NOVEL ANALYTICAL METHODS TO INTERROGATE COGNITION AND PAIN IN THE CENTRAL NERVOUS SYSTEM

by

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(Under the Direction of Michael G. Bartlett)

ABSTRACT

To better study drugs, drug metabolites and endogenous biomarkers associated with the cognition and pain functions of the central nervous system (CNS), it is necessary to have sensitive, specific and robust analytical methods for the quantitation of these analytes, providing important information for studies on the synthesis, metabolism, pharmacokinetics and mechanism of actions. In this dissertation, a series of studies on novel analytical methods and applications to interrogate cognition and pain in the CNS were presented. Chapter 1 is the introduction and describes the layout of the dissertation. Chapter 2 is a literature review of sample preparation methods for quantitation of small-molecule analytes in brain tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS). Chapter 3 described the development and validation of an analytical method for the simultaneous quantitation of cotinine and three of its metabolites in rat plasma and brain tissue. In Chapter 4, the analytical method for cotinine and metabolites was applied to a pharmacokinetic study of cotinine in rats, in support of research on the pro-cognitive effects of cotinine. In Chapter 5, a rapid LC-MS-MS analytical method was developed for the quantification of paclitaxel in rat plasma and brain tissue, which was used for studies on the neuropathic pain caused by paclitaxel or other chemotherapeutic agents.

INDEX WORDS: Bioanalysis, Quantitation, High performance liquid chromatography, Mass spectrometry, LC- MS/MS, Plasma, Brain, Cognition, Pain.

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DEDICATION

I would like to dedicate this dissertation to my sweet and loving parents, my father Shubing Li and my mother Kun Lyu, for their dedicated love, support and encouragement to me in the past 26 years. I must thank them for giving me strength, confidence and faith to success, when I was facing difficulties and frustrations in scientific research. I would also like to thank my lovely girlfriend Linna Yan, for her company and spiritual support.

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CHAPTER 1

INTRODUCTION

The central nervous system (CNS) is the part of the nervous system consisting of the brain and spinal cord, which is responsible for the integration of information obtained from and the control of the activities of different parts of the body. As the primary organ in the CNS of higher animals, brain has multiple functions and control of a variety of complicated behaviors in human beings and animals. Cognition is one of the most primary functions of brain, which can be defined as a comprehensive mental process that includes attention, memory, language, calculating, reasoning, problem solving and decision making. Sensation of pain is another important function of the CNS, which is responsible for the perception of damaging stimuli to the body and plays a key role in the self-protection system of human beings and animals. Both cognition and pain are critical functions of the CNS, which have garner great attention and interest from scientific researcher in the fields of physiology, pathology and pharmacology, toxicology.

A lot compounds, including drugs, drug metabolites and endogenous biomarkers have closed interactions with the CNS. Information about the distribution of drugs, kinetics of drug metabolites and generation of endogenous biomarkers in the CNS is of great importance in revealing the mechanisms of the interactions between these molecules and the CNS. In order to study these topics, research on analytical methods for the quantitation of analytes of interest in the CNS has been of great interest in diagnostic, non-clinical and pre-clinical studies. Research on quantitative bioanalytical methods involves the effective extraction of analytes from the

complex biological samples, specific separation of analytes from interfering impurities, sensitive measurements of analytes with great precision and accuracy, complete method validation for reliability and robustness as well as applications in the real sample quantitation in non-clinical and pre-clinical studies.

In this dissertation, a series of different studies were demonstrated as novel analytical methods to interrogate cognition and pain in the CNS. Chapter 2 is a review of sample preparation methods for quantitation of small-molecule analytes in brain tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS). Literature published under this topic over the past two decades were reviewed, categorized and summarized to obtain important information for future method development work. Chapter 3 described the development and validation of a sensitive, specific and robust analytical method for the simultaneous quantitation of cotinine and three major cotinine metabolites in rat plasma and brain tissue. This method was used to support the studies on the pro-cognitive effects of cotinine and the development of new therapeutic agents for the treatment of Alzheimer's disease. In Chapter 4, this analytical method for cotinine and metabolites was applied to a pharmacokinetic study of cotinine in rat species, revealing important pharmacokinetic information for orally and intravenously dosed cotinine. In Chapter 5, a rapid LC-MS-MS bioanalytical method was developed and validated for the quantification of paclitaxel in rat plasma and brain tissue, which was applied to a study on the neuropathic pain caused by paclitaxel or other chemotherapeutic agents.

