found to be comparable to those where hexane was used as the main component of the mobile phase. In a follow-up communication, Kagan et al., demonstrated the compatibility of ENFB for LC-APCI/MS using the same group of compounds [12]. Detector response for non-polar compounds was found to be stronger for ENFB mobile phases using APCI over reverse-phase mobile phase systems using ESI. For polar compounds, detector responses for both APCI and ESI were comparable [12].

Macrocyclic glycopeptide based chiral stationary phases, teicoplanin [13-15] and vancomycin [16,17] have been used successfully for the enantioselective separation of a variety of chiral compounds. The multi-modal capability of these stationary phases has enabled them to seamlessly integrate with LC-MS detection for reverse phase and polar organic mode separations [14,15, 17]. In addition to these modes, normal phase, containing hexane, can also be utilized for chiral separations using the glycopeptide stationary phases. In the following study, ethoxynonafluorbutane is substituted for heptane for the

enantioselective separation of various compounds using the macrocyclic glycopeptide stationary phases as well as with a recently developed polymeric chiral stationary phase[18].

EXPERIMENTAL

Reagents and Samples

All racemic compounds were obtained from Sigma (St. Louis, MO, USA), except phensuximide and 3a, 4, 5, 6-tetrahydrosuccinimido-(3, 4-b) acenaphthen-10-one which were donated by Astec (Whippany, NJ, USA) and prop-2-ene-1-sulfinyl-benzene, 3-methanesulfinyl-propene, 2-prop-2-ene-1-sulfinyl-ethanol, and diphenylmethyl phenyl sulfoxide which were kindly donated by Prof. William Jenks of Iowa State University. Ethoxynonafluorobutane (HFE) was purchased as Novec Engineered Fluid HFE-7200 from 3M Co. (St. Paul, MN, USA). HPLC grade heptane (Hep), methanol (MeOH) and 2-propanol (IPA) were acquired from Fisher (Pittsburgh, PA, USA). 100 % pure ethyl alcohol (EtOH) was purchased from Apper Alcohol (Shelbyville, KY, USA). All compounds were dissolved in 100 % IPA and diluted to 100 μg ml^{-1} prior to injection.

Apparatus and Instrument Conditions

Experiments were performed on a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system with a photodiode array detector (PDA) coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with an APCI ion source. The degassing system

on the Surveyor LC pump was bypassed as the fine gas-permeable tubing of the degasser was incompatible with ENFB. The MS was operated in positive ion mode using selected ion monitoring (SIM) mode of detection for each compound. Nitrogen (Praxair, Danbury, CT, USA) was used as both sheath and auxiliary gases. Ultra-high purity helium (Linweld, Lincoln, NE, USA) was used as the dampening gas in the ion trap. Sheath and auxiliary gases were 80 and 20 arbs (arbitrary units), respectively. MS parameters were optimized to the following: APCI vaporizer temp = 400.0 degrees Celsius, corona discharge current = 5.00 μA, tube lens offset = 30.0 V, and capillary temp = 200 degrees Celsius. MS data were acquired using Xcalibur software version 3.1 available from Thermo Finnigan.

Separations were carried out at room temperature on 250 x 4.6 mm ID Chirobiotic V or Chirobiotic T chiral columns from Astec (Whippany, NJ, USA) or the SS-PCAP column (developed in-house) [19]. The SS-PCAP (250 x 4.6 mm ID) is a poly (trans-1,2-cyclohexanediamine acrylamide) stationary phase having a particle size of 5 μm and was obtained from Astec. The normal phase mobile phase systems contained ENFB with ethanol, methanol, or IPA as the organic modifier. Mobile phase flow-rates were 1.0 ml min^{-1} unless otherwise noted.

RESULTS AND DISCUSSION

Substituting MS Compatible, Ethoxynonafluorobutane, for Heptane

Ethoxynonafluorobutane was originally developed by 3M Co. as a cleaning solvent and lubricant carrier [20]. The solvent is an azeotropic mixture of $(CF_3)_2CFCF_2OC_2H_5$ and $CF_3CF_2CF_2CF_2OC_2H_5$ with similar properties. The environmentally friendly properties of this solvent include zero ozone depletion potential and a low atmospheric lifetime of 0.77 years [20]. The boiling point and solvent strength of ENFB are similar to those of hexane [11]. However, the viscosity and UV cutoff are slightly lower for hexane. Nevertheless, ENFB has no flashpoint and low flammability making it ideal for use with APCI sources with MS detection. Additionally, according to 3M, it is completely compatible with Teflon, Peek, and Tygon tubing, permitting its use with most LC systems.

The compounds used in this study are shown in Figure 1. All compounds were analyzed at the appropriate $[M+H]^+$ ion with the exception of diphenylmethyl phenyl sulfoxide. The nature of APCI as a hard ionization source caused this particular compound to fragment as shown in Figure 1. The ion that was monitored for this compound by SIM detection was therefore 167 *m/z*.

For comparison purposes, the chiral separations of 5-methyl-5-phenylhydantoin, 3a, 4, 5, 6-tetrahydrosuccinimido-(3, 4-b) acenaphthen-10-one, and fipronil using ENFB (with MS detection) or heptane (with PDA detection) are shown in Figure 2. For similar ratios of heptane or ENFB to modifier, the peak shapes and retention times are comparable regardless of which stationary phase was utilized. This demonstrated that in most cases ENFB can be substituted for heptane with minimal effects on chromatographic retention. However, the substitution of ENFB for heptane appears to slightly decrease the resolution.

A comparison of the chromatographic separation parameters for heptane mobile phases versus ENFB substituted mobile phases is compiled in Table I. The majority of the compounds tested had similar resolutions (Rs) and selectivities (α) upon substitution with ENFB for heptane. The cases in which the resolutions and selectivities were greatly different were attributed to the differences in flow rate between the two methods. Nonetheless, all compounds tested had produced lower peak efficiencies (N) when ENFB-based mobile phases were used. These efficiencies can probably be attributed to a) extra-column band broadening as a result of interfacing with the MS detector (which would also occur had the heptane method been interfaced with the MS), and/or b) the higher viscosity of ENFB produces less efficient peaks as a result of poorer mass transfer due to lower diffusion rates.

Limits of Detection for APCI-MS versus PDA detection

The structures of the compounds analyzed in this study were non-polar and were therefore not conducive to ionization by ESI. However, with the assistance of chemical ionization as in APCI, these compounds can form gas phase ions for MS analysis. Table II lists the limits of detection (LOD) and linearity for LC-PDA detection compared to LC-APCI-MS detection. For diphenylmethyl phenyl sulfoxide, the limit of detection is similar for both PDA and MS detection while the sensitivity (as defined by IUPAC is the slope of the dose response curve [21]) is approximately 1.5 times higher for MS detection. For diaminocyclohexane acrylamide, the sensitivity is 3 times higher and the limit of detection is an order of magnitude lower for MS over PDA detection.

Our findings demonstrated that although these compounds have excellent chromophores, their ionization efficiency was sufficient to not only allow MS detection but in the case of diaminocyclohexane acrylamide offer superior detection capabilities with the use of MS. The low surface tension of ENFB [20] allows for ions to be easily desolvated and may also contribute to the reasonable ionization efficiencies of the compounds analyzed.

Effect of Modifier on Chromatographic Parameters

The miscibility of hexane or heptane with certain organic solvents such as methanol is limited, whereas ENFB is completely miscible with a variety of solvents including methanol, ethanol, and 2-propanol providing for greater flexibility in method development. The type of organic modifier, however, can affect the chromatographic parameters of chiral separations. Figure 3 shows examples of three compounds separated on different stationary phases using ethanol, 2-propanol, or methanol as the organic modifier. Methanol provided for high peak efficiencies, but the worst resolutions, for all three compounds while, IPA provided the exact opposite trend, i.e., low efficiencies and high resolutions. With peak efficiencies of 1400+ theoretical plates, moderate selectivities, and baseline or near baseline resolutions, the use of ethanol as the organic modifier often was the best compromise.

Effect of Modifier on APCI-MS Sensitivity

In addition to chromatographic efficiency, resolution, and selectivity, the type of organic modifier can affect APCI-MS sensitivity. The effect of modifier on MS sensitivity was tested for 5-methyl-5-phenylhydantoin. This compound was chosen because it could be separated on the Chirobiotic V using 100% modifier, without any ENFB. The dose response curves for ethanol, methanol, and IPA are shown in Figure 4. The response for the first eluting peak was charted for all three modifiers. The sensitivities for methanol and ethanol were nearly identical; the curves had slopes of approximately 4000. The sensitivity of IPA, however, clearly was much lower, more than half that of the other modifiers. While methanol and ethanol have similar values for surface tension, the surface tension of IPA is greater [22]. The desolvation efficiencies of IPA < methanol ≅ ethanol may contribute to the difference observed for MS sensitivity.

Flow-rate and Sensitivity for APCI

MS detector response is proportionally to the total number of molecules being detected per unit time, making them mass flow-dependent detectors [23]. Therefore, it is possible that flow rate can factor highly in MS detector sensitivity and response [24-26]. In order to determine the dependence of sensitivity on flow rate, standards of α-methyl-α-phenyl succinimide were separated on the Chirobiotic T using flow rates of 1.0 ml min^{-1} and 0.5 ml min^{-1}. The dose response curves are shown in Figure 5. Peak 1 and peak 2 are the first and second eluting enantiomers, respectively. The sensitivity at the lower flow rate was less than two-fold higher than that of the higher flow rate. This observed sensitivity difference is very small compared to that of ESI-MS detection which was previously reported to be nearly an

order of magnitude higher at lower flow rates [17]. Clearly, flow rate has less of an impact on sensitivity for APCI than ESI. Unlike ESI, prior to entering the atmospheric pressure probe chamber, all of the solvent is vaporized into gas by the APCI probe. This reduces the negative effect of high flow rate on the efficiency of ion sampling and thereby allows APCI to be compatible with high flow rates [25].

CONCLUSIONS

In this study, ethoxynonafluorobutane was found to be a viable alternative to heptane for normal phase enantiomeric separations. ENFB's chemical characteristics, such as having no flashpoint and low flammability, made it especially attractive for use with APCI-MS detection. ENFB substituted mobile phases provided comparable resolutions and selectivities for all the compounds tested, although peak efficiencies were considerably lower than heptane-rich mobile phase methods. The limits of detection and sensitivities for ENFB/MS detected compounds were either comparable or better than those of heptane/PDA detection. The miscibility of ENFB with most common organic solvents made it suitable for method development. Ethanol, as a compromise organic modifier, was found to provide better selectivities than methanol and better efficiencies than IPA mobile phase modifiers. Additionally, methanol and ethanol afforded better sensitivities for APCI-MS than IPA as an organic modifier. Higher flow rates were found to not impact sensitivity greatly.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Structures and molecular weights for compounds analyzed.

Figure 2. Examples of ENFB-substituted and heptane mobile phase chiral separations of selected compounds. A) 5-methyl-5-phenylhydantoin enantiomers separated on the Chirobiotic T stationary phase using ENFB with MS detection (top panel) and heptane with PDA detection (bottom panel). B) 3a, 4, 5, 6-tetrahydrosuccinimido-(3, 4-b) acenaphthene-10-one enantiomers separated on the Chirobiotic V stationary phase using ENFB with MS detection (top panel) and heptane with PDA detection (bottom panel). C) fipronil enantiomers separated on the SS-PCAP stationary phase using ENFB with MS detection (top panel) and heptane with PDA detection (bottom panel). Separation conditions are listed in Table I.

Figure 3. Effect of organic modifier on chromatographic parameters. A) diaminocyclohexane acrylamide enantiomers separated on the SS-PCAP stationary phase using EtOH (top panel), IPA (middle panel) or MeOH (bottom panel) as the organic modifier. B) 4-benzyl-2-oxazolidinone enantiomers separated on the Chirobiotic V stationary phase using EtOH (top panel), IPA (middle panel) or MeOH (bottom panel) as the organic modifier. C) 2-(prop-2-ene-1-sulfinyl)-ethanol enantiomers separated on the Chirobiotic T stationary phase using EtOH (top panel), IPA (middle panel) or MeOH (bottom panel) as the organic modifier. Rs, resolution; α, selectivity; N_1, peak efficiency for the first eluting peak. All flow rates were 1.0 ml min^{-1}.

Figure 4. Effect of modifier on APCI-MS sensitivity for 5-methyl-5-phenylhydantoin using the Chirobiotic V stationary phase. All separations were carried out without ENFB using 100 % organic modifier. Linearity of EtOH curve, $y = 3987.4x - 328021$, $r^2 = 0.9909$; linearity of MeOH curve, $y = 3886.8x + 17482$, $r^2 = 0.9905$; linearity of IPA curve, $y = 1706.9x - 61573$, $r^2 = 0.9900$

Figure 5. Dependence of sensitivity on flow rate for α-methyl-α-phenyl succinimide using the Chirobiotic T stationary phase. Peak 1 and peak 2 are the first and second eluting peaks, respectively. Linearity of peak 1 curve for 0.5 ml min^{-1} flow rate, $y = 4525.8x + 139452$, $r^2 = 0.9855$; linearity of peak 1 curve for 1.0 ml min^{-1} flow rate, $y = 2621.3x - 82405$, $r^2 = 0.9942$. Linearities of peak 2 were similar to those of peak 1 for both flow rates.

Table I. Comparison of chromatographic parameters for heptane versus ethoxynonafluorobutane substituted mobile phases

#	Compound Name	Heptane M.P.	Flow Rate (ml/min)	Chromatographic Parameters†				ENFB M.P.*	Chromatographic Parameters‡			
				k'_1	Rs	N_1	α		k'_1	Rs	N_1	α
Chirobiotic V												
1	phensuximide	70:30 Hep: EtOH	0.5	2.15	1.56	8500	1.11	95:5 ENFB:EtOH	5.47	1.41	1200	1.15
2	5-methyl-5-phenylhydantoin	100 % EtOH	1.5	1.00	2.48	2600	1.61	100 % EtOH	0.88	1.60	600	1.58
3	4-benzyl-2-oxazolidinone	70:30 Hep: EtOH	1	2.98	2.55	6500	1.20	70:30 ENFB: EtOH	2.18	1.63	1500	1.30
4	3a,4,5,6-tetrahydrosuccinimido(3,4-b) acenaphthen-10-one	75:25 Hep: EtOH	1	5.88	1.50	4500	1.14	75:25 ENFB: EtOH	4.63	1.44	2300	1.17
5	diphenylmethyl phenyl sulfoxide	90:10 Hep: EtOH	1	2.10	1.28	7400	1.09	90:10 ENFB: EtOH	3.86	1.56	2900	1.16
Chirobiotic T												
6	prop-2-ene-1-sulfinyl-benzene	90:10 Hep: EtOH	1	5.98	1.73	6900	1.11	95:5 ENFB:EtOH	8.75	1.22	3400	1.10
7	3-methanesulfinyl-propene	75:25 Hep: EtOH	1	6.96	1.89	5300	1.13	75:25 ENFB: EtOH	5.40	1.44	3600	1.13
8	2-(Prop-2-ene-1-sulfinyl)-ethanol	75:25 Hep: EtOH	1	6.81	2.96	5800	1.23	75:25 ENFB: EtOH	7.53	1.58	1800	1.17
9	α-methyl-α-phenyl succinimide	50:50 Hep: EtOH	1	0.95	1.44	4600	1.25	60:40 ENFB: EtOH	1.75	1.60	1900	1.23
10	5-methyl-5-phenylhydantoin	50:50 Hep: EtOH	1	2.65	4.81	1000	3.01	50:50 ENFB:EtOH	3.62	4.38	400	2.49
11	α,α-dimethyl-β-methylsuccinimide	70:30 Hep: EtOH	1	1.48	1.18	7800	1.10	95:5 ENFB:EtOH	6.00	1.13	4900	1.10
SS-PCAP												
12	oxazepam	50:50 Hep: EtOH	1.5	5.28	3.62	1800	1.51	40:60 ENFB: EtOH	5.60	1.83	1600	1.25
13	1,1'-bi-2-naphthol	50:50 Hep: EtOH	1	5.16	3.00	3300	1.29	50:50 ENFB:EtOH	3.29	2.59	2600	1.32
14	fipronil	80:20 Hep: EtOH	1	2.32	2.86	4800	1.21	80:20 ENFB:EtOH	2.68	1.59	1100	1.25
15	3,4-dihydroxyphenyl-α-propylacetamid	50:50 Hep: EtOH	1	3.73	1.39	2800	1.17	60:40 ENFB: EtOH	9.70	1.59	1100	1.24
16	diaminocyclohexane acrylamide	90:10 Hep: EtOH	1	0.88	2.24	5000	1.32	90:10 ENFB: EtOH	2.76	1.62	1400	1.25

† parameters using PDA detection; ‡ parameters using MS detection

$k'_1 = (t_1-t_0)/t_0$; $N_1 = 16(t_1/w_1)^2$; $Rs = 2(t_2 - t_1) / (w_1 + w_2)$; $\alpha = (t_2 - t_0) / (t_1 - t_0)$ where t_2 and t_1 are the retention times and w_2 and w_1 are the baseline peak widths of the second and first peak, respectively, and where t_0 is dead time.

* all flow ratee were 1.0 ml/min for mobile phases containing ENFB

Table II. Limits of Detection for Selected Compounds

Compound	SIM (m/z)	Column	LC-PDA			LC-APCI-MS		
			Linearity	r^2	LOD*	Linearity	r^2	LOD*
diphenylmethyl phenyl sulfoxide	167	V	y = 206790x + 4E+6	0.9854	1 µg/ml	y = 341868x + 1E+6	0.9975	1 µg/ml
diaminocyclohexane acrylamide	223	SS-PCAP	y = 1E+06x + 3E+06	0.9989	5 µg/ml	y = 3E+06x - 827829	0.9993	500 ng/ml

*LOD, limit of detection based on signal to noise ratio = 3

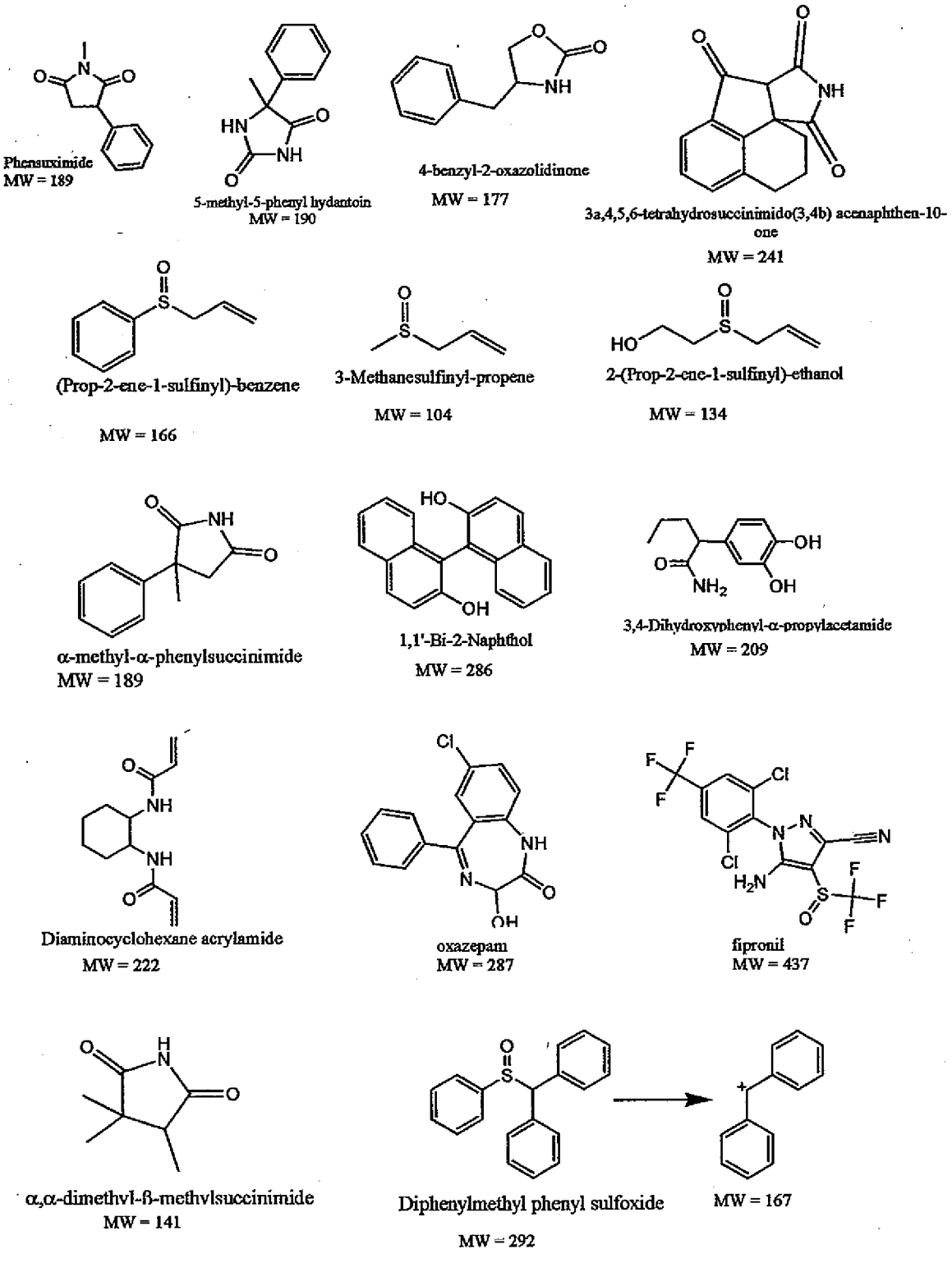
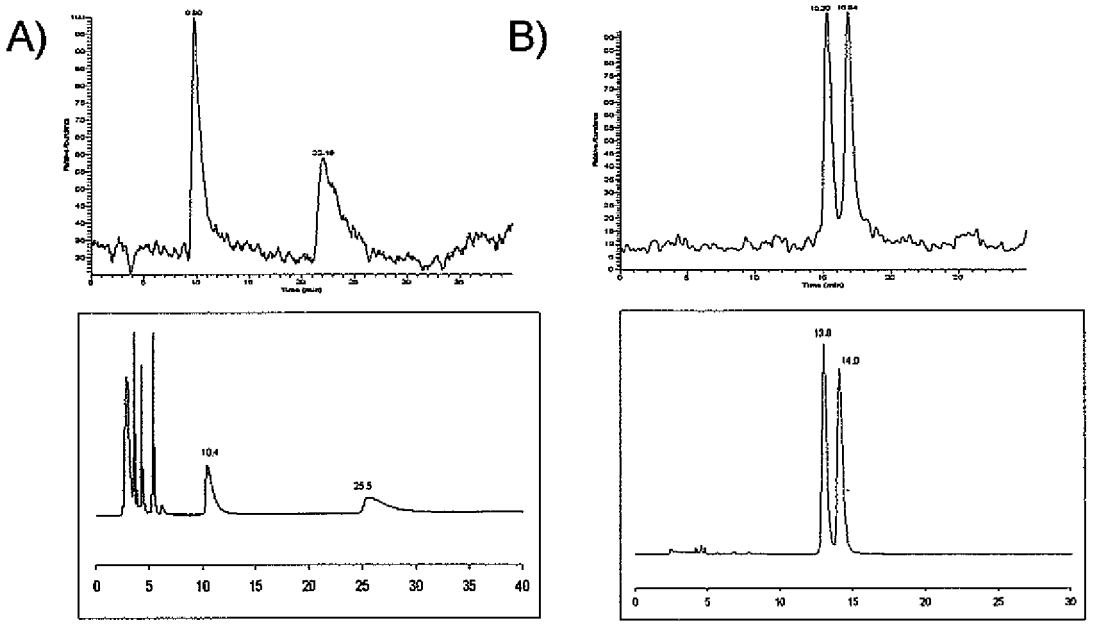
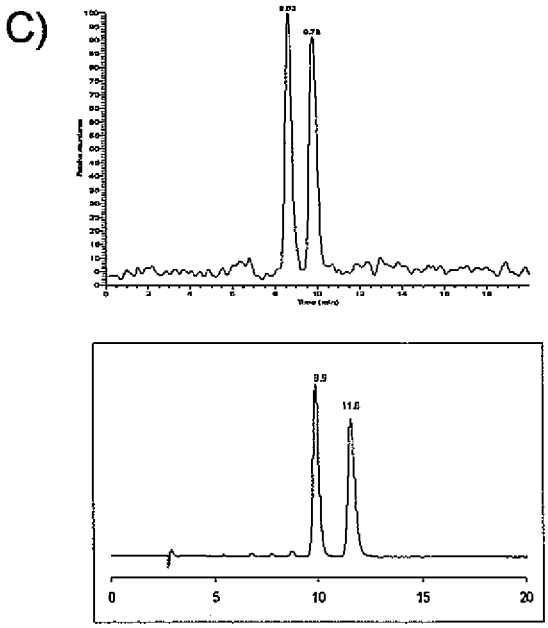

Phensuximide
MW = 189
5-methyl-5-phenyl hydantoin
MW = 190
4-benzyl-2-oxazolidinone
MW = 177
3a,4,5,6-tetrahydrosuccinimido(3,4b) acenaphthen-10-one
MW = 241
(Prop-2-ene-1-sulfinyl)-benzene
MW = 166
3-Methanesulfinyl-propene
MW = 104
2-(Prop-2-ene-1-sulfinyl)-ethanol
MW = 134
α-methyl-α-phenylsuccinimide
MW = 189
1,1'-Bi-2-Naphthol
MW = 286
3,4-Dihydroxyphenyl-α-propylacetamide
MW = 209
Diaminocyclohexane acrylamide
MW = 222
oxazepam
MW = 287
fipronil
MW = 437
α,α-dimethyl-ß-methylsuccinimide
MW = 141
Diphenylmethyl phenyl sulfoxide
MW = 292
MW = 167

Figure 1.



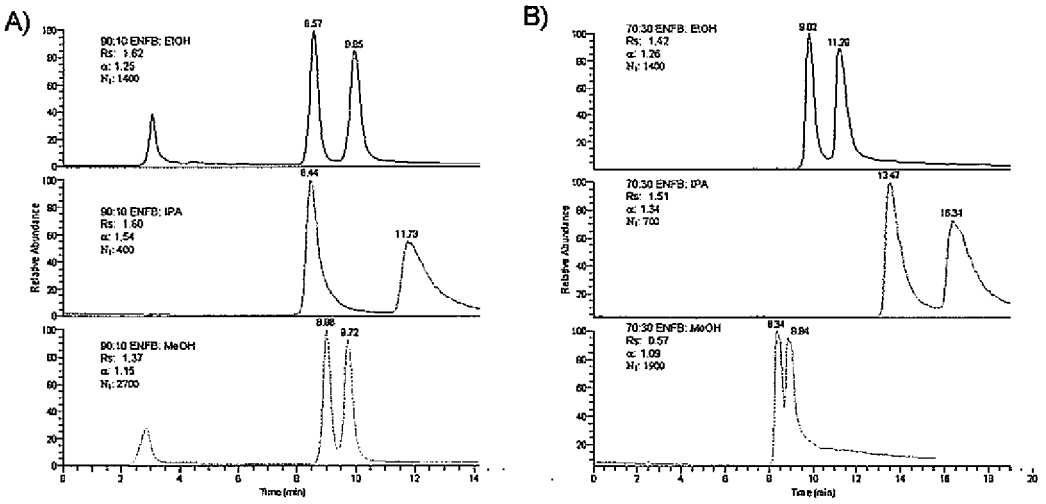
5-methyl-5-phenylhydantoin

3a, 4, 5, 6-tetrahydrosuccinimido-(3,4-b) acenaphthen-10-one



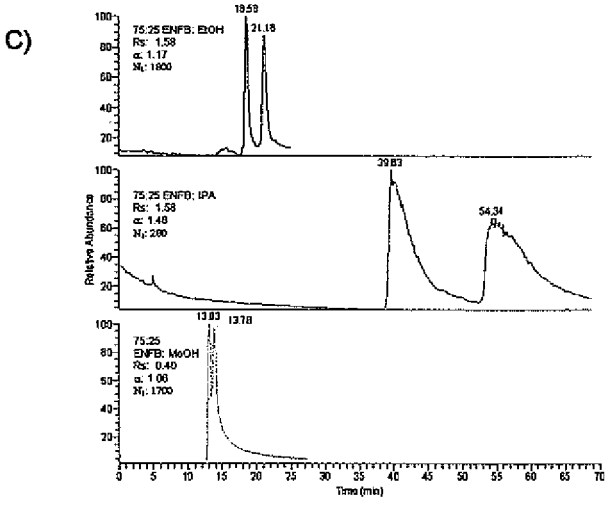
fipronil

Figure 2.



Diaminocyclohexane acrylamide

4-benzyl-2-oxazolidinone



2-(Prop-2-ene-1-sulfinyl)-ethanol

Figure 3.

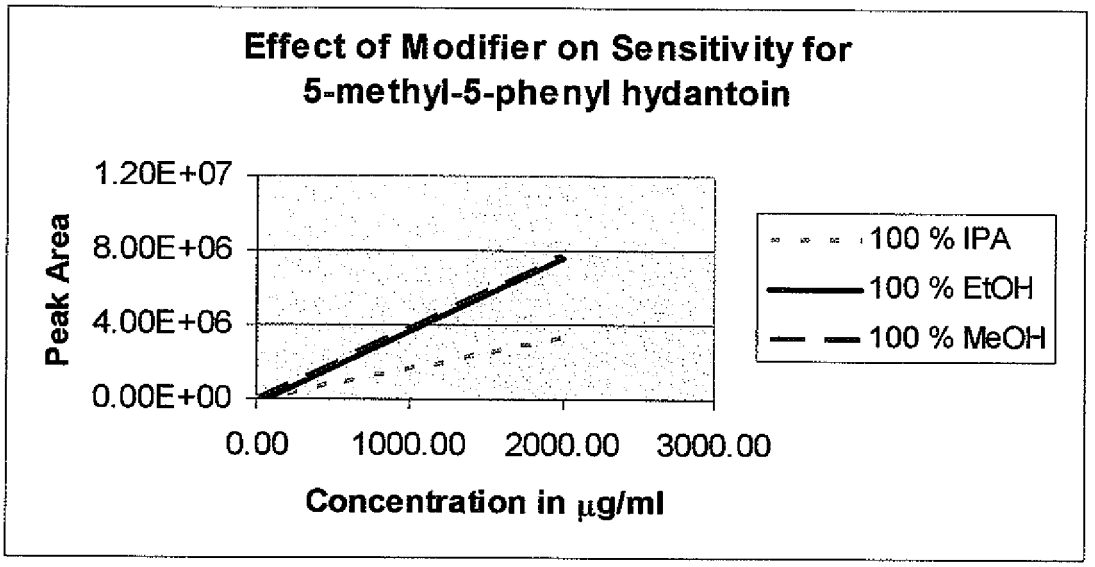

Effect of Modifier on Sensitivity for
5-methyl-5-phenyl hydantoin
Peak Area
1.20E+07
8.00E+06
4.00E+06
0.00E+00
0.00
1000.00
2000.00
3000.00
Concentration in µg/ml
100 % IPA
100 % EtOH
100 % MeOH

Figure 4.

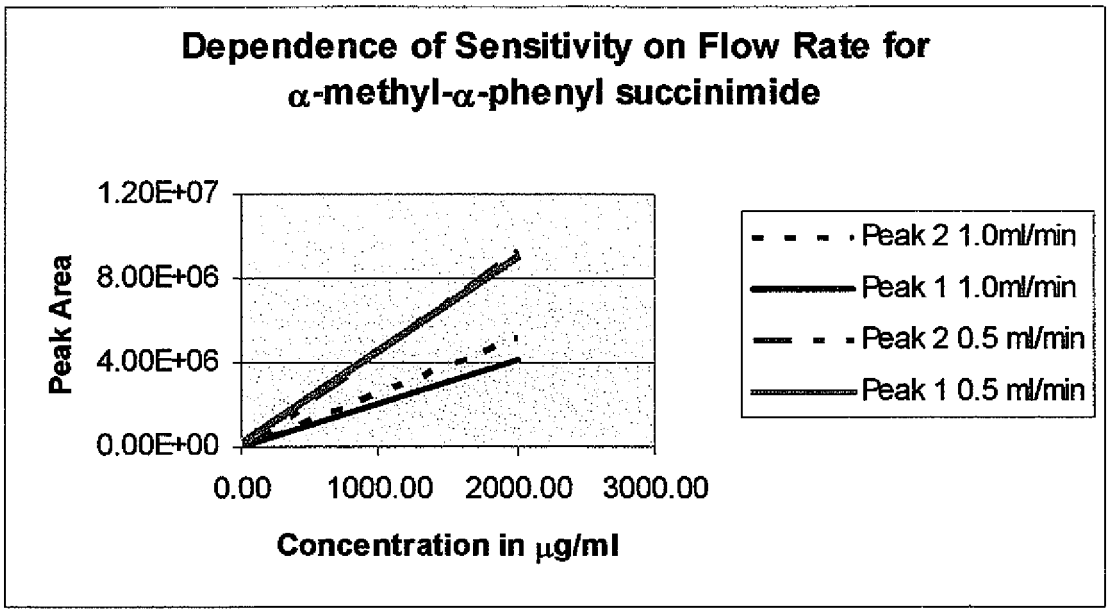

Dependence of Sensitivity on Flow Rate for
α-methyl-α-phenyl succinimide
1.20E+07
8.00E+06
4.00E+06
0.00E+00
Peak Area
0.00
1000.00
2000.00
3000.00
Concentration in μg/ml
Peak 2 1.0ml/min
Peak 1 1.0ml/min
Peak 2 0.5 ml/min
Peak 1 0.5 ml/min

Figure 5.

CHAPTER 4. ANALYSIS OF NATIVE AMINO ACID AND PEPTIDE ENANTIOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/ATMOSPHERIC PRESSURE CHEMICAL IONIZATION MASS SPECTROMETRY

A paper published in the *Journal of Mass Spectrometry*[2]

Meera J. Desai and Daniel W. Armstrong

ABSTRACT

High performance liquid chromatography coupled to atmospheric pressure chemical ionization (APCI) mass spectrometry was used for the separation and detection of amino acid and peptide enantiomers. With detection limits as low as 250 pg, twenty-five amino acids enantiomers were baseline resolved on the Chirobiotic T chiral stationary phase. APCI demonstrated an order of magnitude better sensitivity over electrospray ionization (ESI) for free amino acids and low molecular weight peptides at the high LC flow rates necessary for rapid analysis. As peptide chain length increased (peptides with MW $\geq$ 300 Da), however, ESI proved to be the more ideal atmospheric pressure ionization (API) source. A mobile phase consisting of 1 % (w/w) ammonium trifluoroacetate in methanol and 0.1 % (w/w) formic acid in water increased the sensitivity of the APCI method significantly. A step gradient was then used to simultaneously separate all 19 native protein amino acid enantiomers in less than 20 minutes using extracted ion chromatograms.

INTRODUCTION

There has long been an emphasis on the analysis of free amino acids and peptides primarily due to their significant role in biological function and processes. Since the majority of amino acids are chiral, the separation and detection of amino acid and peptide enantiomers has become an essential part of these systematic analyses. Determination of the relative amounts of D- and L-amino acids is now mandated for the characterization of various biological, environmental, or pharmaceutical samples.[1] In addition, specific and often unusual D- and L-amino acids are finding increasing use in the fields of peptide design and protein engineering.[2]

Traditional chiral separation and detection techniques such as high-performance liquid chromatography (HPLC) with UV/Vis detection can be cumbersome for the enantiomeric analysis of most amino acids. UV detection at lower wavelengths, such as 205 nm, has been employed to overcome this problem. However, many of these methods suffer from an increased background due to mobile phase absorption.[3,4] Therefore, most HPLC methods use derivatization procedures either pre- or post-column to enhance the detection response for UV/Vis, fluorescence, or electrochemical detection.[5-11] Mass spectrometric detection, however, can be used for the detection of amino acid enantiomers without the need for derivatization. In fact, Hemmermeister and co-workers recently reported that native amino acids actually had better sensitivity than their dansyl chloride derivatized counterparts for MS detection.[12]

Converting existing chiral LC methods to chiral LC-MS methods is not entirely straightforward. Many chiral methods use the normal phase mode for the separation of

enantiomers. Normal phase solvents, such as hexane and heptane, in high quantities, are often incompatible with electrospray ionization (ESI) sources. In addition to solvent type, buffer volatility is a significant issue when converting reverse phase methods. When replacing non-volatile buffers with MS compatible additives, chiral chromatographic resolution often suffers. In order to achieve enantiomeric resolution with sensitive MS detection mobile phase composition must be systematically selected.

The use of HPLC for the chiral separation, identification, and characterization of amino acids has proven to be quite successful with the aid of chiral resolving agents,[13-16] chiral additives,[17,18] or chiral stationary phases (CSPs).[19,20] Chiral resolving agents form dynamic diastereomeric complexes with chiral analytes allowing for their separation on many chromatographic stationary phases.[19,20] Resolving agents and chiral additives do not allow for the collection of pure samples after separation. When coupled to mass spectrometric detection, these agents and/or additives can complicate the mass spectrum or possibly suppress ionization. Chiral stationary phases, on the contrary, allow for the direct separation and detection of enantiomers, simplifying the interpretation of mass spectral data.[19,20]

A number of papers have reported the successful use of teicoplanin stationary phases for the LC separation of amino acid enantiomers.[21-26] Teicoplanin is a macrocyclic glycopeptide with numerous chiral centers.[21] Its basket-like structure as well as the presence of both a single primary amine and carboxylic acid group contribute to the enantioselectivity of teicoplanin.[23,24] This chiral stationary phase is able to separate both native and derivatized amino acids primarily in the reverse phase mode, making it highly compatible to detection with atmospheric pressure ionization mass spectrometry (API-MS).[21-26]

An assortment of publications has demonstrated the use of electrospray and pneumatically assisted electrospray (ionspray) for amino acid detection and identification.[26-32] Few of these papers considered the unique problems and requirements of enantioselective analysis and characterization. In addition, atmospheric pressure chemical ionization (APCI) has not been thoroughly investigated for the ionization of the proteinic amino acids and peptides. Clearly, there is a need for further exploration of this particular ionization method.

A variety of amino acids are found in most clinical and environmental samples. Therefore, the ability to separate multiple amino acids both chirally and achirally in a simultaneous analysis is also of fundamental interest. Many techniques have demonstrated the concurrent achiral separation of various amino acids.[33-35] In order to resolve amino acids from one another, these non-chiral methods all suffer from tremendously long retention times. The resolution of all the native amino acids and their respective enantiomers would require greater separation selectivity than is currently practical resulting in even longer retention times. The use of MS detection, however, can overcome these problems. Mass spectrometric detection provides a second dimension of discrimination enabling the extraction of specific m/z values from unresolved amino acids, thereby significantly reducing analysis time.[26]

In this investigation, HPLC/APCI-MS with a teicoplanin CSP was used for the simultaneous enantiomeric separation of all 19 native chiral amino acids. This technique was also utilized in the separation of di- and tri-peptides.

EXPERIMENTAL

Chemicals and reagents

Ammonium trifluoroacetate (NH_4TFA) reagent was purchased from Aldrich (Milwaukee, WI). Racemic amino acid and peptide standards were obtained from Sigma (St. Louis, MO). Phe-phe-phe was obtained from ICN Biomedicals, Inc.(Aurora, OH). HPLC grade methanol (MeOH) and water were acquired from Fisher (Pittsburgh, PA). Formic acid and 200 proof ethyl alcohol (EtOH) were purchased from J.T. Baker (Phillipsburg, NJ) and Apper Alcohol (Shelbyville, KY), respectively. All amino acids and peptides were dissolved in 50:50 (methanol: water) and diluted to 10 ug ml^{-1} for injection.

Instrumentation

Experiments were performed on a Thermo Finnigan (San Jose, CA) Surveyor LC system coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with ESI and APCI ion sources. The MS was operated in positive ion mode using single ion monitoring (SIM) detection. Nitrogen (Praxair, Danbury, CT) was used as both the sheath and auxiliary gases. Ultra-high purity helium (Linweld, Lincoln, NE) was used as the dampening gas in the ion trap.

ESI conditions. Sheath and auxiliary gases were 90 and 60 arbs (arbitrary units), respectively. MS parameters were optimized to the following: source voltage = 4.50 kV, capillary voltage = 10.0 V, tube lens offset = 0.0 V, and capillary temp = 270 degrees Celsius. *APCI conditions.* Sheath and auxiliary gases were 80 and 10 arbs, respectively. MS parameters were optimized to the following: APCI vaporizer temp = 350.0 degrees Celsius, corona discharge current = 5.00 μA, tube lens offset = 30.0 V, and capillary temp = 200 degrees Celsius.

HPLC-UV/Vis analyses were performed on a Shimadzu (Kyoto, Japan) LC 10A chromatograph (SCL-10A controller, LC-10AT pumps, and SIL-10A auto-sampler) with a SPD-10A UV/Vis detector and a Shimadzu C-R6A integrator. UV/Vis detection was performed at 210 nm.

All separations were carried out at room temperature on 250 x 4.6 mm ID or 250 x 2.0 mm ID Chirobiotic T (teicoplanin) and Chirobiotic TAG (teicoplanin-aglycone) chiral columns from Astec (Whippany, NJ). Non-chiral peptides studies were carried out on a 100 x 4.6 mm ID C-18 column also obtained from Astec. Solvent systems were methanol: water, ethanol: water, or 1% (w/w) NH_4TFA in methanol: 0.1 % (w/w) formic acid in water at varying concentrations. Mobile phase flow rates varied from 200 to 800 µl min^{-1}.

Simultaneous enantiomeric amino acid separation. Using full scan MS ranging from 87 m/z to 207 m/z the total ion chromatogram for the mixture of all 19 chiral proteinic amino acids was collected. Using Xcalibur™ 1.3 software, provided by Thermo Finnigan, extracted ion chromatograms were reconstructed at the appropriate m/z for each amino acid. A step gradient was used to separate the mixture of amino acids. A mobile phase consisting of 80 % A : 20 % B (A: 1% NH_4TFA in MeOH; B: 0.1 % formic acid in water) was run from 0.0 to 8.50 minutes. At 8.51 to 17.00 the mobile phase was switched to 20% A: 80 % B and then switched back to the original 80:20 (A:B) ratio at 17.01 minutes until the end of the chromatographic run.

RESULTS AND DISCUSSION

Enantiomeric separations of individual amino acids

Twenty-five individual amino acids were separated and detected with LC-MS using the Chirobiotic T (teicoplanin) CSP, of which all were baseline resolved or better ($Rs \geq 1.5$). Two amino acids, 2-pyrrolidone-5-carboxylic acid and carnitine, were separated using the Chirobiotic TAG (teicoplanin aglycone) column.

The majority of amino acids could be separated and detected in the reverse-phase mode using either ethanol: water or methanol: water mobile phases. Reverse-phase solvents such as these are highly compatible with mass spectrometric detection.[36] In fact, the separation and detection of proline enantiomers could be achieved with simply 100 percent water as the mobile phase. Basic amino acids such as lysine, arginine, histidine, and ornithine would not elute from the CSP without the use of additives. The anionic sites of teicoplanin strongly attract the positively charged basic amino acids.[21] Previously, nonvolatile buffer systems such as sodium phosphate have been used to elute these basic analytes.[21,22] However, it is well known that these types of buffers can cause contamination of API sources and, therefore, are not suitable for MS detection.[36] In addition, non-volatile buffers may decrease the sensitivity of MS analysis. The volatile additives, ammonium trifluoroacetate and formic acid, facilitated the elution of the basic amino acids from the teicoplanin column. Table 1 catalogs the results of all the individual amino acid separations using several different mobile phases with APCI-MS detection.

Amino acids were detected at the m/z equivalent to the $[M + H]^+$ ion of each analyte. Although APCI commonly results in the fragmentation of labile amino acids, this occurrence was not observed under these conditions. In solution at pH 7, neutral amino acids are zwitterionic, acidic amino acids are negatively charged, and basic ones are positively charged. Despite this fact, all these analytes, regardless of acidity or basicity, ionized to the

$[M+H]^+$ ion for both APCI and ESI. Comparing the gas-phase proton affinities (PAs) of the solvent system to the analyte explain this interesting phenomenon. The gas-phase PAs of methanol, ethanol, and water are 184.9, 190.3, and 173.0 kcal mol^{-1}.[37] The proton affinities of all native amino acids were previously reported to be greater than 200 kcal mol^{-1}.[38] Therefore, amino acids in the gas-phase are far better proton acceptors than the solvent system allowing for the formation of the $[M+H]^+$ ion for all amino acids and peptides analyzed.

Limits of detection

The limits of detection for UV/Vis (at 210 nm) were compared to those for SIM-MS for selected amino acids. Separation conditions were kept constant with both detection systems for each individual amino acid. Detection limits as low as 250 pg were achieved by MS. Table 2 shows a summary of experimental detection limits of UV/Vis and MS for various neutral, acidic, and basic amino acids.

Sensitivity of ESI vs. APCI

Selecting the appropriate ionization source for analytes is essential to achieving low detection limits. Analyte type is an important criterion for choosing probe type. ESI is generally used for thermally labile, polar, and larger molecular weight analytes. APCI is useful for analyzing smaller, volatile but thermally stable compounds. Thurman recently found that APCI had better sensitivity for non-polar, neutral and less basic analytes whereas ESI performed best for polar, cationic and anionic compounds.[39] The polarity of amino acids

varies significantly as does their acidity and basicity, so one cannot discern which probe is the more suitable based on these criteria alone.

HPLC conditions also factor into the choice of API probe type. As it is well known, APCI sources can handle much higher flow rates than ESI sources. Assisted ESI sources, such ionspray, have been able to overcome this limitation to some degree.[40] In addition to flow rate restrictions, high water containing mobile phases reduce sensitivity by making solvent evaporation and ion desorption more difficult for unassisted electrospray ionization.[41] In APCI, however, eluents are vaporized in the probe prior to ionization allowing for the use of most types of LC solvents.

The sensitivity of ESI was compared to that of APCI for all amino acids and peptides. Using identical mobile phase compositions and flow rates, varying concentrations of amino acids (0.05, 0.10, 0.50, 5.0, 10.0 $\mu l\ ml^{-1}$) were separated and ionized by APCI and ESI sources. Peak area versus concentration was plotted for calibration purposes. For every free amino acid tested, both APCI and ESI had essentially equivalent limits of detection. The sensitivity (defined by IUPAC as the slope of the dose response curve),[42] however, was an order of magnitude higher for APCI over ESI at the flow rates utilized. The linearity of the calibration curves was also significantly better with APCI ($r^2 > 0.999$) compared to ESI ($r^2 < 0.970$). The relatively high flow rate of this method (800 $\mu l\ min^{-1}$) most likely contributes to the poor linearity of the calibration curves for ESI. In fact, it was observed that lowering the flow rate to a more ESI compatible one (<300 µl/min) made the sensitivity of the ESI method to comparable to that of APCI at 800 µl/min. The major drawback of using such a low flow rate is of course the significant increase retention times for the enantiomeric separation.