CHAPTER 2

LITERATURE REVIEW

A REVIEW OF SAMPLE PREPARATION METHODS FOR QUANTITATION OF SMALL-MOLECULE ANALYTES IN BRAIN TISSUE BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

Pei Li, Michael G. Bartlett. Accepted by *Analytical Methods*.
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Abstract

Concentration measurements are one of the most important and fundamental approaches in preclinical and clinical studies of small-molecule drugs, metabolites and biomarkers, since information about the absorption (drug), synthesis (biomarker), distribution, metabolism and elimination can be obtained by determining the concentrations of target analytes in biological fluids or tissue samples. Among all the bioanalytical techniques, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been widely used, due to its high sensitivity, selectivity and reproducibility. Attention has been paid to the quantitation of small-molecule analytes in brain tissue samples by LC-MS/MS, because the important information about brain concentrations obtained via such studies can be used to interpret the distribution and function of target chemicals in the central nervous system (CNS). In order to be analyzed by LC-MS/MS, brain tissue samples need to be properly obtained and carefully prepared into an LC-MS/MS compatible form. The choice made here will which greatly influence the sensitivity and robustness of the method. As a result of the vital function and complex composition of brain tissue, sample collection and preparation can be very challenging. In this review, we summarize the current techniques for the collection and preparation of brain tissue samples, which can be used as a reference for future method development for quantitation of small-molecule analytes by LC-MS/MS.

Key words

Review, LC-MS/MS, Brain tissue, Sample preparation

1. Introduction

Bioanalysis, defined as the quantitative measurements of xenobiotics and biotics in biological matrices, has been of great importance in drug discovery, research and development, because of the crucial information it provides on drug absorption, distribution, metabolism and elimination (ADME). Though the development of large-molecule drugs and biomarkers has been rapidly growing recently, small-molecule drugs, which account for over 90% of FDA-approved drugs according to the DrugBank 3.0 database, as well as small-molecule drug metabolites and biomarkers are still the most studied analytes in bioanalysis. Different techniques have been used for the quantitative bioanalysis of small molecules, including liquid chromatography with ultraviolet detector (LC-UV), liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), among which liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been the most widely used and most reliable tool, due to its high sensitivity, specificity, precision and accuracy. Typically, a high performance liquid chromatography (HPLC) or ultra high performance liquid chromatography (UHPLC) system is used to separate the analytes from the processed biological matrices based on the specific interactions between the analytes and the analytical LC column. The LC eluent is then directly introduced to a mass spectrometer for the detection and quantitation of the analytes, using a multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) function. During this process, collision-induced dissociation (CID) is often used to generate fragment ions from the precursor ion, which provides a specific precursor-product ion transition (or multiple ion transitions) for the instrument to record, integrate and quantify. With the specificity and sensitivity provided by both the LC column and the MRM function, LC-MS/MS can serve as one of the most suitable tools for small-molecule quantitation in most scenarios.