Therefore, a compromise must be made between the best sensitivity achieved by MS detection, and the best overall chromatographic response.

Figure 1 illustrates the difference in MS detector response for phenylalanine between ESI (2A) and APCI (2B) at 400 µl/min. The SIM chromatograms at 166 m/z shown are on the same scale. The small difference in retention times of D- and L-phenylalanine between ESI and APCI probes can be attributed to differences in the post-column tubing volume.

APCI continued to have better sensitivity over ESI for the various di-peptides tested. However, as peptide chain length increased the sensitivity of APCI over ESI became less apparent even at high flow rates. This observation is believed to be a result of the increasing molecular weight of the peptides. Figure 2 shows the decreasing signal to noise values for peptides as molecular weight increases using APCI. Gly-thr was detected with the best signal to noise ratio while leu-gly-phe, a peptide which is 159 Da heavier than gly-thr, had the worst signal to noise level of those shown.

In order to further investigate the sensitivity dependence on analyte mass for ESI and APCI, a series of gly-, leu-, and phe-peptides were tested with both ion sources. The gly peptide series (gly, di-gly, tri-gly, tetra-gly, penta-gly, and hexa-gly) initially showed an increase in analyte response going from gly to tri-gly for both ESI and APCI. The low molecular weight of glycine residues may contribute to this observation. The response for APCI began to decrease for the glycine series after tetra-gly, whereas the MS response for ESI increased following the addition of a fourth glycine residue. The leucine (leu, di-leu, and tri-leu) and phenylalanine peptide series (phe, di-phe, and tri-phe) showed a similar trend. As the chain length of the peptides increased the magnitude of the MS response for ESI overtook that of APCI. The crossover point in the relative sensitivity of APCI and ESI

occurs between 200 and 300 Da for these particular peptides (Figure 3). As reported by Harrison, the gas phase proton affinities of gly_1 to gly_6 increase negligibly[38], therefore, it is believed that in this case, molecular weight is a stronger determining factor than basicity (as Thurman et al suggest)[39] for choosing API source type. In general, it can be said that APCI is more sensitive for peptides of MW $\leq$ 200 Da and ESI is more sensitive for peptides of MW $\geq$ 300 Da under these experimental conditions. Analytes between 200-300 Da have similar sensitivities for both APCI and ESI.

Effect of additives on sensitivity

Additives are often employed to aid in ionization for API-MS techniques. In addition to being volatile so as not to plug up the sample orifice, the additives should not form strong ion pairs, and they should be used to control pH to aid in the protonation or deprotonation of analytes. When converting LC methods to LC-MS methods, one of the most important criteria is enhancing ionization without losing resolution and selectivity.

In this study, most amino acids and peptides could be eluted from the CSP and ionized by both ESI and APCI without the use of additives with relatively low detection limits. However, it was found that with the addition of the reagents, ammonium trifluoroacetate and formic acid, the sensitivity for all amino acids tested increased by an order of magnitude. Figure 4 shows the calibration curves for D- and L-leucine with and without additives using APCI-MS. The slope of the dashed lines was approximately 1×10^7 with r^2 values greater than 0.999. In comparison, the slope of the solid lines was approximately 1×10^6 with r^2 values less than 0.978. Once again, the detection limits remained comparable with and without additives. At the limit of detection, the sample run

without additives had a higher signal to noise than the sample run with additives in the mobile phase. This could be attributed to the enhancement of ionization not only of the analyte but also of the background noise by the reagents. Moreover, the use of additives did not affect the resolution or peak shape of the separated enantiomers.

Simultaneous enantiomeric separation of all 19 native amino acids by LC/APCI-MS

The ability to separate enantiomers of all the protein amino acids in a single run would be of great use to biochemical science, specifically in the areas of protein and peptide analysis.[21] It is even more desirable to develop a method which does not require derivatization as this can add extra steps to the sample preparation of actual samples.[26] It would also be beneficial to reduce the time required to do a separation of this kind. Mass spectrometric detection provides the solution for both of these challenges.

Achiral chromatographic resolution is not necessary for compounds with different m/z values. Leucine and isoleucine, being isomers of each other, have the same m/z, as do lysine and glutamine (m/z = 147). Chromatographic resolution of these analytes from each other prior to MS analysis was therefore necessary.

As a result of increased sensitivity and the fact that additives were necessary for the elution of basic amino acids from the teicoplanin column, the mobile phase used contained ammonium trifluoroacetate and formic acid reagents. All amino acids enantiomers with the exception of proline could be separated under these conditions. Proline eluted in a single peak at a retention time of 10.65. Therefore a step gradient was employed in order to separate all 19 native amino acids. Figure 5 shows the extracted ion chromatograms of the amino acids for a single run with a total analysis time of less than 20 minutes. L-proline now

eluted at 11.75 minutes with D-proline eluting at 18.05 minutes. Unfortunately, the D-forms and L-forms of leucine and isoleucine could not be fully separated from one another. Asparagine and glutamic acid enantiomers co-elute with proline and leucine/isoleucine enantiomers, respectively. The ionization of asparagine and glutamine could be suppressed by the presence of those co-eluting species resulting in poor peak shape of the reconstructed ion chromatograms.[43,44]

CONCLUSION

HPLC coupled with mass spectrometry has shown to be a viable alternative to traditional detection techniques for the separation of underivatized amino acids and peptides. The sensitivity of APCI for free amino acids was found to be far better than that for electrospray ionization at high flow rates, with detection limits at the nano-gram and lower levels. Peptides of $\geq$ 300 Da had greater sensitivity when ESI was employed. The sensitivity of the APCI method was further enhanced through the use of volatile additives. A mixture of the 19 native amino acids was separated using this method of which each amino acid in the mixture was baseline separated into its D- and L-forms in less than 20 minutes. With such low limits of detection, application of this method to the analysis of biological and environmental samples is definitely possible.

ACKNOWLEDGEMENTS

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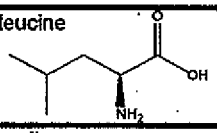
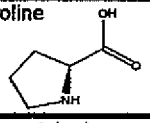
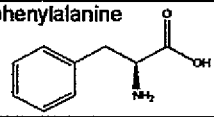
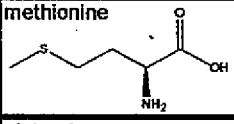
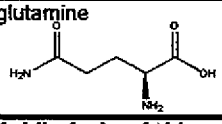
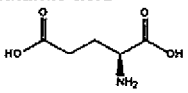
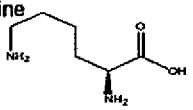
Table 1. Results of individual amino acid chiral separations

Compound Number	Compound Name	Molecular Weight	Chirobiotic T/TAG	Mobile Phase Composition	Chromatography Results	
					Rs (Resolution)	α (Selectivity)
1	alanine	89	T	50:50 (100% EtOH: 100% H_2O) 0.4ml/min	1.72	1.46
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	2.40	1.60
2	serine	105	T	85:15 (100 % EtOH : 100% H_2O) 0.4 ml/min	1.55	1.63
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	1.75	1.19
3	proline	115	T	100% H_2O 0.4 ml.min	2.13	1.85
4	valine	117	T	50:50 (100% EtOH: 100% H_2O) 0.4ml/min	1.62	1.30
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	2.52	1.37
5	norvaline	117	T	50:50 (100% EtOH: 100% H_2O) 0.2ml/min	3.40	2.21
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	5.29	2.41
6	threonine	119	T	70:30 (100% EtOH: 100% H_2O) 0.8ml/min	1.94	1.22
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	1.76	1.19
7	cysteine	121	T	50:50 (100% EtOH: 100% H_2O) 0.8ml/min	2.60	1.19
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	8.22	2.42
8	isoleucine	131	T	50:50 (100% EtOH: 100% H_2O) 0.4ml/min	1.67	1.21
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	4.11	1.64
9	leucine	131	T	50:50 (100% EtOH: 100% H_2O) 0.4ml/min	2.26	1.64
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	3.90	1.60
10	norleucine	131	T	80:20 (100% EtOH: 100% H_2O) 0.2ml/min	3.87	2.38
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	4.72	1.84
11	asparagine	132	T	50:50 (100 % MeOH : 100 % H_2O) 0.8 ml/min	3.50	1.22
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	5.31	1.56
12	aspartic acid	133	T	100% MeOH 1.0 ml/min	1.51	1.58
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	2.67	1.28
13	glutamine	146	T	50:50 (100 % MeOH : 100 % H_2O) 0.8 ml/min	2.55	1.18
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	5.44	1.43
14	lysine	146	T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	6.08	1.50
15	glutamic acid	147	TAG	80:20 (100% MeOH: 100% H_2O) 1.0ml/min	1.54	1.77
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	4.41	1.39
16	methionine	149	T	25:75 (100% EtOH: 100% H_2O) 0.4ml/min	1.50	1.37
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	5.75	1.70
17	histidine	155	TAG	60:40 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.2ml/min	2.23	1.24
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	1.53	1.13
18	phenylalanine	165	T	50:50 (100% EtOH: 100% H_2O) 0.4ml/min	1.53	1.40
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	3.44	1.39
19	arginine	174	T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	4.96	1.64
20	tyrosine	181	T	55:45 (100% EtOH: 100% H_2O) 0.2ml/min	1.58	1.38
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	3.24	1.34
21	tryptophan	204	T	20:80 (100% EtOH: 100% H_2O) 0.4ml/min	1.30	1.26
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	3.33	1.38
Other Amino Acids						
22	2 - pyrrolidone - 5 carboxylic acid	129	TAG	50:50 (100% EtOH: 100% H_2O) 0.2ml/min	1.33	1.19
				80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	1.43	1.13
23	ornithine	132	TAG	50:50 (1.0 % NH4TFA in MeOH: 0.1% formic acid in H2O) 0.4ml/min	1.69	1.36
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	3.33	1.38
24	cis-2-aminocyclohexane carboxylic acid	143	T	90:10 (100% EtOH: 100% H_2O) 0.2 ml/min	1.27	1.22
25	carnitine	161	TAG	70: 30 (100 % MeOH : 100 % H_2O) 0.2 ml/min	1.85	1.66
26	DOPA	197	TAG	50:50 (100% EtOH: 100% H_2O) 0.2ml/min	6.44	8.25
			T	80:20 (1.0 % NH_4TFA in MeOH: 0.1% formic acid in H_2O) 0.8ml/min	6.86	1.63
27	acetyl carnitine	203	T	62.5:37.5 (100 % MeOH : 100 %H_2O) 0.4 ml.min	1.71	1.16

Rs = $2(t_2 - t_1) / (w_1 + w_2)$; where t_2 and t_1 are the retention times and w_2 and w_1 are the baseline peak widths of the second and first peak, respectively. $\alpha = (t_2 - 1.2) / (t_1 - 1.2)$; 1.2 was experimental dead time

Table 2. Comparison of Detection Limits

#	Compound	Detection Limits		Separation Conditions
		UV/Vis*	MS**	
	Neutral Amino Acids			
1	leucine 	10 µg S/N_1= 12 S/N_2= 7	500 pg S/N_1= 11 S/N_2= 15	50/50 MeOH: H_2O 0.8 ml/min Chirobiotic T
2	proline 	5 µg S/N_1= 8 S/N_2= 4	1 ng S/N_1= 11 S/N_2= 7	100% H_2O 0.8 ml/min Chirobiotic T
3	phenylalanine 	100 ng S/N_1= 6 S/N_2= 3	1 ng S/N_1= 3 S/N_2= 3	80/20 A:B[ψ] 0.8 ml/min Chirobiotic T
4	methionine 	5 µg S/N_1= 10 S/N_2= 6	250 pg S/N_1= 6 S/N_2= 4	80/20 A:B[ψ] 0.8 ml/min Chirobiotic T
5	glutamine 	10 µg S/N_1 = 6 S/N_2= n/a	10 ng S/N_1=6 S/N_2= 3	80/20 A:B[ψ] 0.8 ml/min Chirobiotic T
	Acidic Amino Acid			
6	glutamic acid 	1 µg S/N_1= 4 S/N_2= 4	10 ng S/N_1= 3 S/N_2= 3	80/20 A:B[ψ] 0.8 ml/min Chirobiotic T
	Basic Amino Acid			
7	lysine 	10 µg S/N_1= 6 S/N_2= 6	10 ng S/N_1= 3 S/N_2= 2	80/20 A:B[ψ] 0.8 ml/min Chirobiotic T

*detection at 210nm

**APCI/MS SIM at $[M+H]^+$ for each amino acid

[ψ]A: 1.0% NH_4TFA in MeOH; B: 0.1 % Formic Acid in H_2O

S/N_1, S/N_2 : Signal to Noise ratios of peaks 1 and 2, respectively

FIGURE LEGENDS

Figure 1. Comparison of ESI vs. APCI for D- and L-phenylalanine. Both ion chromatograms are on the same scale. SIM mode at m/z 166, mobile phase: 50:50 MeOH: water, flow rate 0.4 ml min^{-1}, Chirobiotic T 250 x 4.6 mm ID.

Figure 2. LC/APCI-MS separation of di- and tri-peptides. All separations were achieved on Chirobiotic T 250 x 4.6 mm ID with a flow rate of 0.8 ml min^{-1}, SIM mode at $[M+H]^+$ ion. (A) gly-thr MW = 176.2, mobile phase: 50:50 MeOH: water. (B) gly-asn MW = 189.2, mobile phase: 50:50 MeOH: water. (C) gly-leu-ala MW = 259.3, mobile phase: 60:40 MeOH: water. (D) leu-gly-phe MW = 335.4, mobile phase: 60:40 MeOH: water.

Figure 3. Mass dependence on sensitivity for APCI and ESI. Phe, di- and tri-phe, leu, di-and tri-leu, and gly, di-, tri-, tetra-, penta- and hexa-gly were analyzed using both APCI and ESI sources. Solid curves represent APCI-MS response; dashed curves represent ESI-MS response.

Figure 4. Effect of additives on sensitivity for D- and L-leucine. Mobile phase: 50:50 MeOH: water (with and without additives), additives: 1.0 % NH_4TFA in MeOH and 0.1% formic acid in water, flow rate 0.8 ml min^{-1}, Chirobiotic T 250 x 4.6 mm ID column.

Figure 5. Simultaneous separation of 19 chiral amino acids in less than 20 minutes using LC-APCI/MS. Total ion chromatogram (TIC) and extracted ion chromatograms are shown.

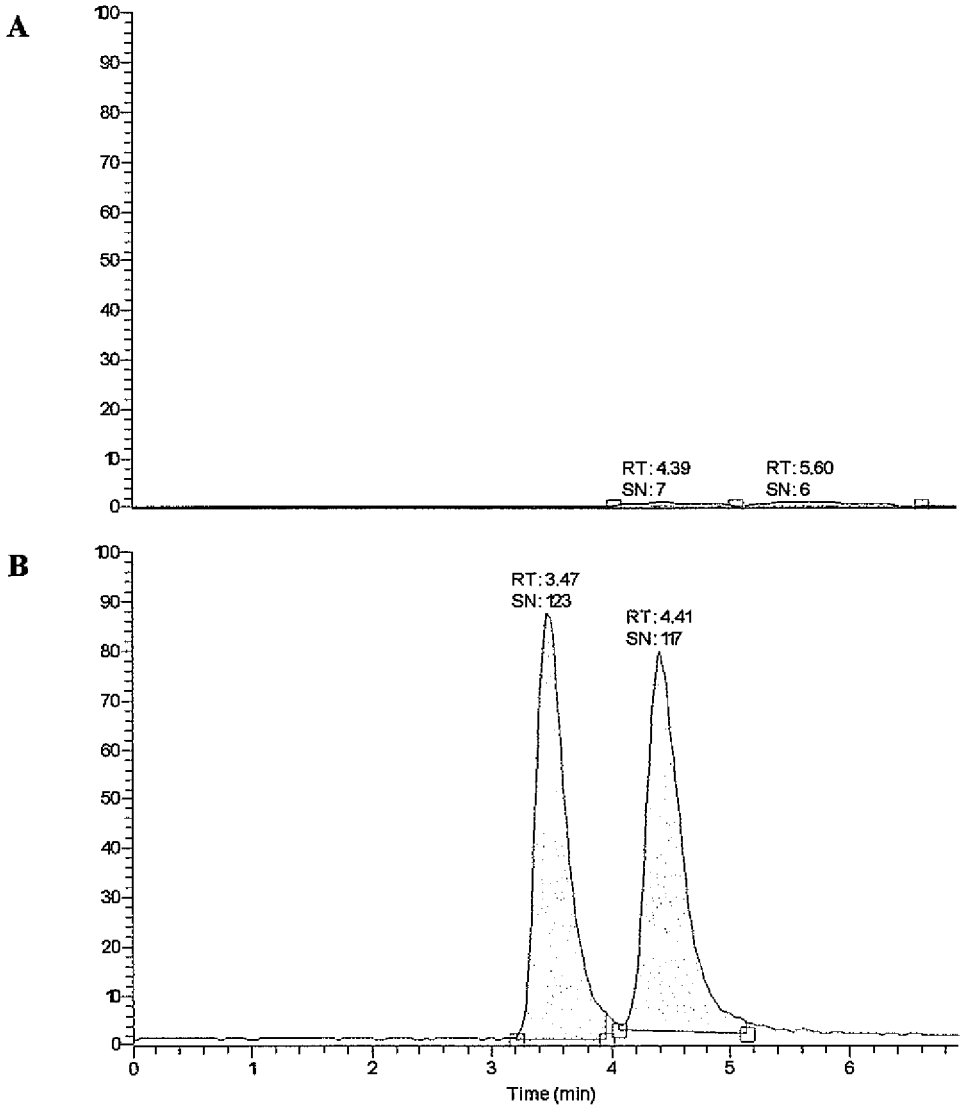


Figure. 1

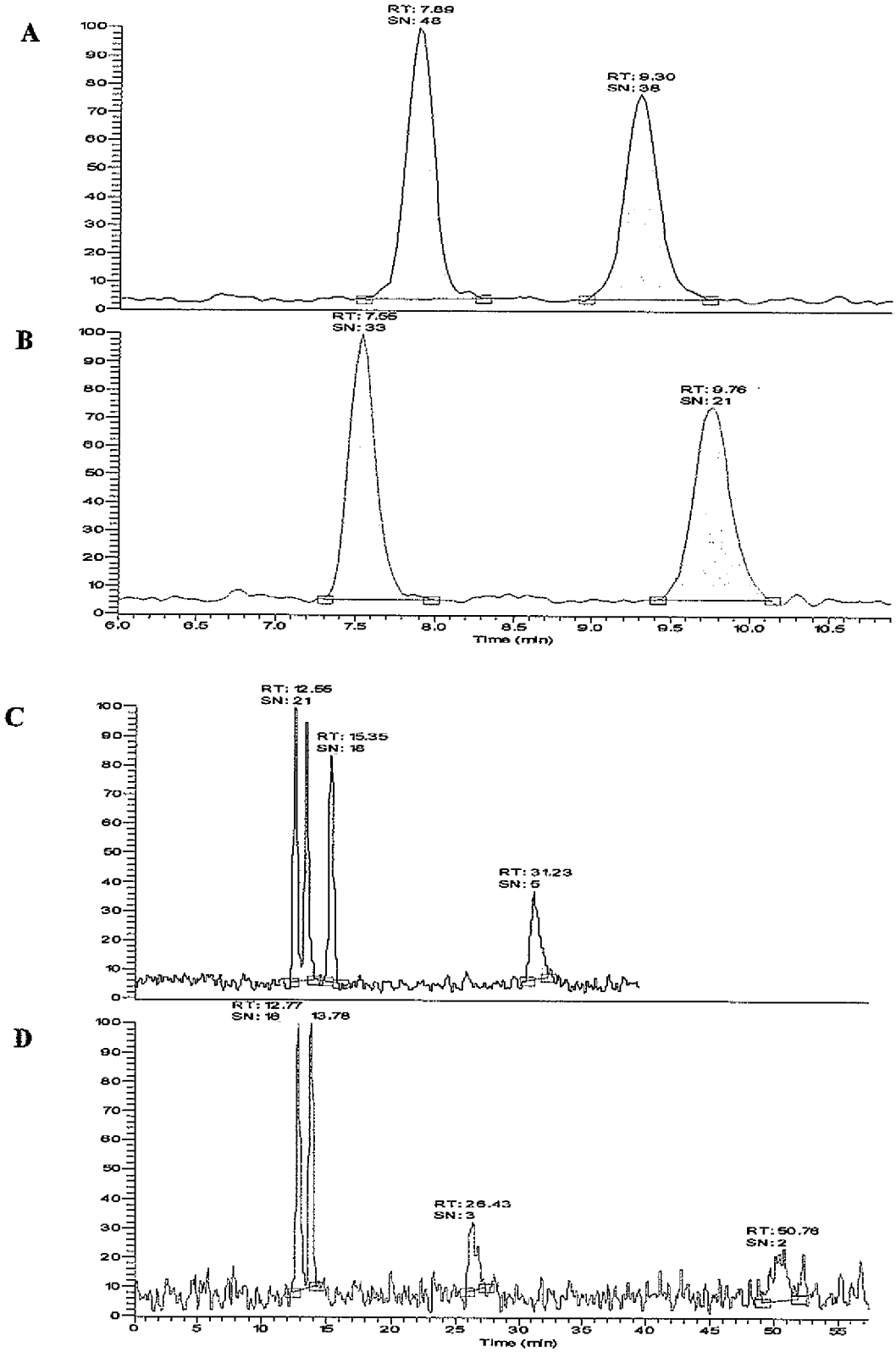

A
RT: 7.89
SN: 48
RT: 9.30
SN: 38
B
RT: 7.55
SN: 33
RT: 9.76
SN: 21
Time (min)
C
RT: 12.55
SN: 21
RT: 15.35
SN: 18
RT: 31.23
SN: 5
D
RT: 12.77
SN: 18
13.78
RT: 26.43
SN: 3
RT: 50.76
SN: 2
Time (min)

Figure. 2

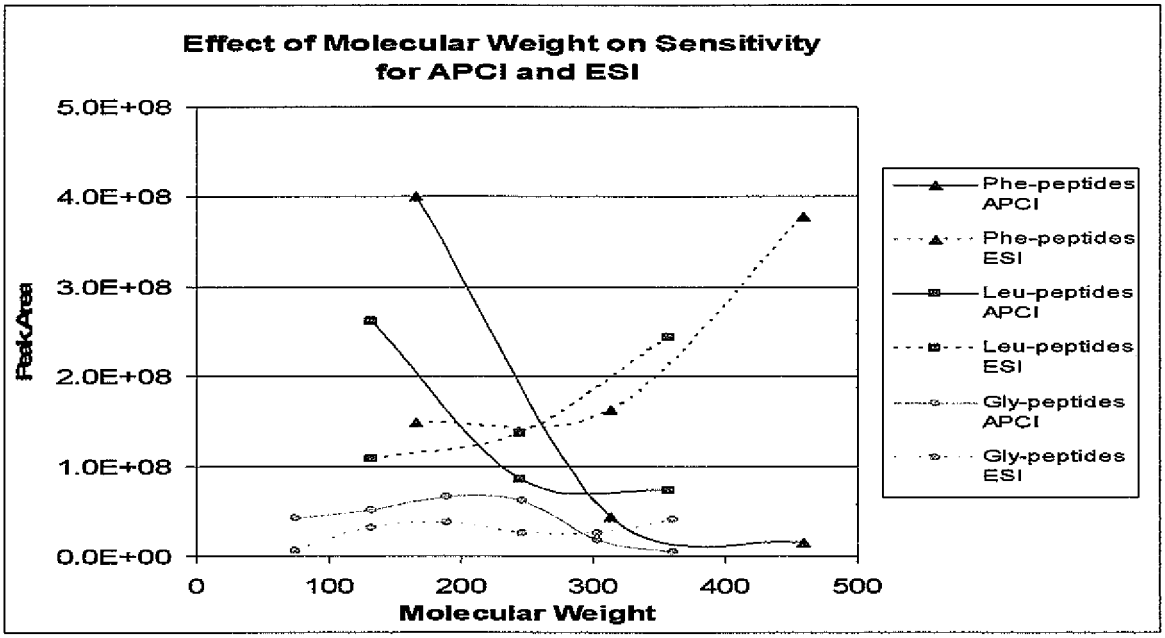

Effect of Molecular Weight on Sensitivity for APCI and ESI
5.0E+08
4.0E+08
3.0E+08
2.0E+08
1.0E+08
0.0E+00
Peak Area
0
100
200
300
400
500
Molecular Weight
Phe-peptides APCI
Phe-peptides ESI
Leu-peptides APCI
Leu-peptides ESI
Gly-peptides APCI
Gly-peptides ESI

Figure 3.

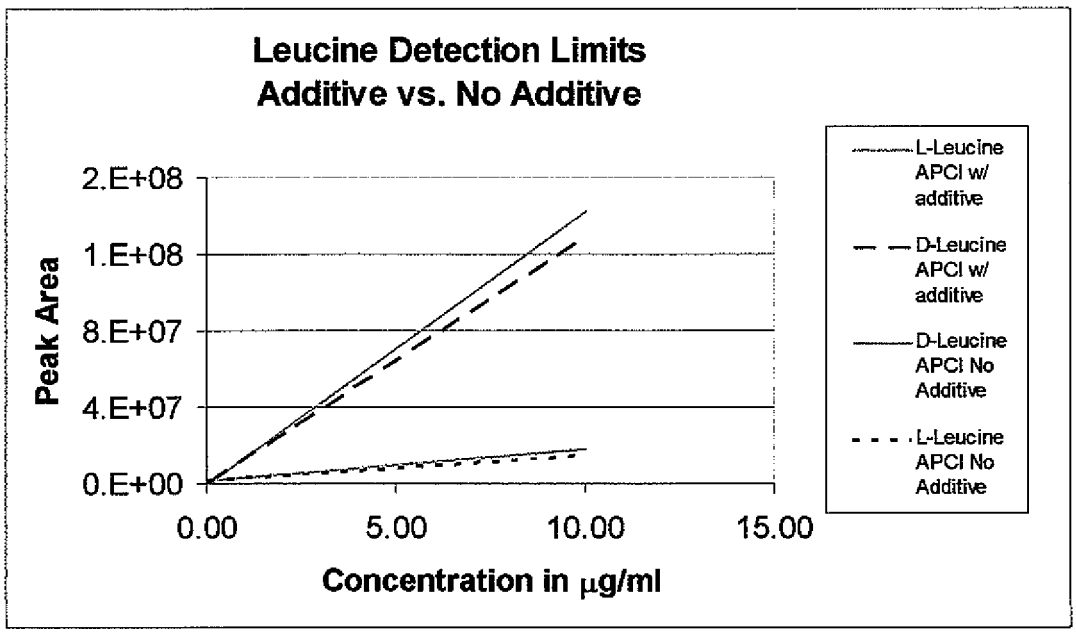

Leucine Detection Limits
Additive vs. No Additive
Peak Area
2.E+08
1.E+08
8.E+07
4.E+07
0.E+00
0.00
5.00
10.00
15.00
Concentration in µg/ml
L-Leucine APCI w/ additive
D-Leucine APCI w/ additive
D-Leucine APCI No Additive
L-Leucine APCI No Additive

Figure. 4

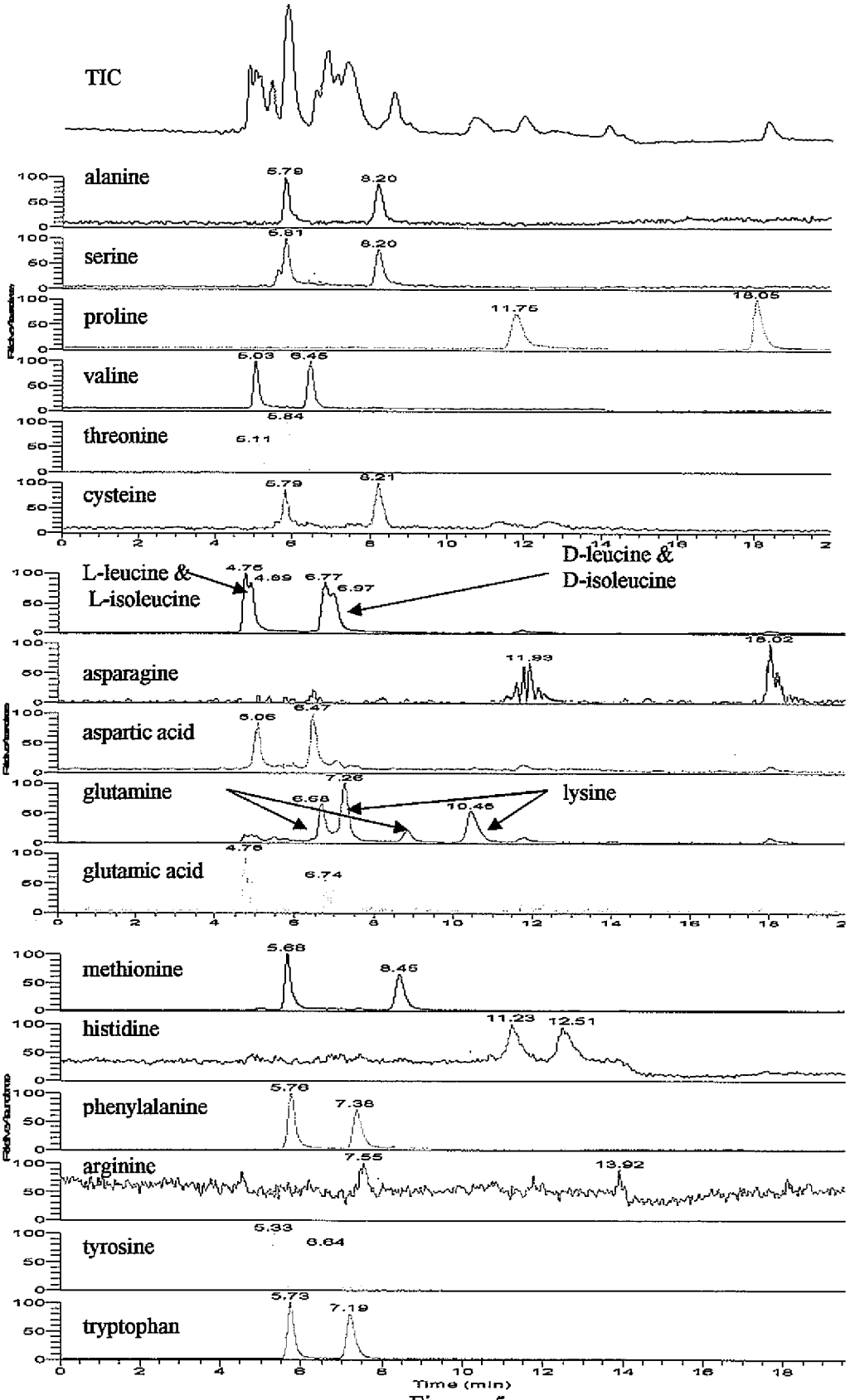

TIC
alanine
5.79
8.20
serine
5.81
8.20
proline
11.75
18.05
valine
6.03
6.45
5.84
threonine
6.11
cysteine
5.79
8.21
L-leucine &
L-isoleucine
4.76
4.89
6.77
6.97
D-leucine &
D-isoleucine
asparagine
11.93
18.02
aspartic acid
6.06
6.47
glutamine
7.26
6.68
10.46
lysine
4.76
glutamic acid
6.74
methionine
5.68
8.46
histidine
11.23
12.51
phenylalanine
5.76
7.38
arginine
7.55
13.92
tyrosine
5.33
6.64
tryptophan
5.73
7.19
Time (min)

Figure. 5

CHAPTER 5. ANALYSIS OF DERIVATIZED AND UNDERIVATIZED THEANINE ENANTIOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/ATMOSPHERIC PRESSURE IONIZATION-MASS SPECTROMETRY

A paper published in *Rapid Communications in Mass Spectrometry*[3]

Meera J. Desai and Daniel W. Armstrong

ABSTRACT

Theanine, a naturally occurring non-proteinic amino acid found in tea leaves, has demonstrated wide-ranging physiological activity, from lowering blood pressure to enhancing the anti-tumor activity of chemotherapeutic drugs. The chiral nature of theanine suggests that enantiospecificity plays a significant role in its various pharmacological functions. Using the Chirobiotic T (teicoplanin) chiral stationary phase, native and derivatized theanine enantiomers were separated and detected via HPLC coupled to atmospheric pressure ionization-mass spectrometry (API-MS). With the use of flow rates compatible with each ionization source, native theanine standards achieved excellent sensitivity and detection limits (10 ng/ml) for both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). Optimum sensitivity and detection limits for derivatized theanine standards were achieved using ESI-MS. The enantiomeric composition of six commercially available L-theanine samples was evaluated using the high-flow APCI-MS method and confirmed with photodiode array detection. Five of the six products

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contained significant amounts of D-theanine. Only one product, SunTheanine® appeared to contain only the L-theanine enantiomer.

INTRODUCTION

Tea is the most popular beverage consumed worldwide. The tea plant is grown in over 30 different countries and is available in many different varieties and flavors. There are three main types of tea: green, Oolong, and black tea. Green teas are subject to minimal oxidation, whereas Oolong and black teas are allowed to partially and extensively oxidize, respectively.[1] The taste of all types of teas can be attributed to the presence of many amino acids, in particular 5-N-ethylglutamine (found only in the free amino acid form), also known as theanine. Theanine was first identified by Sakato to be one of the major chemical constituents of green tea leaves.[2] Later, Cartwright, et al. demonstrated the presence of theanine in other forms of teas as well.[3] Ekborg-Ott et al. found a variety of black tea to have the highest concentration of theanine of the 17 teas tested.[4] Theanine reportedly makes up approximately 1-2% of the dry weight of tea.[5,6] The only other known natural source of theanine is the mushroom *Xerocomus badius*.[7]

Recently, there has been an increased interest in the pharmacological effects of theanine. A number of papers have documented the inhibition of peroxidation of low-density lipoproteins (LDL) by tea extracts.[8,9] Active oxygen species, which are known to cause significant damage to cells, are thought to be taken up by theanine and other tea components. In addition, theanine is thought to play a major role in preventing neuronal death.[9-11] Kakuda reported that neuronal death induced by glutamic acid was suppressed with the introduction

of theanine.[9,11] These findings prove extremely significant for the treatment and/or prevention of ischemia and reperfusion injury (stroke).

Theanine has also been shown to have an affect on blood pressure and hypertension in rats. In a controlled study, Yokogoshi reported dose-dependent reduction of blood pressure in hypertensive rats during theanine administration, but no change when the related amino acids, glutamine and glutamate, were introduced.[12,13] The mechanism by which theanine decreases blood pressure is not well understood. It is known, however, that levels of various neurotransmitters, such as serotonin and dopamine, can affect blood pressure and even heart rate.[13] Some theories suggest that theanine actually alters the levels of these neurotransmitters in the brain.[12-16] A number of studies support this theory by measuring neurotransmitter levels in rats after theanine exposure.[12, 14-16] It was found that levels of serotonin and 5-hydroxyindole acetic acid decreased significantly,[14] while those of tryptophan[14] and dopamine increased upon administration of theanine.[15,16] Neurotransmitters such as these are known to have various physiological functions. Specific regulation of these levels could possibly be used in the treatment of neurological diseases, such as Parkinson's and schizophrenia.

A recent study using electroencephalography (EEG) also showed the inhibiting affect of theanine on caffeine stimulation.[17] When mice were given equal molar amounts of caffeine and theanine, the stimulatory affects of caffeine were significantly reduced. In fact, at 10:1 ratios of theanine to caffeine, stimulation was found to be completely quenched.[17] Theanine has also been found to boost immunity[18-19] as well as improve the anti-tumor activity of various chemotherapeutic drugs,[20-28] and reduce tumor growth[29,30] and metastasis.[31]

Clearly, the physiological effects of theanine are varied and significant. Theanine, like most amino acids, is chiral. Therefore, it is quite possible that the pharmacological effects of one enantiomer over another may vary significantly. A few papers have reported the quantitative HPLC analysis of theanine in tea extracts[32] as well as in rat serum, tissue, and urine.[33,34] These methods all suffer from the inability to separate theanine into its D- and L-forms. This was remedied by a method developed by Ekborg-Ott et al.[4] In this procedure, theanine samples from tea extract were derivatized with 9-fluorenylmethyloxycarbonyl glycyl chloride (FMOC-Gly-Cl) reagent, and its enantiomers separated using a γ-cyclodextrin (γ-CD, Cyclobond II 2000) column with fluorescence detection. It was found that all teas, regardless of the manufacturing process, contained L-theanine and smaller percentages of D-theanine. The racemization and hydrolysis of theanine in aqueous solution were also evaluated.[4] Increasingly, chiral stationary phases based on macrocyclic antibiotics have been preferred for the enantiomeric separation for both native and derivatized amino acids.[35-38] Neither the enantiomeric separation of theanine on the macrocyclic antibiotic chiral selectors, nor the more sensitive MS detection of theanine, have been reported to our knowledge.

Recently, several papers have used HPLC and the macrocyclic antibiotic chiral selectors coupled with MS detection for the enantiomeric analysis of various chiral compounds, including amino acids.[39-44] MS detection was found to be quite sensitive for analyzing the underivatized amino acid enantiomers. However, derivatization is often necessary to extract target amino acids from biological samples, such as blood and urine.[45-50] The present study demonstrates the efficacy of MS detection for the analysis of underivatized

and derivatized theanine samples. The enantiomeric composition of commercially available theanine samples was also evaluated using this MS method.

EXPERIMENTAL

Chemicals and reagents

Racemic theanine, D-theanine, and L-theanine standards, were prepared in-house using a previously reported procedure.[4] HPLC grade methanol (MeOH) and ultra-pure HPLC-grade water were acquired from Fisher (Fair Lawn, NJ, USA) and Alfa Aesar (Ward Hill, MA, USA), respectively. Ammonium trifluoroacetate (NH_4TFA) reagent was purchased from Aldrich (Milwaukee, WI, USA). Formic acid was obtained from J.T. Baker (Phillipsburg, NJ, USA). All underivatized theanine samples were dissolved in water and diluted to 20 μg/ml for injection.

Commercially available L-theanine samples were obtained from Amax Nutra Source (City of Industry, CA, USA), Honson Industries, Ltd. (Markham, ON, Canada), HWBT (Zheda Technology & Trading Company, Hangzhou, China), Shengma Bio & Chem Co. (Shanghai Waygain Import and Export Co., Ltd., Shanghai, China), Tans (Zhejiang Zhongjin Environmental Protection Corp., Hangzhou, Zhejiang, China), and SunTheanine®(Taiyo International, Inc., Edina, MN, USA).

Derivatization of theanine

2.0 mg/ml solutions of 9-fluorenylmethyloxycarbonyl chloride (FMOC) and 5-dimethylamino-1-naphthalene-sulfonyl chloride (dansyl), both purchased from Aldrich, were prepared in HPLC-grade acetonitrile (Fisher). Derivatization procedures were similar to those previously reported.[51,52] A 0.1 M borate buffer solution, pH 7.8, prepared using ACS-

grade borate (Fisher), was used to formulate a 2.0 mg/ml stock solution of racemic theanine. For each derivatization reagent, 490 µl of borate buffer, 490 µl of acetonitrile, 10 µl of theanine stock solution, and 10 µl of the appropriate derivatization solution, were combined. The mixtures were vortexed for a few seconds and then allowed to react for at least 10 minutes before analysis. Solution concentrations were approximately 20 µg/ml.

AccQ-Fluor Reagent Kit™, containing 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent powder, acetonitrile (AccQ-Fluor Reagent Diluent), and borate buffer, was purchased from Waters (Milford, MA, USA). The use of this derivatization method for enantioselective amino acid analysis was first reported by Pawlawski.[53] The AQC reagent powder was reconstituted using 1 ml of the Reagent Diluent. The reagent solution was vortexed for 10 seconds and then placed on a 55°C heating block for 10 minutes. 10 µl of the theanine stock solution, 70 µl of AccQ-Fluor borate buffer, and 20 µl of the reagent solution were added to a sample vial. The mixture was vortexed for 10 seconds, and once again placed on a 55°C heating block for 10 minutes. The solution was then diluted to 20 µg/ml.

Instrumentation

Experiments were performed using a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system with a photodiode array detector (PDA) coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with ESI and APCI ion sources. The MS was operated in positive ion mode using single ion monitoring (SIM) detection. SIM was chosen over SRM (single reaction monitoring) as the background ("chemical noise") levels for SIM were not a limiting parameter. Nitrogen (Praxair, Danbury, CT, USA) was used as

both the sheath and auxiliary gases. Ultra-high purity helium (Linweld, Lincoln, NE, USA) was used as the damping gas in the ion trap.

ESI conditions. Sheath and auxiliary gases were 50 and 40 (arbitrary units), respectively. MS parameters were optimized to the following: source voltage 4.50 kV, capillary voltage 10.0 V, tube lens offset = 0.0 V, and capillary temp 270 °C. *APCI conditions.* Sheath and auxiliary gases were 80 and 20 (arbitrary units), respectively. MS parameters were optimized to the following: APCI vaporizer temp 400.0 °C, corona discharge current 5.00 μA, tube lens offset 30.0 V, and capillary temperature 200 °C.

All separations were performed at room temperature on a 250 x 4.6 mm Chirobiotic T (teicoplanin) chiral column from Astec (Whippany, NJ, USA). Solvent systems used were either reverse phase (methanol:water with additives) or polar organic mode (methanol with additives) for the derivatized and underivatized theanine samples, respectively.[40] Mobile phase flow rates were 0.4 ml/min or 0.8 ml/min.

RESULTS AND DISCUSSION

Enantiomeric separation of underivatized and derivatized theanine standards

Racemic theanine standards were derivatized using FMOC, dansyl, and AQC reagents as per the procedures described previously. Figure 1 shows the structures and molecular masses of native theanine, FMOC-theanine, dansyl-theanine, and AQC-theanine derivatives. Using the Chirobiotic T stationary phase, underivatized theanine was separated using an APCI-MS compatible reversed phase method (*vide infra*). A baseline separation of the enantiomers was obtained in less than 9 minutes. SIM detection was used to monitor the $[M+H^+]$ ion for theanine at *m/z* 175.0.

This method was also evaluated for the separation of the derivatized theanine standards. However, different mobile phase compositions were needed to achieve the optimum separation conditions for each of the derivatives. FMOC- and dansyl-theanine were best separated in the reverse-phase mode using 1.0 % NH_4TFA in MeOH: 100 % water at 30:70 and 35:65 ratios, respectively. The elimination of 0.1 % formic acid from the previous method seemed to improve the separation. SIM detection at *m/z* 397 and 408 was used for FMOC- and dansyl-theanine, respectively. AQC-theanine, however, achieved the best separation using polar-organic mode (containing no water). SIM detection of AQC-theanine at *m/z* 345 was used. Figure 2 shows the baseline separations for the native and derivatized theanine standards.

It was found that the APCI sensitivity of the derivatized theanine standards was lower than that for the underivatized theanine standard (data not shown). For comparison, the MS signal for the theanine standard dissolved in water was compared to the signal for the theanine stock solution used for making the derivatives (dissolved in borate buffer). Figure 3 shows the peak area counts (AA) for both enantiomers of each sample. The peak areas of the theanine stock solution are far less than those for the theanine standard dissolved in water, as are the signal to noise ratios (S/N). It is therefore possible that the borate buffer used in the derivatization procedure affected the sensitivity of the APCI-MS method for FMOC-, dansyl-, and AQC-theanine. As a result, electrospray ionization was evaluated for both the derivatized and underivatized theanine standards.

Using flow rates compatible with ESI, the sensitivity and detection limits for underivatized and derivatized theanine standards were assessed for ESI-MS detection. The ESI sensitivity of the underivatized theanine was improved by lowering the flow rate from

0.8 ml/min to 0.4 ml/min, whereas the reduction of flow rate for the AQC-theanine method had no effect on the sensitivity. This observation is likely due to fact that the mobile phase of the AQC- method contains no water. It is generally understood that, although water easily supports the formation of ions, its surface tension and solvation energy make desorption more difficult. Table 1 shows the sensitivity and the detection limits for all the theanine standards. The underivatized theanine had at least an order of magnitude better sensitivity than all the theanine derivatives, with a limit of detection of 10 ng/ml. (Sensitivity as defined by IUPAC is the slope of the dose-response curve.)[54] These results are consistent with those of Hemmermeister and co-workers, who found that native amino acids actually had better sensitivity than their dansyl chloride derivatized counterparts for ESI-MS detection.[48] Of the derivatives, AQC-theanine had the best sensitivity and detection limit for ESI-MS detection, once again most likely due to the use of polar organic LC mode.

Enantiomeric composition of commercially available L-theanine samples

As the pharmacological activity of theanine continues to be of interest to researchers, a variety of companies have begun marketing theanine as a nutraceutical and/or food/beverage additive. When extracted from tea leaves, theanine is predominately found in the L-form, as are most naturally occurring amino acids.[4] However, when synthesized L-theanine may not be the only enantiomer formed. Using the HPLC/APCI-MS method, the enantiomeric composition of commercially available theanine samples was evaluated. All products were marketed as L-theanine. Figure 4 illustrates the results for 5 of the 6 theanine products tested. All 5 samples show significant amounts of D-theanine present. In fact, all of the products appear to be racemic, within experimental error.

Figure 5 shows the results for the SunTheanine® product as compared with the L-theanine standard. This was the only commercially available product tested which showed no substantial amount of D-theanine. These results were confirmed using data obtained from the photodiode array detector on the LC system.

CONCLUSIONS

The HPLC/API-MS system provides excellent sensitivity and detection limits for the analysis of underivatized theanine. Using lower flow rates, the sensitivity of ESI was comparable to that of APCI at the higher flow rates for the native theanine. The sensitivity of derivatized theanine standards was improved with ESI-MS detection, but overall proved less sensitive than the underivatized theanine.

Using the high-flow APCI-MS method, the enantiomeric composition of six commercially available L-theanine products was evaluated and confirmed with PDA detection. Five of the six products appeared to be racemic. Only the SunTheanine® sample was the pure L-theanine enantiomer.

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FIGURE CAPTIONS

Figure 1. Structures and molecular masses of theanine, FMOC-theanine, Dansyl-theanine, and AQC-theanine.

Figure 2. Optimized enantiomeric separation of theanine, AQC-theanine, FMOC-theanine, and Dansyl-theanine. (a) LC/APCI-MS, SIM: *m/z* 175, Mobile phase conditions: 80:20 (1.0 % NH_4TFA in MeOH: 0.1 % formic acid in H_2O) 0.8 ml/min. (b) LC/ESI-MS, SIM: *m/z* 345, Mobile phase conditions: 30:70 (1.0 % NH_4TFA in MeOH: 100 % MeOH) 0.8 ml/min. (c) LC/ESI-MS, SIM: *m/z* 397, Mobile phase conditions: 30:70 (1.0 % NH_4TFA in MeOH: 100 % H_2O) 0.4 ml/min. (d) LC/ESI-MS, SIM: *m/z* 408, Mobile phase conditions: 35:65 (1.0 % NH_4TFA in MeOH: 100 % H_2O) 0.4 ml/min.

Figure 3. Comparison of theanine standard solution and theanine stock solution. RT: retention time, AA: peak area count, SN: signal to noise ratio. Separation conditions for both: LC/APCI-MS, SIM: *m/z* 175, Mobile phase conditions: 80:20 (1.0 % NH_4TFA in MeOH: 0.1 % formic acid in H_2O) 0.8 ml/min. (a) 20 µg/ml racemic theanine standard dissolved in 100% water. (b) 20 µg/ml racemic theanine stock solution dissolved in borate buffer used in derivatization procedure.

Figure 4. Enantiomeric composition of commercially available theanine samples compared to theanine standards. Retention times are slightly shifted from previously run standards due to day-to-day ambient temperature fluctuations. Separation conditions for

all standards and samples: LC/APCI-MS, SIM: *m/z* 175, Mobile phase conditions: 80:20 (1.0 % NH_4TFA in MeOH: 0.1 % formic acid in H_2O) 0.8 ml/min.

Figure 5. Enantiomeric composition of SunTheanine® as compared to L-theanine standard. Separation conditions for both: LC/APCI-MS, SIM: *m/z* 175, Mobile phase conditions: 80:20 (1.0 % NH_4TFA in MeOH: 0.1 % formic acid in H_2O) 0.8 ml/min.

Table 1. Sensitivity and Detection Limits of Underivatized and Derivatized Theanine

Compound	SIM (m/z)	Method	Linearity	r^2	LOD
Theanine	175	80:20 (A:B) 0.4 ml/min ESI	y = 34818x + 2E+07	0.9922	10 ng/ml
AQC-theanine	345	30:70 (A:100% MeOH) 0.8 ml/min ESI	y = 6192.3x - 3E+06	0.9998	500 ng/ml
FMOC-theanine	397	30:70 (A:100% H_2O) 0.4 ml/min ESI	y = 1256.5x + 2E+06	0.9889	500 ng/ml
Dansyl-theanine	408	35:65 (A:100% H_2O) 0.4 ml/min ESI	y = 345.03x + 242094	0.9923	1 µg/ml

A: 1.0 % NH_4TFA in MeOH, B: 0.1 % formic acid in H_2O, LOD: Limit of Detection

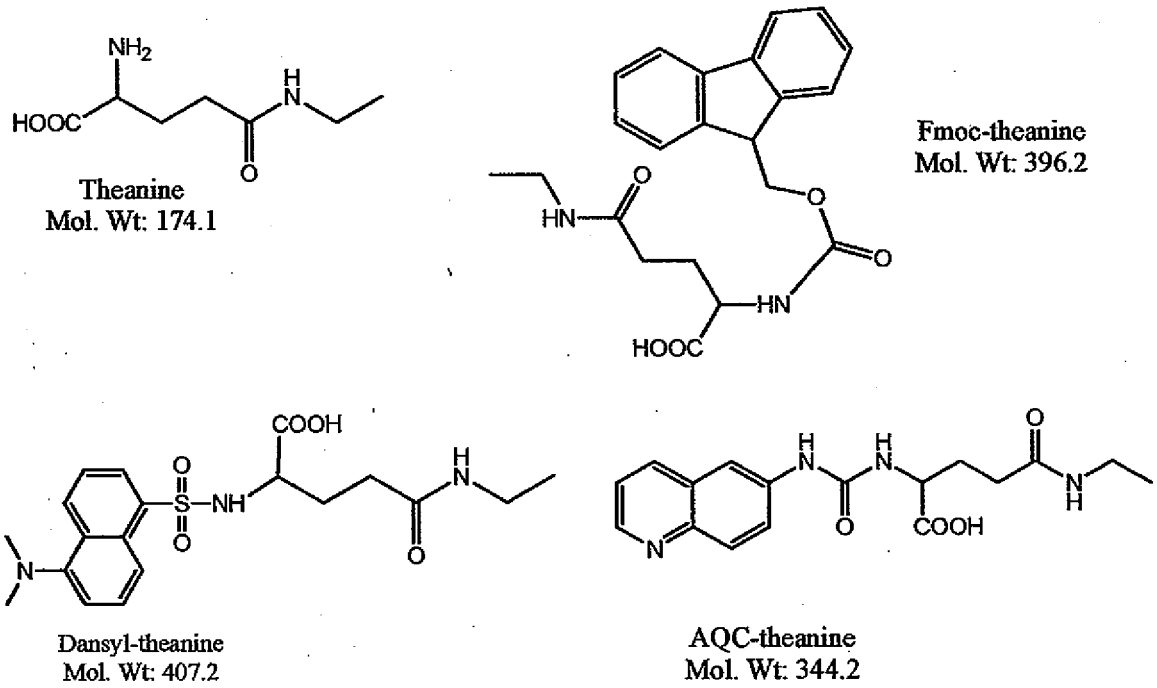
NH2
HOOC
H
N
O
Theanine
Mol. Wt: 174.1
HN
O
O
O
HN
HOOC
Fmoc-theanine
Mol. Wt: 396.2
COOH
O
S
O
NH
H
N
O
N
Dansyl-theanine
Mol. Wt: 407.2
H
N
H
N
O
O
N
H
COOH
N
AQC-theanine
Mol. Wt: 344.2

Figure 1

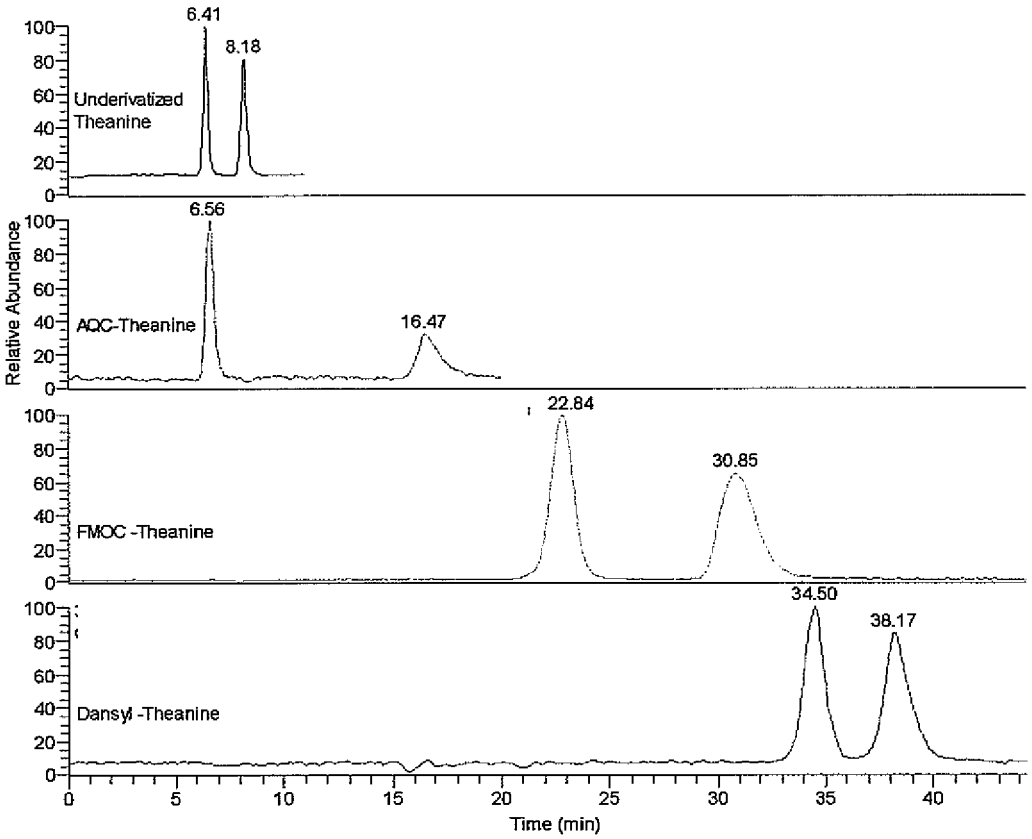
6.41
8.18
Underivatized Theanine
6.56
AQC-Theanine
16.47
22.84
30.85
FMOC -Theanine
34.50
38.17
Dansyl -Theanine
Relative Abundance
Time (min)

Figure 2

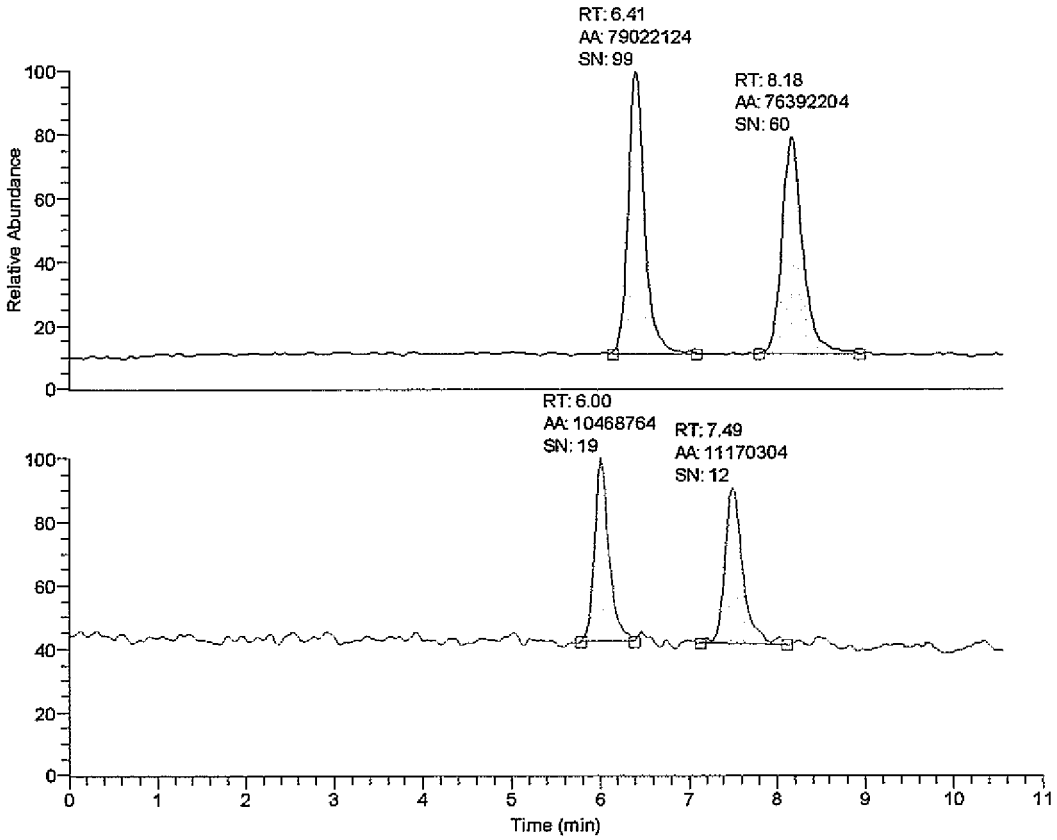
RT: 6.41
AA: 79022124
SN: 99
RT: 8.18
AA: 76392204
SN: 60
RT: 6.00
AA: 10468764
SN: 19
RT: 7.49
AA: 11170304
SN: 12
Relative Abundance
Time (min)

Figure 3

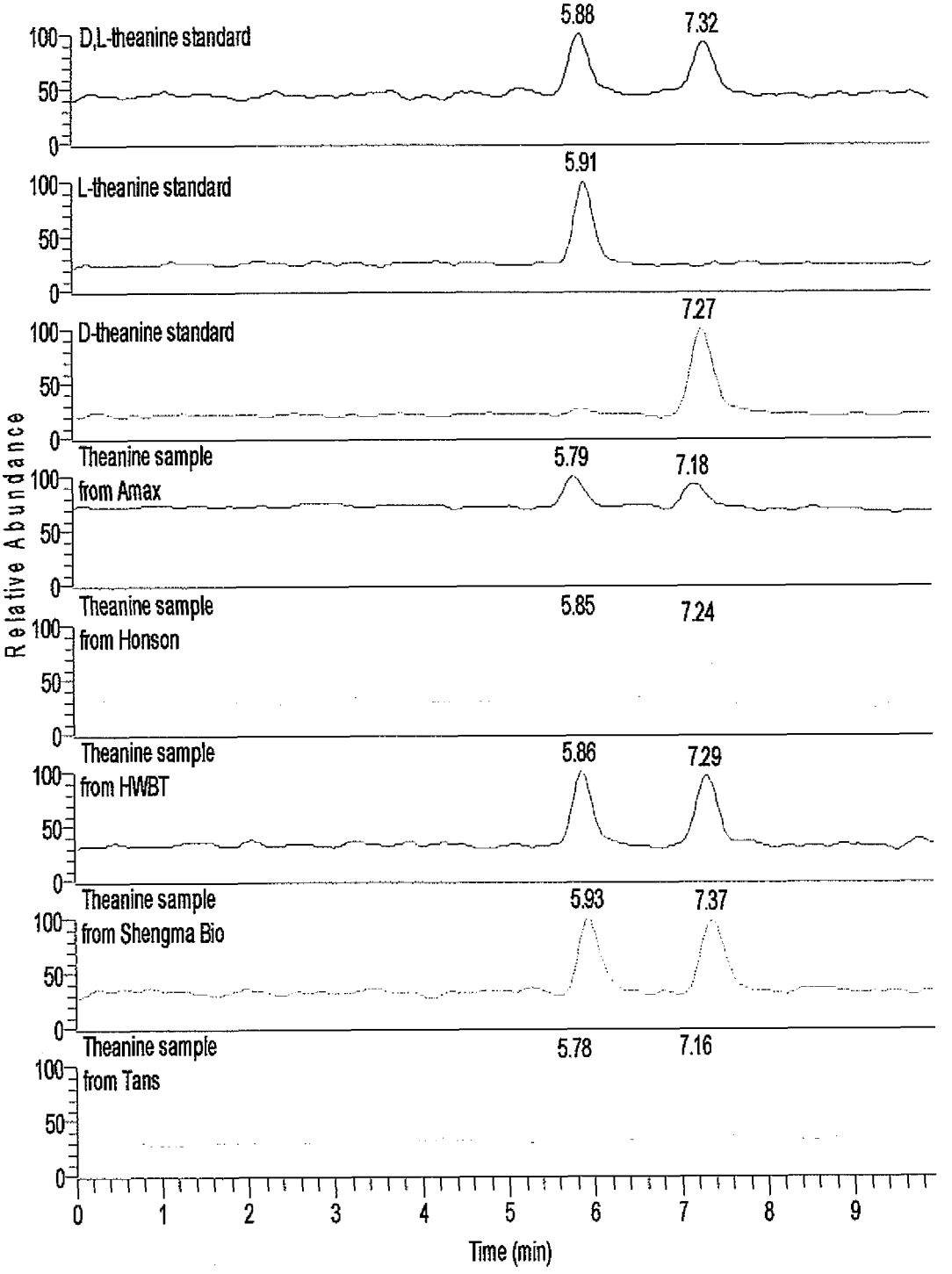
D,L-theanine standard
5.88
7.32
L-theanine standard
5.91
D-theanine standard
7.27
Theanine sample from Amax
5.79
7.18
Theanine sample from Honson
5.85
7.24
Theanine sample from HWBT
5.86
7.29
Theanine sample from Shengma Bio
5.93
7.37
Theanine sample from Tans
5.78
7.16
Relative Abundance
100
50
0
0 1 2 3 4 5 6 7 8 9
Time (min)

Figure 4

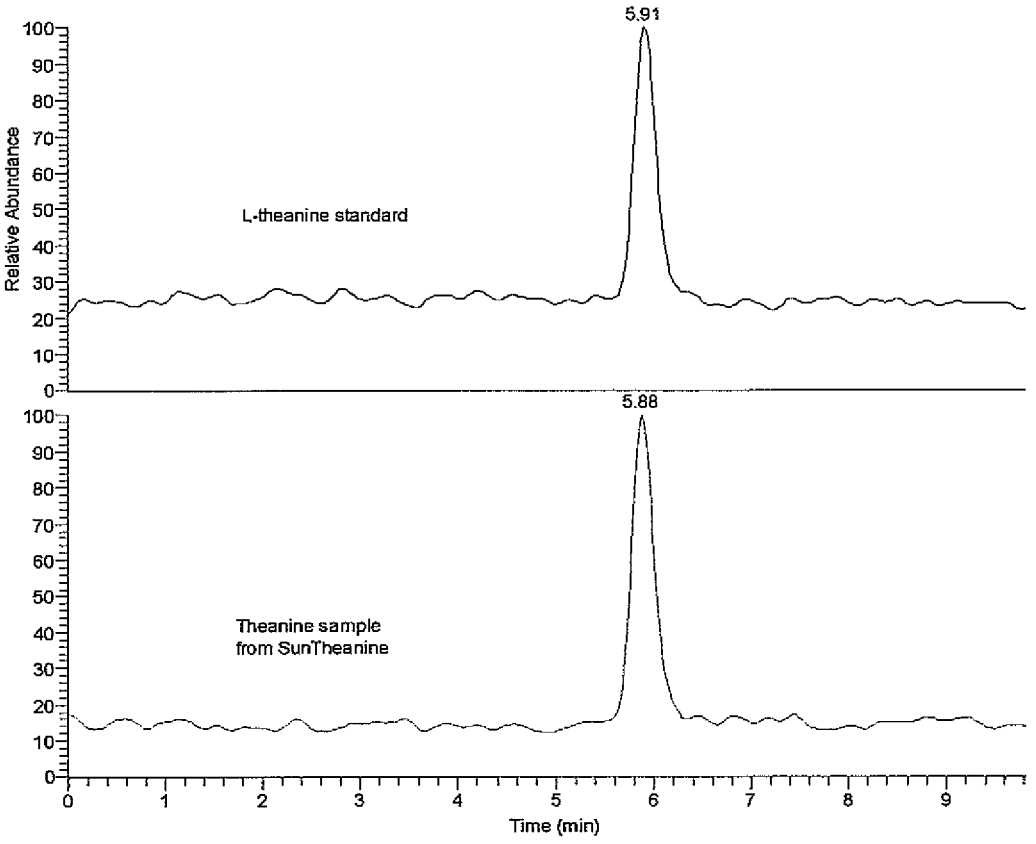
5.91
L-theanine standard
5.88
Theanine sample
from SunTheanine
Relative Abundance
Time (min)

Figure 5

CHAPTER 6. PHARMACOKINETICS AND PHARMACODYNAMICS OF THEANINE ENANTIOMERS IN RATS

A paper submitted to the *British Journal of Pharmacology*

Meera J. Desai, Manmeet S. Gill, Walter H. Hsu, Daniel W. Armstrong

SUMMARY

1. Theanine, first discovered in tea, is a chiral non-proteinic amino acid that has been shown to have cardiovascular, neurological, and oncological benefits and is being considered as a therapeutic/medicinal agent and additive in consumer products. The stereochemical effects of theanine on biological systems have not been investigated.

2. The present study evaluated the pharmacokinetics and pharmacodynamics of D-theanine, L-theanine, and D,L-theanine in plasma and urine using liquid chromatography coupled to electrospray ionization mass spectrometry in rats after oral and i.p. administration.

3. Oral administration data indicated that gut absorption of D-theanine was far less than that of L-theanine. However, after i.p. administration, plasma theanine concentrations of L- and D-theanine were similar. This indicated that D- and L-theanine exhibit a competitive effect with respect to intestinal absorption.

4. Regardless of the route of administration, oral or i.p., the presence of the other enantiomer always decreased theanine plasma concentrations. This further indicated that D,L-theanine also exhibits a competitive effect with respect to urinary reabsorption.

5. Data on urinary concentrations of D-theanine suggested that the D-isomer may be eliminated with minimal metabolism.

6. Our results indicated that the rate of L-theanine metabolism into ethylamine is increased in the presence of D-theanine. L-theanine appeared to be preferentially reabsorbed and metabolized by the kidney while D-theanine was preferentially excreted.
7. Clearly, the bioequivalencies of D,L-theanine and its enantiomers were found to be quite different from one another. Consequently, the efficacy of commercial theanine products containing D-theanine, L-theanine, or D,L-theanine may be quite different.

Introduction

Since ancient times, tea has been touted as having numerous health benefits. Only recently, have scientists been able to identify one of its major components, theanine, responsible for these physiological functions. Theanine, N-ethylglutamine, was first isolated from green tea leaves in the late 1940s (Sakato, 1949) but has also been found in other tea varieties as well (Ekborg-Ott et al., 1997). Free, unbound theanine makes up nearly 2% of the dry weight of tea (Goto et al., 1994). There is more theanine in tea than all other free amino acids combined. It is found naturally in only one other known source, the mushroom *Xerocomus badius* (Casimir et al., 1960).

The pharmacological benefits of theanine are numerous ranging from prevention of neuronal death (Nagasawa et al., 2004) to reduction of tumor growth (Zhang et al., 2002) and enhancement of antitumor activity of chemotherapeutic drugs (Sugiyama & Sadzuka, 2003). Theanine has also been shown to inhibit the peroxidation of low-density lipoproteins (LDL) (Yokozawa & Dong, 1997) and reduce hypertension and blood pressure in rats (Yokogoshi & Kobayashi, 1998). The mechanism by which blood pressure is reduced is hypothesized to be related to the effect of theanine on neurotransmitters, such as dopamine and serotonin

(Yokogoshi & Terashima, 2000). In addition, ethylamine, a major metabolite of theanine, has been found to dramatically boost immunity (Kamath et al., 2003).

Like most amino acids, theanine is chiral, and its pharmacological activity may vary depending on which enantiomer is present. As interest in the pharmacology of theanine continues to grow, the development of methods which allow for the separation and detection of theanine enantiomers is needed. Few articles have reported the use of high-performance liquid chromatography (HPLC) to analyze theanine in tea extracts (Ding et al., 2002) and biological fluids (Terashima et al., 1999; Unno et al., 1999), but all of these methods were unable to distinguish between D- and L-theanine isomers. In addition, these methods required either amperometric detection or fluorescent derivatization of theanine for detection. Recently, several studies have used mass spectrometry (MS) methods with high sensitivities and low detection limits to separate native amino acids without the need for derivatization (Petritis et al., 2001; Desai & Armstrong, 2004a; Desai & Armstrong, 2004b).

Using a similar HPLC-MS method developed in our laboratory, we recently evaluated the enantiomeric composition of six commercially available L-theanine products (Desai & Armstrong, 2004c). As a result of the varied pharmacological benefits of theanine, a number of commercial companies have begun to distribute it as a nutraceutical and/or food/beverage additive. Five of the six products tested were found to be racemic mixtures (which contain equivalent amounts of both D- and L-theanine). Only one product, SunTheanine®, appeared to be the pure L- isomer. Since it is possible that the pharmacology, pharmacokinetics, and metabolism can vary depending on the enantiomer, it is of concern that commercial products, marketed as pure L-theanine, are actually racemates. Therefore, determination of the

differences and/or similarities in the pharmacokinetics of L-theanine, D-theanine, and racemic D,L-theanine is essential.

To our knowledge, no reports concerning the pharmacokinetics of D-theanine and D,L-theanine have been published. One previous study did monitor the metabolism of what was assumed to be L-theanine in rats (Unno et al., 1999). However, this study exhibited a number of short comings: a) dosage was not based on weight; b) blood samples for all time points were obtained by cardiac puncture of rats while they were anesthetized; c) the time-dependent appearance of theanine was only measured for a single dose; and d) urine was collected only after 24 hours and not at hour-time intervals. In the following study the pharmacology, absorption, and excretion of D-theanine, L-theanine, and racemic D,L-theanine were evaluated *in vivo* using male Sprague-Dawley rats, addressing the deficiencies of the previous L-theanine paper. The objectives of our study were to determine if a) D- and L-theanine have similar pharmacokinetic properties; b) the metabolism of the racemate is similar to that of the individual D- and L- enantiomers; and c) the presence of the other enantiomer in the racemic mixture affects GI absorption, metabolism, and urinary excretion.

Methods

Chemicals. Racemic theanine was obtained from HWBT Zheda Technology & Trading Company (Hangzhou, China) as a dietary supplement. L-theanine (SunTheanine®) was purchased from Taiyo International, Inc. (Edina, MN). D-theanine was synthesized in-house using a previously reported procedure (Ekborg-Ott et al., 1997). HPLC grade methanol and acetonitrile were acquired from Fisher (Fair Lawn, NJ). Ultra-pure HPLC-grade water was purchased from Alfa Aesar (Ward Hill, MA). Ammonium trifluoroacetate

(NH_4TFA) was obtained from Aldrich (Milwaukee, WI) and formic acid was purchased from J.T. Baker (Phillipsburg, NJ). D^5-L-glutamic acid was used as the internal standard and was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Ethylamine hydrochloride standard was obtained from Fluka (Steinheim, Switzerland). 9-fluorenylmethyloxycarbonyl chloride (Fmoc) and boric acid were purchased from Aldrich and Fisher, respectively.

Animals. Male Sprague-Dawley rats, weighing 300-500g, were purchased from Harlan Global (Indianapolis, IN). The animals were housed in a facility with a 12-hour light/dark cycle with food and water available ad libitum then food was withheld 14 hours before theanine administration. All animal studies were approved by the Committee on Animal Care of Iowa State University.

Plasma Theanine Study. The pharmacology of D-theanine, L-theanine, and racemic theanine was evaluated using male Sprague-Dawley rats. Rats were dosed orally with 0.25 g kg^{-1}, 0.5 g kg^{-1} and 1.0 g kg^{-1} doses and intra-peritoneally (i.p.) with 0.5 g kg^{-1} and 1.0 g kg^{-1} doses. Dosages were chosen for the following reasons, a) to maintain solubility of theanine in water without super-saturation, b) to stay within the detection limits of this assay, and c) for comparison purposes, doses were kept similar to the previous study using L-theanine (Unno et al., 1999). For each dose, 0.25, 0.5, and 1.0 g kg^{-1}, theanine solutions were dissolved in water to yield concentrations of 37.5 mg ml^{-1}, 75.0 mg ml^{-1}, and 150 mg ml^{-1}, respectively. The volume of solution administered to each rat was based on its weight and volumes administered were between 2.0 and 3.3 ml for both oral and i.p. applications. Oral theanine treatments were delivered using a syringe fitted with a feeding tube inserted directly into the stomach. Blood samples were taken from the saphenous vein according to a

previously described procedure (Hem et al., 1998) at 0, 15, 30, 60, 120, 240, and 360 minutes after administration. Samples were collected in 50 µl heparinized micro-hematocrit tubes from Fisher Scientific (Pittsburgh, PA) and sealed with a tube sealing compound prior to centrifugation using an IEC MB micro-hematocrit centrifuge from Damon/International Equipment Company (Needham Heights, MA). The plasma portion was emptied into centrifuge tubes and proteins were precipitated by adding ice-cold acetonitrile in a 3:1 solvent to sample ratio (Polson et al., 2003). The mixture was then kept at 4°C prior to centrifugation for 10 minutes at 2600g. Samples were stored at -20°C until LC-MS analysis.

Urinary Theanine Study. Male Sprague-Dawley rats were housed individually in metabolic cages. Prior to theanine administration, urine was collected after withholding food for ~ 14 hours. Rats were orally dosed with 0.5 g kg^{-1} for both D- and L-theanine and with 1.0 g kg^{-1} for racemic theanine. Urine was also collected after i.p. administration of 1.0 g kg^{-1} racemic theanine. Samples were taken at 0, 60, 120, 240, and 360 minutes after administration when available. An aliquot of 0.25 ml of urine was added to Ultrafree-MC 5kD centrifugal filter devices (Millipore, Belford, MA). The tubes were then centrifuged for 45 minutes at 2600g (Carducci et al., 1996). Samples were stored at -20°C and thawed prior to LC-MS analysis.

LC-MS Analyses. Experiments were performed using a Thermo Finnigan (San Jose, CA) Surveyor LC system with a photodiode array detector (PDA) coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with an electrospray ionization (ESI) source. Data acquisition was controlled by Xcalibur version 3.1 software (Thermo Finnigan). The MS was operated in positive ion mode using single ion monitoring (SIM) detection for both theanine and the internal standard, D^5-L-glutamic acid at 175 and 153 m/z,

respectively. Nitrogen (Praxair, Danbury, CT) was used as both the sheath and auxiliary gases. Ultra-high purity helium (Linweld, Lincoln, NE) was used as the damping gas in the ion trap. Electrospray conditions were as follows: sheath and auxiliary gases 50 and 40 (arbitrary units), respectively, source voltage 4.50 kV, capillary voltage 10.0 V, tube lens offset 0.0 V, and capillary temperature 270 °C.

Analyses of theanine in plasma and urine were performed at room temperature on a 250 x 4.6 mm Chirobiotic T (teicoplanin) chiral column from Astec (Whippany, NJ). For urine samples the Chirobiotic T column was fitted with a 2.0 cm x 4.0 mm ID teicoplanin guard column. Mobile phase conditions were isocratic at 80:20 (1.0 % NH_4TFA in methanol: 0.1 % formic acid in water) at 0.4 ml min^{-1}. For plasma and urine samples, 10 µl of supernatant or filtrant, 5 µl of internal standard (1mg ml^{-1} D^5-L-glutamic acid), and 35 µl of water were added to an autosampler-compatible centrifuge tube of which 2 µl was injected onto the LC-ESI/MS system.

Measurement of Theanine Metabolite, Ethylamine, in Plasma. After oral administration of rats with 0.5 g kg^{-1} for both D- or L-theanine or with 1.0 g kg^{-1} for racemic theanine, blood samples taken at 0, 15, 30, 60, 120, 240, and 360 (previously evaluated for the presence of theanine) were analyzed for ethylamine by LC coupled to fluorescence detection. The molecular weight of ethylamine, 45 Da, was lower than the MS m/z cutoff making MS detection unsuitable. Therefore, ethylamine was derivatized using Fmoc reagent. Frozen plasma samples containing acetonitrile were thawed to room temperature and re-centrifuged for 10 minutes at 2600g. An aliquot of 10 µl of the supernatant was placed into an autosampler vial to which 490 µl of acetonitrile, 490 µl of 0.1 M borate buffer (pH 7.1)

and 10 µl of 4.0 mg ml^{-1} Fmoc reagent were added (Carpino et al., 1986). Samples were vortexed for 10 sec and allowed to react for 30 minutes prior to analysis. 2 µl of each sample was injected onto a 10 cm x 4.6 mm ID C-18 column available from Astec for LC-fluorescence analysis. The previously mentioned Surveyor LC system was coupled to a Shimadzu RF-10Axl fluorescence detector (Kyoto, Japan), excitation: 266 nm; emission: 315 nm. The mobile phase conditions were isocratic at 75:25 (methanol: water) at 0.4 ml min^{-1}.

Data Analyses. All theanine plasma and urine concentrations were calculated using a calibration curve derived from the ratio of theanine standards to the 100 µg ml^{-1} internal standard. Concentration of ethylamine was calculated using a calibration curve derived from Fmoc - derivatized ethylamine standards. Data are shown as means ± s.e.mean and were subjected to Student's *t*-test and the significance level was set at $p < 0.05$. The plasma concentration-time data were analyzed using Origin software Microcal version 7.0 (North Hampton, MA). The area under the plasma concentration-time curve (AUC) was integrated using trapezoidal rule.

Results

Plasma Concentrations of D-theanine, L-theanine, and Racemic Theanine after Oral Administration. In order to compare the pharmacokinetics of each theanine isomer to the racemate, rats were individually administered either a 0.5 g kg^{-1} dose of D-theanine, L-theanine, or a 1.0 g kg^{-1} dose of racemic D,L-theanine orally. The racemic theanine dose was chosen to be double that of the individual L- and D- doses for purposes of comparative analysis. Figure 1 shows blank and D,L-theanine spiked plasma samples. No theanine signal

was detected for the blank plasma (Fig. 1A) only that of the internal standard was observed. After spiking plasma with D,L-theanine (Fig. 1B and 1C), we were able to separate and detect L- and D-theanine enantiomers along with the internal standard. This optimized method was used to evaluate plasma samples after theanine administration.

After individual D-theanine and L-theanine oral administration, the maximum L-theanine concentrations in plasma were consistently higher than those of D-theanine when given equivalent doses of each enantiomer. The L-theanine concentrations were more than three times those of D-theanine. This relationship was found to be dose-dependent (Fig. 2) when rats were given 0.5 g kg^{-1} doses and 0.25 g kg^{-1} doses. At the lower dosage, L-theanine concentrations were nearly five times higher than those of D-theanine. The maximum plasma concentrations were obtained between 30 and 60 minutes after administration for both theanine doses.

When rats were given a 1.0 g kg^{-1} (50/50) racemic mixture of D,L-theanine, the maximum plasma concentrations for L-theanine were again higher than those for D-theanine. Figure 3 shows the plasma concentration-time curves for racemic D,L-theanine. The maxima still occurred approximately 60 minutes after administration. Figure 4A and B show a comparison of the L- and D-theanine curves with and without the presence of the other enantiomer. For the L-theanine curves, the maximum was significantly lower for the racemic dose, but the overall shape of the curves was similar (Fig. 4A). The maximum levels for D-theanine in the presence of L-theanine (racemic dose) were also significantly lower than D-theanine given alone (Fig. 4B).

Plasma Concentrations of D-theanine, L-theanine, and Racemic Theanine after Intraperitoneal Injection. In order to investigate the role of the gut in absorption of theanine, rats

were administered D-theanine, L-theanine, or D,L-theanine via i.p. injection. The same dosing scheme (both volume and amount) was used for i.p. administrations for comparison purposes. Figure 5 shows the plasma concentration-time curves for i.p. administration of racemic theanine. Individual L- and D-theanine i.p. curves are shown in Figure 6 A and B, respectively. As can be seen, maximum levels were achieved between 15 and 30 minutes and did not recede as quickly as the oral dosing method. A comparison of the maximum plasma concentrations and AUCs for oral versus i.p. administration is compiled in Table 1.

The AUC of the i.p. L-theanine curve was not significantly altered from the oral administration method; whereas the D-theanine AUC was significantly higher and statistically similar to that of L-theanine for the i.p. administration procedure. Similar to the oral administration method, the AUCs for the racemic i.p. dose were still significantly lower than the individual i.p. D- and L-theanine doses. There appeared to be no difference between the AUCs induced by i.p. and oral administration of racemic L-theanine, whereas the i.p. racemic D-theanine AUC was approximately 4 times higher than that of oral racemic D-theanine. Interestingly, for both the oral and i.p. administrations, the AUC for D-theanine alone was 3 times higher than that of D-theanine in the presence of L-theanine (racemic dose). All reported differences and similarities for AUC and C_{max} were found to be statistically significant.

Urinary Excretion of D-theanine, L-theanine, and Racemic D,L-theanine after Oral Administration. In order to investigate the metabolic fate of theanine, urine samples were collected from rats dosed with 0.5 g kg^{-1} D-, or L-theanine, or 1.0 g kg^{-1} racemic D,L-theanine. Samples were collected up to 6 hours after administration. Interestingly, the initial

theanine peak concentration occurred between 1 and 2 hours for racemic theanine and L-theanine, while the individual D-theanine dose generated a concentration peak between 2 and 4 hours. This observation is illustrated by the urine concentration-time curves shown in Figures 7 and 8. There was significantly more D-theanine in the urine at 2 hours when D- was given in the presence of L-, than when D-theanine was administered alone (4300 ± 50 μg ml^{-1} compared to 19 ± 5.0 μg ml^{-1}). However, the overall maximum D-theanine urine concentration achieved in 6 hours was similar for both the racemate and the individual D-theanine dose. Table 2 shows the maximum theanine levels achieved during this time interval. The urine levels of the individual L-theanine dose were significantly lower than that of the racemic L-theanine dose.

Urinary Excretion of Racemic D,L-theanine after Intra-peritoneal Injection. For the purposes of comparative analysis, the urinary excretion profiles of the racemate were also evaluated using i.p. administration. After i.p. administration of 1.0 g kg^{-1} racemate the C_{max} for L- and D-theanine were 860 ± 110 μg ml^{-1} and 6400 ± 700 μg ml^{-1}, respectively. There was still considerably more D-theanine excreted than L-theanine with the i.p. administration ($p < 0.05$). In comparison to the oral racemic administration, these maximum urinary concentrations of D- and L-theanine achieved in 6 hours were not statistically different. Urine concentration-time curves for i.p. administration are shown in Figure 9.

Measurement of Theanine Metabolite, Ethylamine, in Plasma.

Theanine is metabolized in the kidney to glutamic acid and ethylamine (Tsuge, et al. 2003). Therefore, measurement of ethylamine concentrations in plasma provided further insight into the metabolism of all forms of theanine. The amount of ethylamine produced

was measured using LC-coupled to fluorescence detection. The ethylamine plasma concentration-time curves are shown in Figure 10. Ethylamine concentrations did not diminish over the six-hour experimental time frame for both the individual L-theanine and D-theanine administration (Fig. 10 A). However, as can be seen in Figure 10 B, the racemic theanine administration produced a very different ethylamine concentration-time curve. The maximum plasma concentrations ($\pm$ s.e.mean) of ethylamine achieved during this time interval for L-theanine, D-theanine, and the racemate were 420 ± 42 $\mu g\ ml^{-1}$, 11 ± 2 $\mu g\ ml^{-1}$, and 400 ± 43 $\mu g\ ml^{-1}$, respectively. The amount of ethylamine produced by both the racemate and the individual L-theanine doses were statistically indistinguishable and were also significantly higher than the amount produced by the individual D-theanine dose.

Discussion

The naturally occurring non-proteinic amino acid, theanine, has demonstrated vast pharmacological activity from decreasing LDL levels (Yokozawa & Dong, 1997) to boosting immunity (Kamath et al., 2003). Previous studies have shown that in addition to L-theanine small amounts of D-theanine are present in various types of teas (Ekborg-Ott et al., 1998). As interest in theanine's nutraceutical properties developed, more and more companies have begun to manufacture and distribute theanine products. Recent work in our laboratory has shown that certain marketed L-theanine brands largely consist of a 50/50 mixture of both L- and D-theanine (Desai & Armstrong, 2004c). Considering the possibility that each isomer may have different pharmacological, metabolic, pharmacokinetic, and pharmacodynamic

properties, this finding is troublesome. Thus, the absorption, metabolism, and excretion profiles were investigated *in vivo* for D-theanine, L-theanine, and racemic D,L-theanine.

The overall results obtained for oral theanine administration of L-theanine were similar to those previously reported (Unno et al., 1999). The plasma D-theanine concentrations, however, were significantly lower than those of L-theanine, indicating that there is chiral discrimination in the absorption of the D-isomer. The relative maxima of L- and D-theanine were also found to be dose-dependent, negating the possibility of a saturation effect.

After oral administration of the racemate, the overall maxima for both L-theanine and D-theanine were significantly and markedly reduced as compared to individual L- and D-doses. This suggested that the presence of the other enantiomer altered the pharmacokinetics of theanine. The profile of the L-theanine plasma concentration-time curve for the racemate was similar to that of individually dosed L-theanine, indicating comparable kinetic profiles. The shapes of the D-enantiomer curves, however, were quite different. Although the maximum was lower, D-theanine from the racemate was eliminated from the plasma much more slowly than the D-isomer administered alone, suggesting that the presence of L-theanine altered the pharmacokinetics of the D-enantiomer. These oral administration data clearly indicate the presence of stereoselective pharmacokinetics and pharmacodynamics involving theanine. However, at this point it was not clear whether the reason(s) for this was due to stereoselective absorption from the gut, metabolism, or reabsorption/excretion by the kidney.

Previous studies, using a guinea pig model, have compared the intestinal absorption of L-theanine with glutamine, its analogous proteinic amino acid by monitoring transmural

potential difference changes (ΔPD) (Kitaoka et al., 1996). It was found that L-theanine- and glutamine-evoked potential difference changes both conformed to Michaelis-Menten relationship and were statistically similar. However, in the presence of glutamine, the ΔPD of theanine was decreased indicating competition. It was also reported that when Na^+ was eliminated from the luminal solution the ΔPD of both glutamine and L-theanine were inhibited, suggesting that both are transported across the brush-border membrane by a common Na^+-coupled co-transporter (Kitaoka et al., 1996). For this study, in order to determine the role of the gut in theanine absorption, i.p. administrations of D-, L- and racemic D,L-theanine were performed.

The maximum plasma concentrations of L-theanine were similar for both oral and i.p. doses, but those of D-theanine were dramatically increased with i.p. administration. After administration, D-theanine concentrations were statistically equal to those of L-theanine. These findings clearly indicated that the gut plays a major role in inhibiting D-theanine uptake, suggesting that stereospecificity is a factor in amino acid intestinal absorption. In a non-chiral environment, D- and L-theanine are chemically identical so it is unlikely that theanine transport is regulated solely by passive diffusion. As Kitaoka et al. (1996) suggest, intestinal absorption of theanine was likely regulated by active transport which can very well distinguish between D- and L-isomers. Our findings were supported by a study in which intestinal absorption of D,L-amino acid enantiomers were monitored by an *in situ* single pass perfusion (Oguri et al., 1999). The apparent membrane permeability coefficients (P_{app}) for L-isomers of alanine, arginine, and aspartic acid were higher than those for their respective D-isomers indicating higher L-enantiomer uptake.

After i.p. administration of racemic theanine, plasma D-theanine concentrations increased and were similar to those of L-theanine. These results indicated that D- and L-theanine exhibit a competitive effect with respect to GI absorption, suggesting that both D- and L-theanine utilize a common transporter across the brush-border membrane.

Although the previous findings indicated a significant contribution by the GI tract for theanine uptake, overall plasma concentration maxima after i.p. administration of the racemate were still significantly lower than individual L- and D-doses. In fact, racemic L-theanine plasma concentrations after i.p. administration were similar to those of oral administrations, indicating that gut absorption was not the only regulating factor, suggesting that renal reabsorption also plays a role. A previous study utilizing *in vivo et situ* continuous microperfusion of rat kidney tubules, reported similar results, i.e., that D- and L- amino acid reabsorption decreased in the presence of the other enantiomer (Silbernagl & Volkl, 1977). Our findings may corroborate this phenomenon that amino acid reabsorption is regulated by renal function through active transport as well.

The urinary data clearly support the results of the plasma study. After oral administraion of the individual D-theanine dose, the initial urine concentration peaked between 2 and 4 hours. In contrast, large amounts of D-theanine were excreted into the urine soon after administration of racemic theanine indicating that in the presence of L-theanine, the D-isomer is rapidly eliminated with minimal metabolism. Similar findings were reported for the administration of L- and D-phenylalanine (Lehmann et al., 1983). Two other studies hypothesized that the difference in D-amino acid urinary excretion over the respective L-isomer is attributed to preferential kidney reabsorption of the L-enantiomer (Armstrong et al., 1993; Bruckner & Schieber, 2001).

Since theanine is known to metabolize in the kidney to glutamic acid and ethylamine (Tsuge et al., 2003), the results obtained from the plasma ethylamine study should verify the previous data. Both L-theanine and racemic theanine produced similar plasma concentrations of ethylamine whereas administration of D-theanine produced very low plasma ethylamine concentrations. These data provided further merit to the hypothesis that D-theanine is eliminated with minimal metabolism. In addition, although the maximum concentrations of ethylamine were similar for L-theanine and racemic theanine administration, the ethylamine plasma concentration-time profile of D,L-theanine was dramatically different from that of individual L-theanine. It was apparent from this data that the rate of metabolism of L-theanine to ethylamine increased as a result of the presence of D-theanine. This interaction at the metabolism level needs to be further investigated.

In conclusion, the present study evaluated the pharmacokinetic and metabolic behavior of D-theanine, L-theanine, and racemic D,L-theanine via oral and i.p. administration in rats. D-theanine absorption from the GI tract was much less than that of L-theanine. In addition, in the presence of L-theanine, D-theanine uptake was further reduced only for oral administration, indicating that stereospecificity plays a role in theanine intestinal absorption. Plasma concentrations of D- and L-theanine after racemic D,L-theanine administration were consistently lower than D- and L- doses. This suggests that the presence of the other enantiomer inhibits theanine uptake. This was observed for both oral and i.p methods indicating that the kidneys also are a factor in theanine reabsorption. High concentrations of D-theanine were excreted into the urine demonstrating that the D-enantiomer may be eliminated relatively quickly. It was also suggested that the presence of

D-theanine enhances or facilitates the initial production of ethylamine via L-theanine metabolism.

We have shown that there were significant differences among L-, D-, and D,L-theanine GI absorption and urinary excretion. The presence of D-theanine, clearly inhibits L-theanine absorption and vice versa. This indicates, at the very least, that the bioequivalence of D-theanine, L-theanine and racemic theanine are quite different and that the biological behavior and disposition of racemic theanine is more complicated than that of either pure enantiomer. In addition, the pharmacological activity of D-theanine is largely unknown. Therefore, our findings indicate that the efficacy and/or safety of racemic D,L-theanine dietary supplements may be questionable.

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Table 1. Mean maximum theanine plasma concentration (C_{max}) and area under concentration time curve ($AUC^{\dagger}$) for oral dose versus intra-peritoneal administration. Values represent mean ± s.e.mean

Theanine Dose	**Oral Dose** *n = 3*				**i.p. Administration** *n = 3*			
	C_{max} (μg ml^{-1})		**AUC (μg * min ml^{-1})**		**C_{max} (μg ml^{-1})**		**AUC (μg * min ml^{-1})**	
0.5 g kg^{-1} L-theanine	$400 \pm 24^{A,a,b}$		$41200 \pm 7700^{D,g,h}$		$396 \pm 35^{A,B,e}$		$67000 \pm 14300^{D,E,k}$	
0.5 g kg^{-1} D-theanine	$134 \pm 27^{a,c,d}$		$12100 \pm 4600^{g,i,j}$		$358 \pm 11^{B,c,f}$		$50200 \pm 11200^{E,l,f}$	
1.0 g kg^{-1} D,L-theanine	L-theanine	D-theanine	L-theanine	D-theanine	L-theanine	D-theanine	L-theanine	D-theanine
	$143 \pm 3^{C,b}$	21 ± 7^{d}	$22500 \pm 4200^{F,h}$	4200 ± 1600^{j}	$160 \pm 14^{C,e}$	173 ± 17^{f}	$20700 \pm 3900^{F,G,k}$	$16800 \pm 3800^{G,l}$

† AUC calculated using trapezoidal rule with extrapolation to $t = \infty$

^{A,B,C} denotes C_{max} values that are not statistically different $p > 0.05$

^{D,E,F,G} denotes AUC values that are not statistically different $p > 0.05$

^{a,b,c,d,e,f} denotes C_{max} values that are statistically different $p < 0.05$

^{g,h,i,j,k,l} denotes AUC values that are statistically different $p < 0.05$

Table 2. Mean urinary concentration maxima of theanine achieved in six hours after oral administration. Values represent mean ± s.e.mean

Theanine Dose	C_{max} (μg ml^{-1}) *n=4*	
0.5 g kg^{-1} L-theanine	345 ± 70[a]	
0.5 g kg^{-1} D-theanine	4900 ± 1000[b]	
1.0 g kg^{-1} D,L-theanine	L-theanine	D-theanine
	992 ± 151[a]	6500 ± 800[b]

[a] denotes values that are statistically different $p < 0.05$

[b]denotes values that are not statistically different $p > 0.05$

Legends for Figures

Fig. 1. LC-ESI/MS chromatograms for blank and racemic theanine spiked plasma samples. (A) Total ion chromatogram (TIC) for blank plasma containing internal standard only. (B) and (C) Extracted ion chromatograms, m/z 175.0 (B) and m/z 153.0 (C), for a plasma sample spiked with D,L-theanine and internal standard, D^5-L-glutamic acid, respectively. RT and SN denote retention time and signal to noise.

Fig. 2. Plasma concentration-time curves of individually dosed L- and D-theanine after oral administration of 0.5g kg^{-1} and 0.25 g kg^{-1} doses. Values represent mean ± s.e.mean (n = 3). (A) (●) L-theanine 0.5 g kg^{-1} dose, (▪) L-theanine 0.25 g kg^{-1} dose (B) (○) D-theanine 0.5 g kg^{-1} dose, (□) D-theanine 0.25 g kg^{-1} dose

Fig. 3. Mean plasma concentration-time curves for racemic 1.0 g kg^{-1} D,L-theanine oral administration. Values represent mean ± s.e.mean (n = 3). (●) L-theanine, (▪) D-theanine.

Fig. 4. Comparison of mean plasma concentration-time curves for oral administration with and without the presence of the other theanine enantiomer. Values represent mean ± s.e.mean (n = 3). (A) L-theanine (●) L-theanine (0.5 g kg^{-1} L-dose), (▪) L-theanine in the presence of D-theanine (1.0 g kg^{-1} racemic dose) (B) D-theanine (○) D-theanine (0.5 g kg^{-1} D-dose), (□) D-theanine in the presence of L-theanine (1.0 g kg^{-1} racemic dose).

Fig. 5. Mean plasma concentration-time curves for racemic 1.0 g kg^{-1} D,L-theanine i.p. administration. Values represent mean ± s.e.mean (n = 3). (●) L-theanine, (▪) D-theanine.

Fig. 6. Mean plasma concentration-time curves for 0.5 g kg^{-1} individual i.p. administration of L- and D-theanine. Values represent mean ± s.e.mean (n = 3). (A) (●) L-theanine (B) (▪) D-theanine.

Fig. 7. Mean urine concentration-time curves for racemic 1.0 g kg^{-1} D,L-theanine oral administration. Values represent mean ± s.e.mean (n = 4). (●) L-theanine, (▪) D-theanine.

Fig. 8. Mean urine concentration-time curves for 0.5 g kg^{-1} individual oral administration of L- and D-theanine. Values represent mean ± s.e.mean (n = 4). (A) (●) L-theanine (B) (▪) D-theanine

Fig. 9. Mean urine concentration-time curves for racemic 1.0 g kg^{-1} D,L-theanine i.p. administration. Values represent mean ± s.e.mean (n = 3). (●) L-theanine, (▪) D-theanine.

Fig. 10. Mean ethylamine plasma concentration-time curves for 0.5 g kg^{-1} individual oral administration of L-, D-theanine, and 1.0 g kg^{-1} racemic theanine dose. Values represent

mean ± s.e.mean (n = 3). (A) (●) ethylamine from individual L-theanine dose and (▪) ethylamine from individual D-theanine dose (B) (▲) ethylamine from racemic dose.

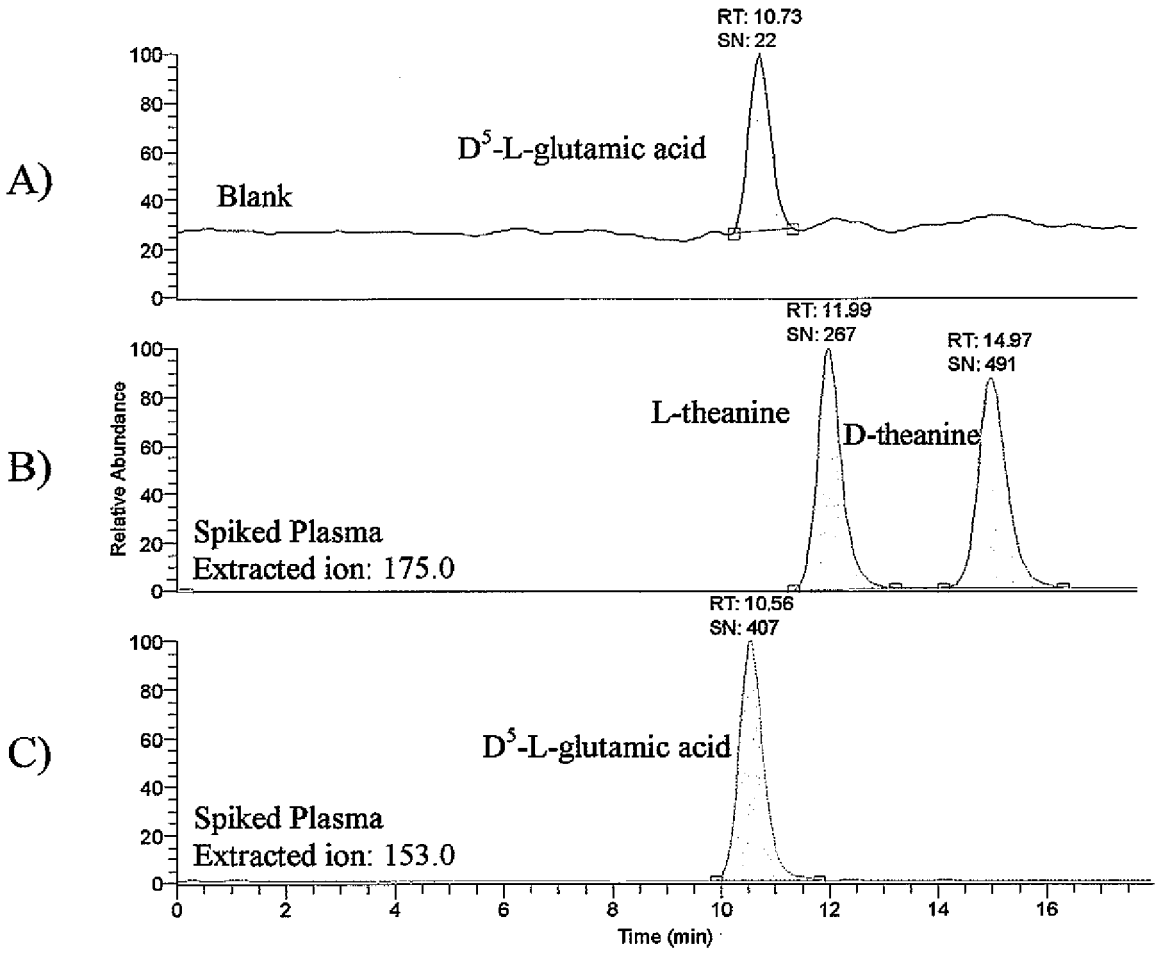
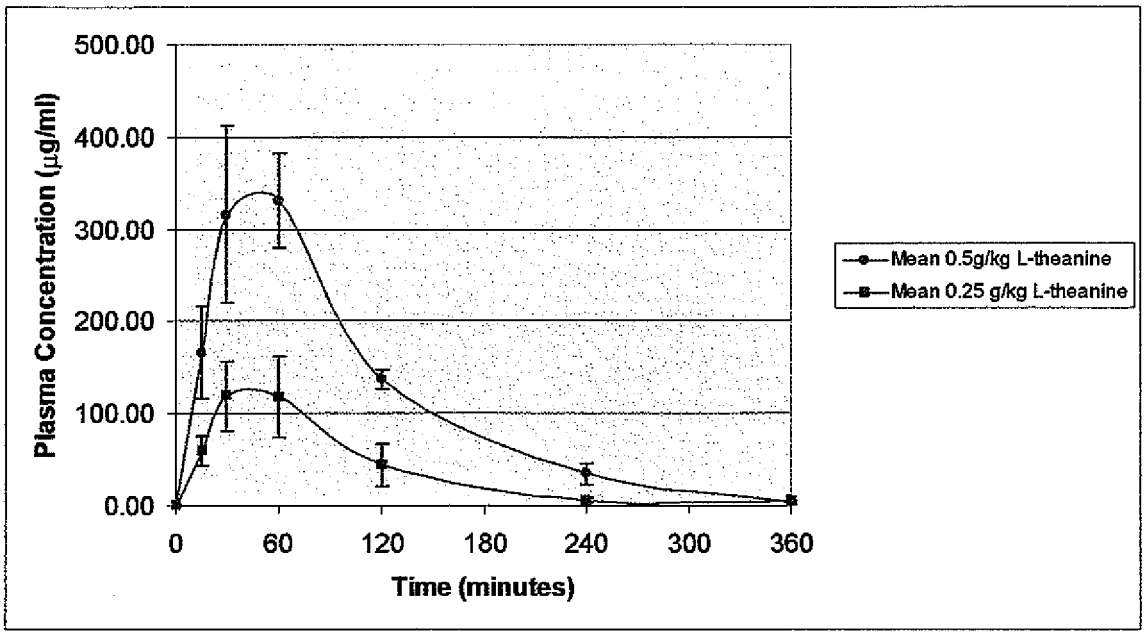

A)
RT: 10.73
SN: 22
D5-L-glutamic acid
Blank
B)
RT: 11.99
SN: 267
RT: 14.97
SN: 491
L-theanine
D-theanine
Spiked Plasma
Extracted ion: 175.0
Relative Abundance
C)
RT: 10.56
SN: 407
D5-L-glutamic acid
Spiked Plasma
Extracted ion: 153.0
Time (min)

Fig. 1.

A)


500.00
400.00
300.00
200.00
100.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)
Mean 0.5g/kg L-theanine
Mean 0.25 g/kg L-theanine

B)

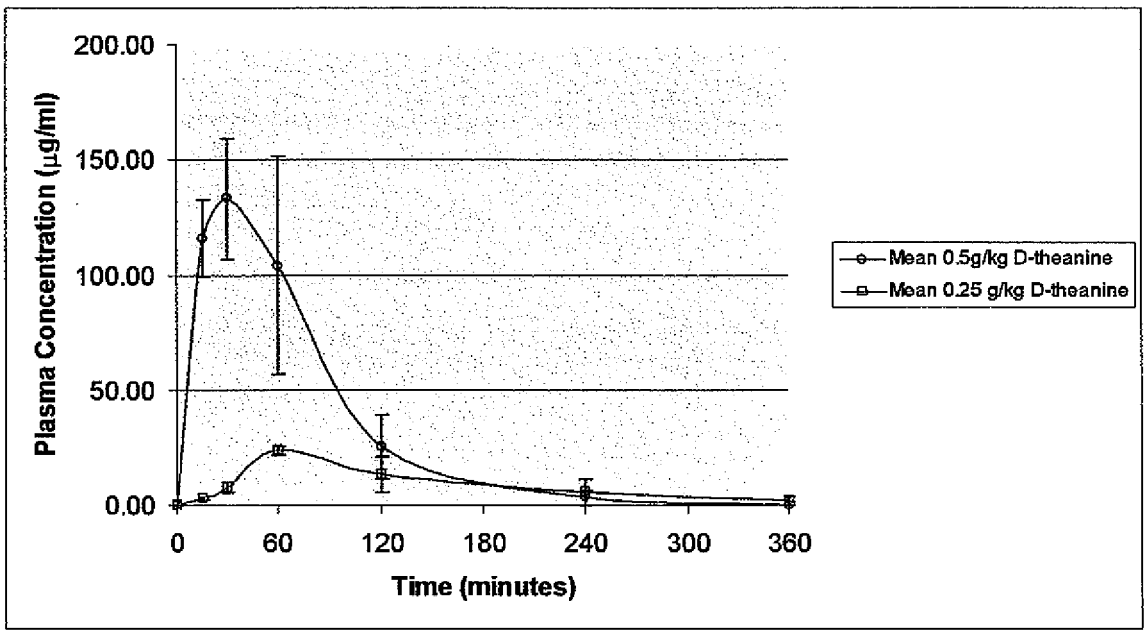

200.00
150.00
100.00
50.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)
Mean 0.5g/kg D-theanine
Mean 0.25 g/kg D-theanine

Fig. 2.

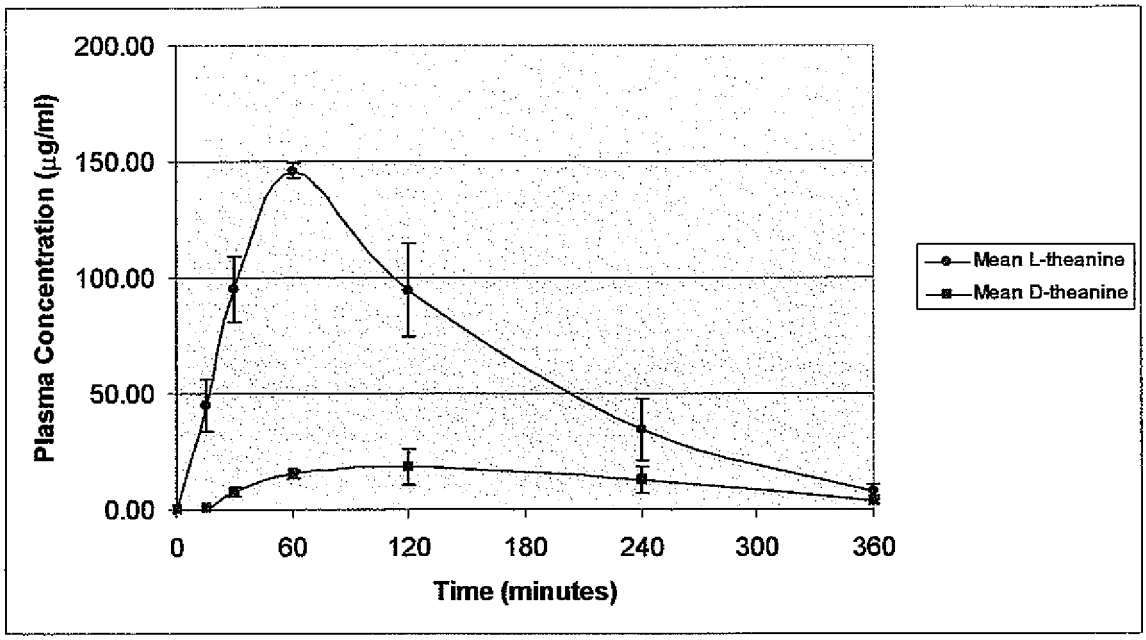
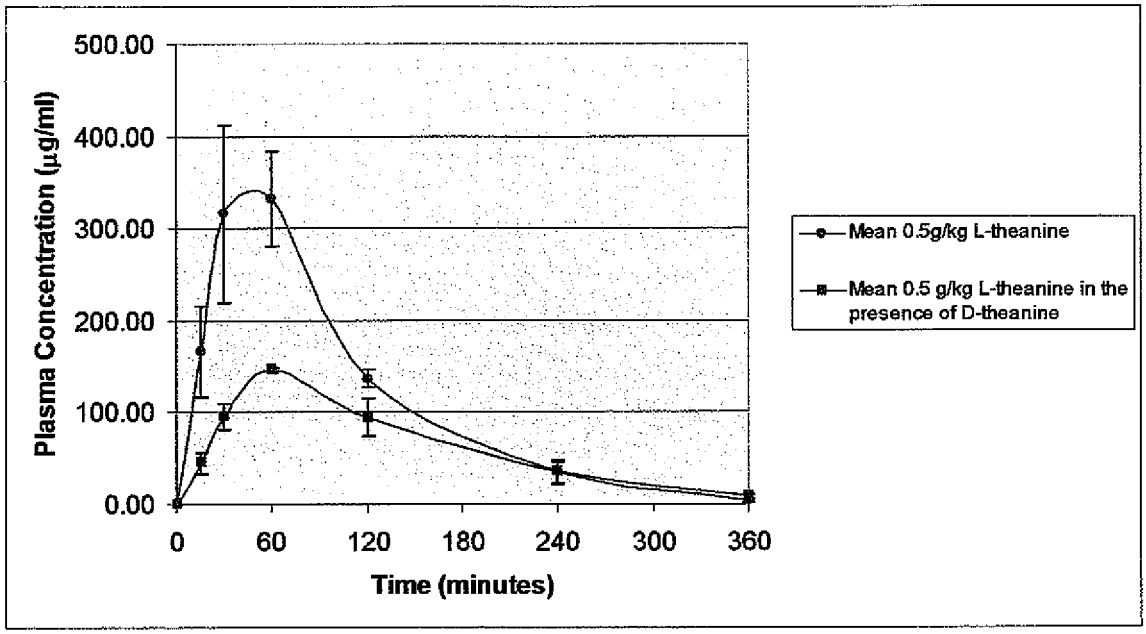

200.00
150.00
100.00
50.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)
Mean L-theanine
Mean D-theanine

Fig. 3.

A)


500.00
400.00
300.00
200.00
100.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)
Mean 0.5g/kg L-theanine
Mean 0.5 g/kg L-theanine in the presence of D-theanine

B)

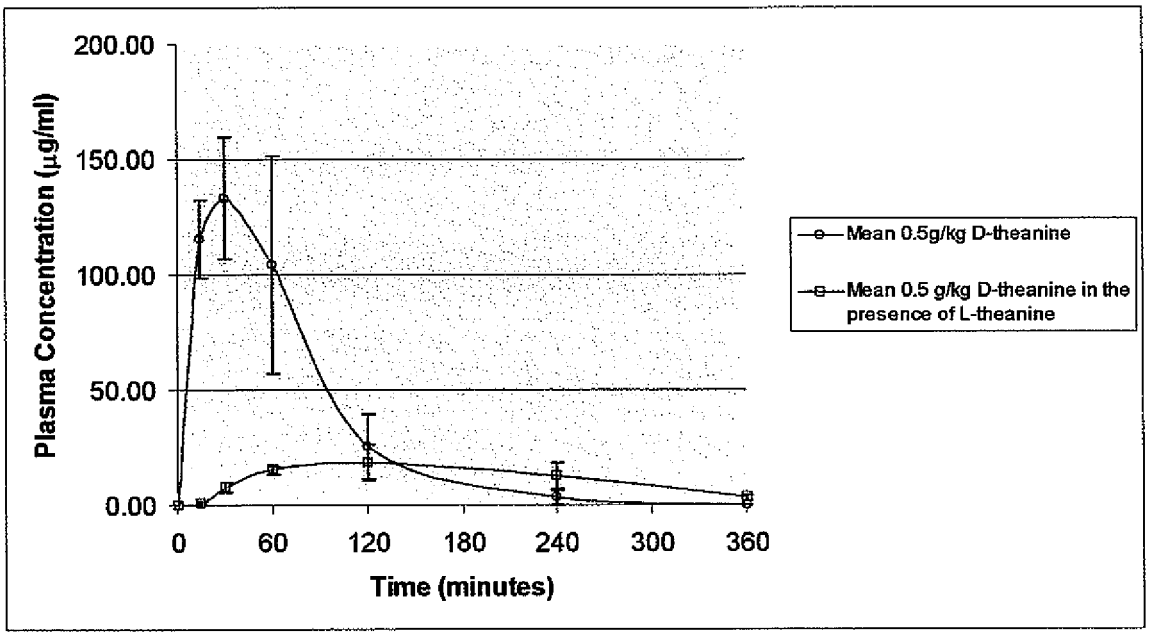

200.00
150.00
100.00
50.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)
Mean 0.5g/kg D-theanine
Mean 0.5 g/kg D-theanine in the presence of L-theanine

Fig. 4.

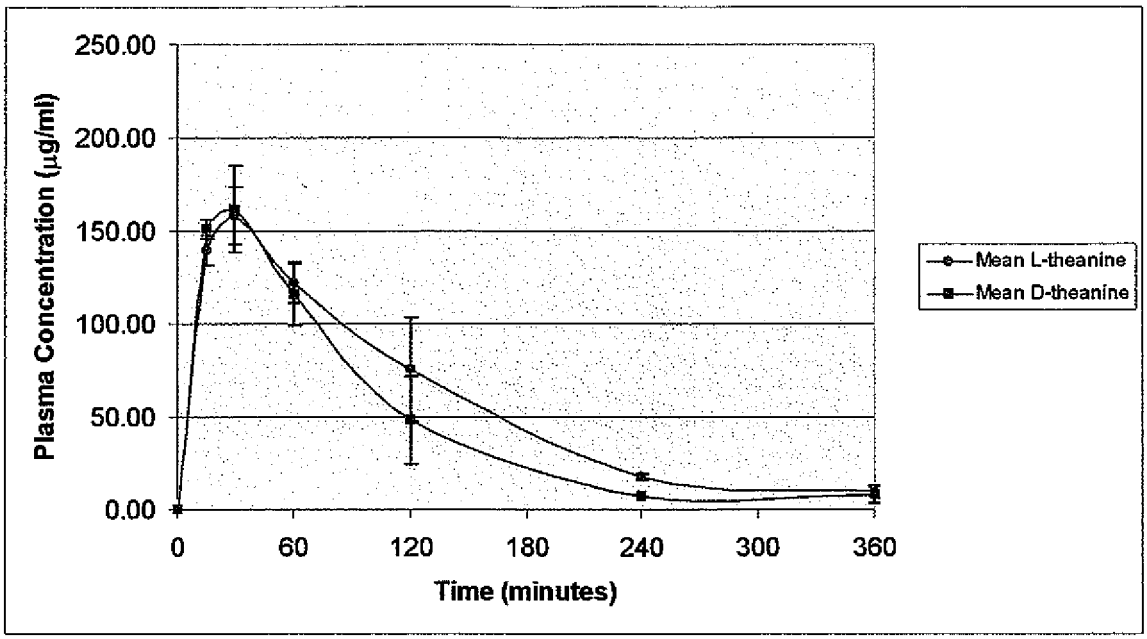
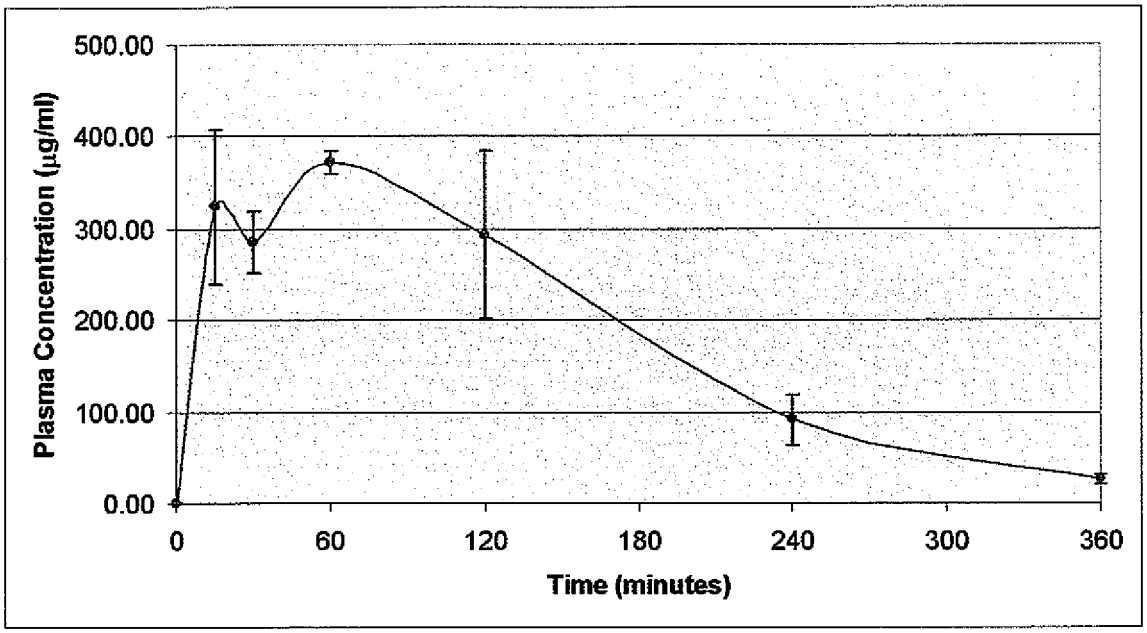

250.00
200.00
150.00
100.00
50.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)
Mean L-theanine
Mean D-theanine

Fig. 5.

A)


500.00
400.00
300.00
200.00
100.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)

B)

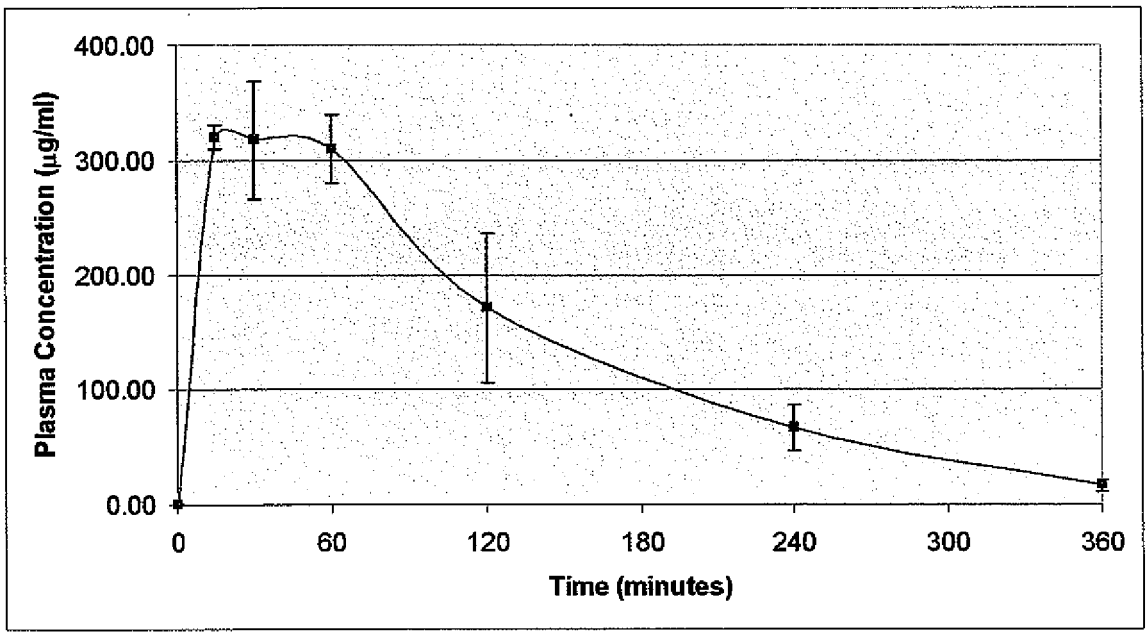

400.00
300.00
200.00
100.00
0.00
Plasma Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)

Fig. 6.

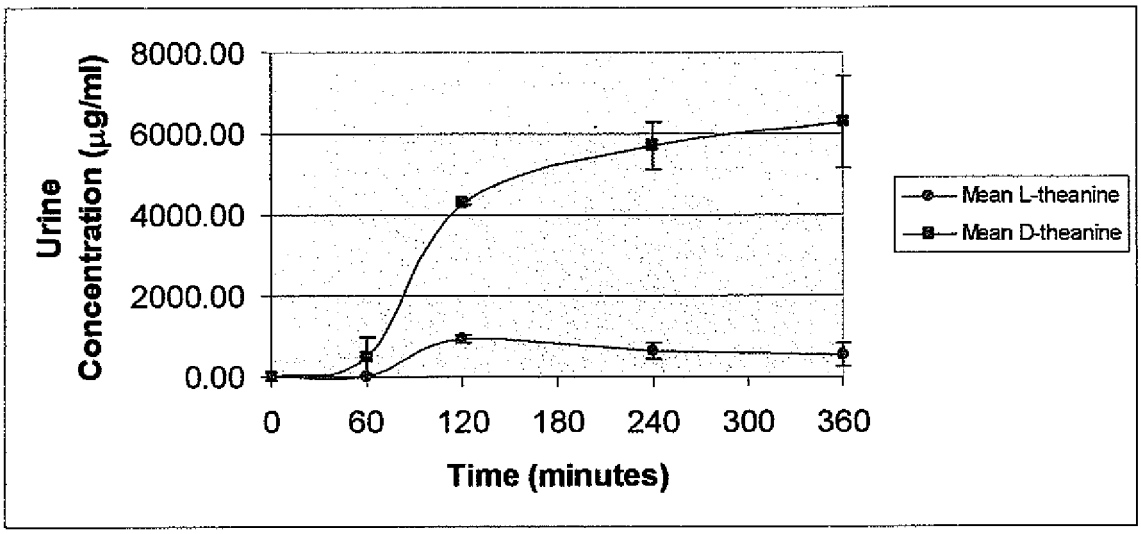
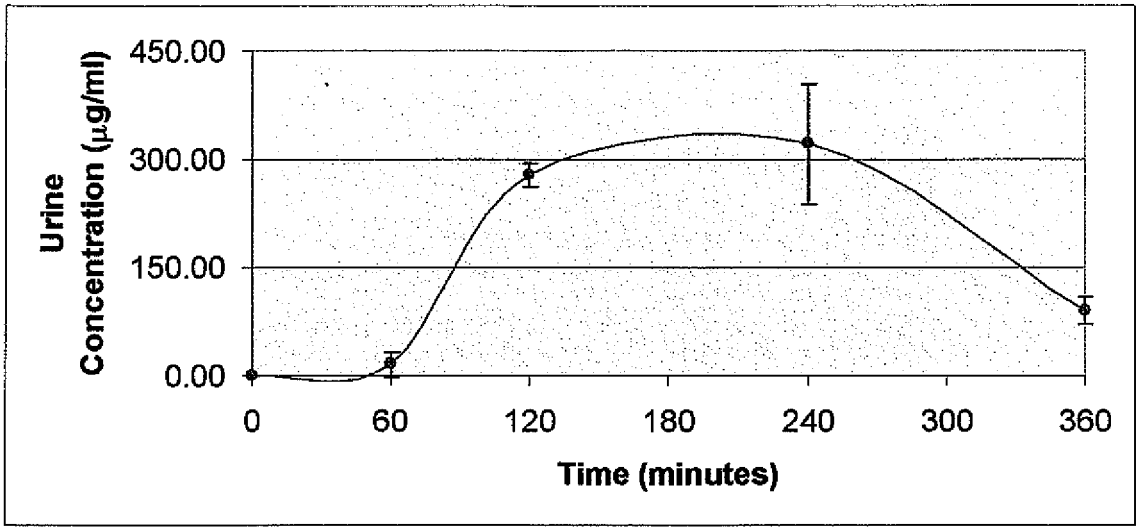

Urine Concentration (µg/ml)
8000.00
6000.00
4000.00
2000.00
0.00
0
60
120
180
240
300
360
Time (minutes)
Mean L-theanine
Mean D-theanine

Fig. 7.

A)


Urine Concentration (µg/ml)
450.00
300.00
150.00
0.00
0
60
120
180
240
300
360
Time (minutes)

B)

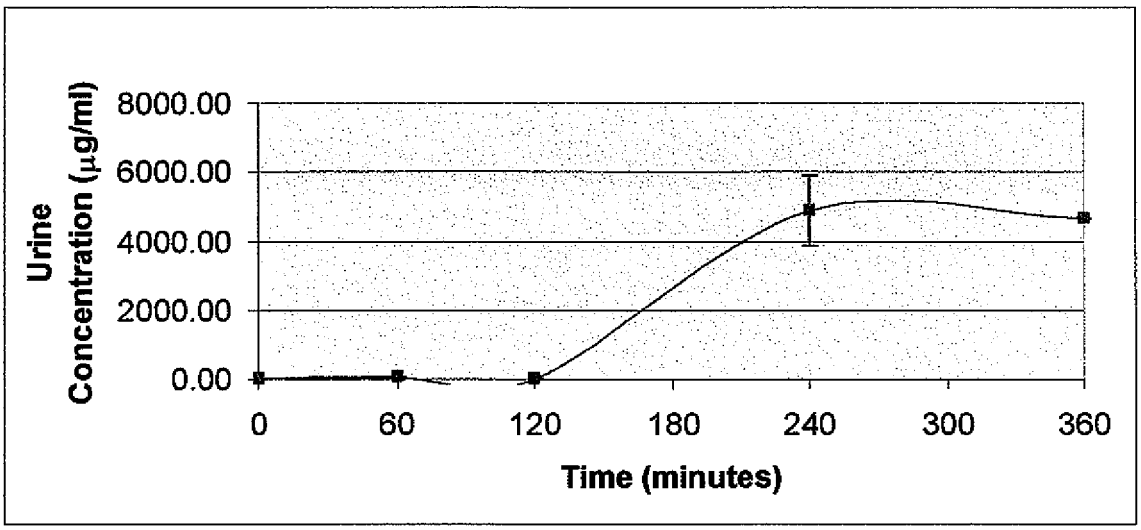

Urine Concentration (µg/ml)
8000.00
6000.00
4000.00
2000.00
0.00
0
60
120
180
240
300
360
Time (minutes)

Fig. 8.

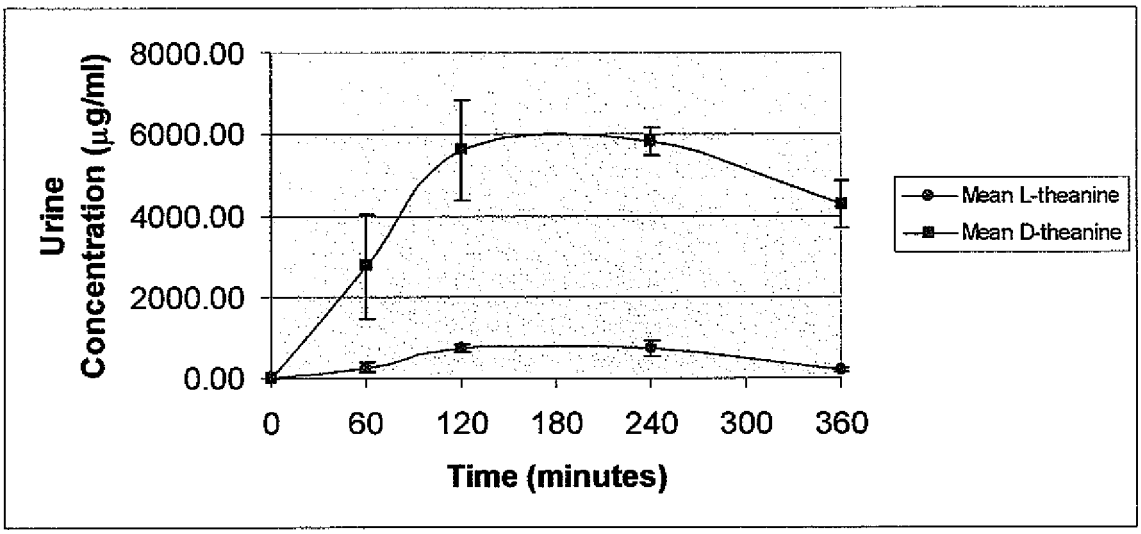
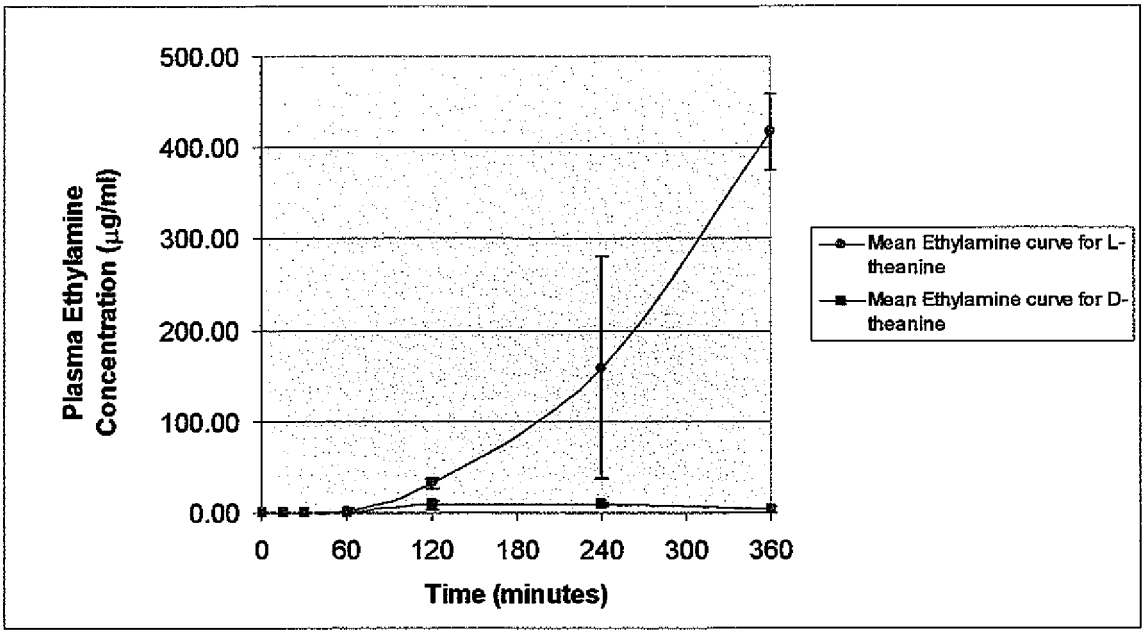

Urine Concentration (µg/ml)
8000.00
6000.00
4000.00
2000.00
0.00
0
60
120
180
240
300
360
Time (minutes)
Mean L-theanine
Mean D-theanine

Fig. 9.

A)


500.00
400.00
300.00
200.00
100.00
0.00
Plasma Ethylamine Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)
Mean Ethylamine curve for L-theanine
Mean Ethylamine curve for D-theanine

B)

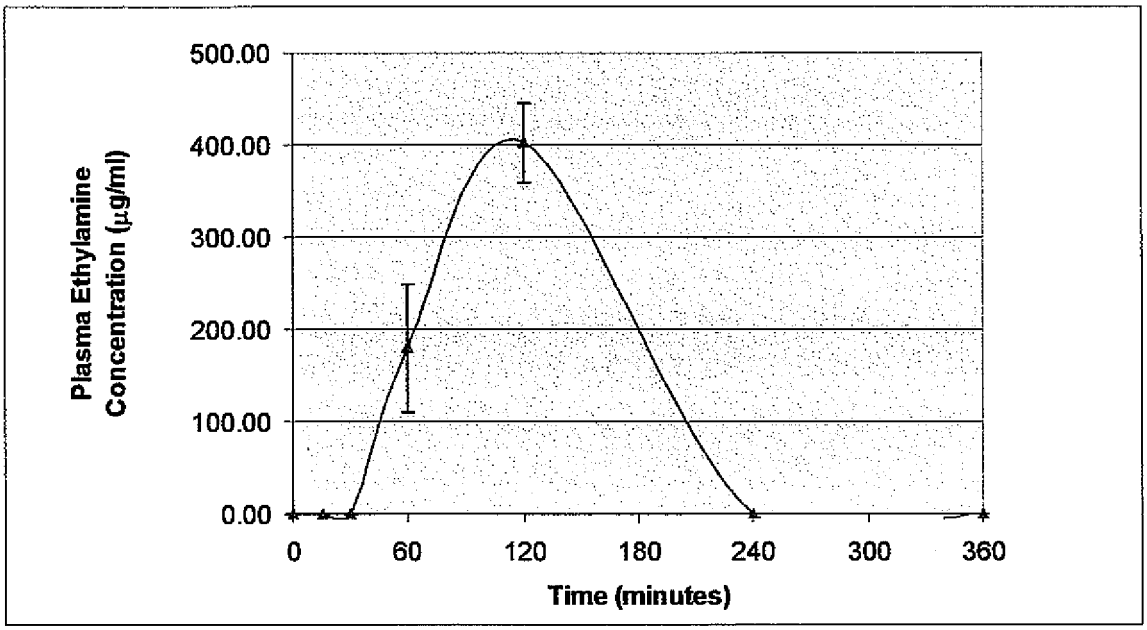

500.00
400.00
300.00
200.00
100.00
0.00
Plasma Ethylamine Concentration (µg/ml)
0
60
120
180
240
300
360
Time (minutes)

Fig. 10.

CHAPTER 7. GENERAL CONCLUSIONS

Separation, characterization, and determination of chirality have long been important criteria for pharmaceutical, biological, and industrial applications. The chiral nature of a compound can affect its function, efficacy, and bioactivity. The sensitivity and selectivity afforded by MS detection has greatly increased the interest in LC-MS methods for enantiomeric analysis.

The work in this dissertation described the development of chiral LC-API/MS methods for the sensitive separation and detection of small molecules. Additionally, the applications of these methods in the analysis of enantiomeric composition and pharmacokinetic and pharmacodynamic studies have also been described.

Our method development studies found that polar organic mobile phases were the most compatible with ESI-MS, providing low limits of detection. It was also shown that water-rich reversed phase methods decreased the ionization efficiency of ESI, while lower flow rates increased the sensitivity by a whole order of magnitude. Additionally, the Chirobiotic coupled column (RVT) was demonstrated to be an important tool for chiral LC-MS method development, providing for the rapid screening of chiral stationary phases.

Normal phase chiral method development proved to be more challenging than reverse phase or polar organic method development in that hexane/heptane mobile phases were completely incompatible with electrospray ionization. Ethoxynonafluorobutane (ENFB), an environmentally friendly fluorocarbon, was used successfully as a substitute for

hexane/heptane mobile phases. Its chromatographic properties were similar to those of hexane/heptane, however, ENFB had no flashpoint and low flammability allowing for facile, worry-free interfacing with APCI-MS detection. Separations using ENFB-substituted mobile phases had comparable resolutions and selectivities, however, the overall peak efficiencies were considerably lower using ENFB. Limits of detection and sensitivity were either comparable or better using ENFB-MS over the heptane-PDA method. Interestingly, flow rate did not affect sensitivity for APCI as greatly as was previously found for ESI-MS detection.

The use of APCI versus ESI was also evaluated for the reverse-phase separation of amino acids and small peptides. The APCI sensitivity of free amino acids was found to be greater than that of ESI at high flow rates (> 0.4 ml min^{-1}). Interestingly, for peptides with molecular weights greater than 300 Da, ESI afforded better sensitivity than APCI. The optimized high-flow APCI method was then used to enantiomerically separate all 19 native amino acids simultaneously in less than 20 minutes. The low limits of detection and excellent sensitivity of this method make it suitable for biological analysis.

Theanine, a free-amino acid found in tea leaves, was enantiomerically separated using the amino acid method previously developed. Theanine had been previously shown to have many pharmacological benefits including reduction of blood pressure and enhancement of chemotherapeutic activity of drugs such as doxorubicin. Using low flow rates the sensitivity of ESI was comparable to APCI for native theanine. Additionally, native theanine had better limits of detection and sensitivity than theanine derivatized with FMOC, AQC, or dansyl

reagents. The native theanine method was then used to evaluate the enantiomeric composition of six commercially available L-theanine products. It was found that five out of the six samples were actually racemic mixtures of both D- and L-theanine rather than the pure L-isomer.

Using the low-flow ESI theanine method, the pharmacokinetics and pharmacodynamics of theanine were evaluated *in vivo* with male Sprague-Dawley rats. The impetus for this study came from the previous finding that five out of the six commercial samples tested were actually racemic. The efficacy of these samples depended on the metabolic fate of D-, L- and/or racemic theanine. Oral administration data demonstrated that the intestinal absorption of L-theanine was higher than that of D-theanine, while i.p. administration provided for similar D-, and L-theanine plasma uptake. This illustrated that there seemed to be a competitive binding effect with respect to gut absorption. Regardless of route, oral or i.p., the presence of the other enantiomeric always decreased overall theanine uptake, demonstrating that there is also a competitive binding effect with respect to reabsorption. Additionally, the urinary data showed that D-theanine was eliminated with minimal metabolism, while the rate of L-theanine metabolism to ethylamine increased in the presence of D-theanine. Clearly, the bioactivity of D-theanine, L-theanine, and racemic D,L-theanine were very different, questioning the efficacy of the commercially available theanine products.

This dissertation described in detail the challenges and difficulties associated with chiral LC-MS method development and provided solutions for overcoming them. The significance of

these methods was clearly illustrated with the evaluation of enantiomeric composition and the pharmacokinetic/pharmacodynamic studies. The presence of more than one enantiomer can have a major impact on the utility of chiral drugs, nutraceuticals, additives and intermediates for synthesis. As the focus of pharmaceutical and industrial research and development continues to be on the formulation of novel chiral entities, the use of LC-MS will continue to evolve and flourish, eventually becoming a vital component of chiral analysis.

ACKNOWLEDGEMENTS

I have always been fortunate to have a mentor in every stage of my academic development. In high school, my algebra teacher, Mr. Ottman, inspired my love of problem solving. In college, my advisor, Dr. Miller, inspired my love of research. And now, in graduate school, Dr. Armstrong has inspired my love of science and what it means to be a good scientist. For four and a half years I have had the honor of learning how to develop and pursue research ideas under his tutelage. For that I will be forever indebted.

I would also like to thank Dr. Yeung, Dr. Houk, Dr. Lin, Dr. Phillips, and Dr. Sheares-Ashby for kindly serving on my committee and providing much appreciated guidance and direction. I would especially like to acknowledge Dr. Walter Hsu for his patience and support. He taught me to think analytically and motivated me to look at challenges from a biological perspective.

I want to thank the members of Dr. Armstrong's research group both past and present for their friendship and support. I would especially like to thank Cliff Mitchell, Jie Ding and Manmeet Gill for their cooperation on various projects. Cliff, you kept me sane and entertained, I will always cherish our friendship. Jie, you have been an amazing colleague and a wonderful scientist, I value our partnership immensely. Manmeet, I forced you to wake up early, but I couldn't have had a better collaborator on such an exciting project.

In addition to mentors and colleagues, a person's success is often a result of the strength and support of their family. I would like to thank my parents, Jay and Bharati Desai. They took a chance on coming to new world leaving their families behind, in order to provide more opportunities for them and their children. As a result of their guidance and unconditional love, my brother and I have achieved great successes in our lives. We owe everything that we are, and have become to them.

I also know that I would not be here today, if not for the love and support of my husband, Jason. When things got difficult, he pushed me, inspired me, and made me a better person and scientist. I am lucky to have not only a companion, but a colleague who always understands where I'm coming from. I thank him for simply being him, allowing me to be me, and loving me for who I am.

Finally, I would like to dedicate this dissertation to the memory of my Uncle Navin. Everyone who ever met him liked him immediately. He had a way of making one feel important and special. He had been a part of every one of my graduations except for this last one. And so I dedicate this to him, in hopes that in some way, this will make him a part of this one as well.