Quantitative bioanalysis of small-molecule drugs or biomarkers by LC-MS/MS has been studied and applied with different types of matrices, especially those related to drug ADME or organ-specific biomarkers, including different types of body fluid (*e.g.* plasma, serum, whole blood, saliva, tears and urine) and organ tissue (*e.g.* kidney, liver, lung and brain tissue). Unlike pure standard solutions or drug formulations, biological samples usually have much more complex biochemical compositions, containing various components like salts, organic small molecules, fibers, proteins and lipids, which may cause a series of issues including LC column degradation, mass spectrometer contamination, signal interference, and most importantly, matrix effects. Matrix effects represent a phenomena of enhancement or suppression of analyte ion intensity caused by coeluting matrix components.[1] Therefore, in order to improve the sensitivity, selectivity and reproducibility while measuring analytes in biological samples, sample preparation, also known as sample pretreatment or sample cleanup, is needed before LC-MS/MS analyses in most occasions. Sample preparation can be considered a pre-analytical separation process in bioanalysis, which mainly involves selective isolation of analytes of interest from the matrix, minimization or elimination of matrix components in processed samples and, if required, enrichment of analytes. Based on the analyte properties and matrix complexities, different sample preparation techniques have been developed, such as dilute-and-shoot, protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE), which are the most traditional and most commonly used ones.[2] Recently, to cope with the demand for improved selectivity, sensitivity and regulations, combinations of these techniques and other innovative sample preparation strategies are becoming increasingly used in bioanalytical practices. An ideal sample preparation method should be able to reduce biological matrices to minimal levels while maintaining the recovery of analytes above 80%. However, due to the

numerous factors affecting matrix removal and analyte extraction (e.g. choice of solvents, ion pairing agents, temperature and buffer pH), the developing work for sample preparation can be very difficult, tedious and labor-intensive, which makes it one of the most significant parts in the development of a whole analytical method. With optimized sample preparation methods and LC-MS/MS conditions, small molecules in biological samples can be measured sensitively, specifically, precisely and accurately.

Among all the common biological matrices studied in bioanalysis, brain tissue has been drawing significant attention from researchers over the last decade. As a specific organ of higher animals, the brain has multiple functions in different brain regions to control a variety of complicated behaviors in human beings and animals. Thus distributions of small-molecule drugs, metabolites or biomarkers in brain tissue are of great pharmacological or physiological importance, due to their direct impact on such brain functions as information processing, body movement control, homeostasis and memory.[3] Quantitative studies of small molecules in brain tissue can serve purposes such as: (1) to study the brain tissue distribution (pharmacodynamics) of drug candidates or different formulations; (2) to study the brain tissue pharmacokinetic profiles of drugs or metabolites; (3) to measure biomarker levels in the brain tissue; and (4) to evaluate the toxicity of chemicals on the brain.

As a special organ with unique functions and anatomy, the brain has a special matrix composition that is different from all other organs, which makes it more challenging for sample preparation development. The biggest challenge for brain tissue analysis is its high lipid composition, which constitutes about one-half of the dry weight of brain tissue. Like other tissues, the brain contains phospholipids, sterols and sphinggolipids; while many other complex lipids, including gangliosides, cerebrosides, sulfatides and phosphoinositides, are also highly

enriched compared to other tissues.[4, 5] Due to the nonpolar phosphate ester group and the long alkyl chain, lipid molecules are usually highly hydrophobic, and may be extracted together with the analytes when sample preparation methods based on hydrophobicity are used. These lipids have a chance of coeluting with the analytes of interest from the LC column and entering the MS. All of the lipids in the brain, most of which are permanently or easily charged in the ion source, generally cause serious matrix effects, especially when an electrospray ionization (ESI) ion source is used. Glycerophosphocholines, a class of lipid molecules with high abundance in the brain tissue, have been specifically believed to broadly cause serious matrix effects in LC-MS/MS analyses.[6] Meanwhile, other components like salts, proteins and carbohydrates in brain tissue can also introduce interferences to the detection of analytes by LC-MS/MS, not only by causing matrix effects, but also by causing other issues, including but not limited to low extraction efficiency, peak distortion and signal interferences in MRM detection. At last, just as all the tissue samples, brain tissue samples need to be properly collected and prepared into an operable physical state, solutions or homogenates in most occasions, in order to be processed in sample preparations and LC-MS/MS analyses. Brain homogenates are problematic due to blockages of the LC system and the irreversible adsorption of impurities on the stationary phase, resulting in elevation of the column backpressure or decreases in column performance, and therefore reduced robustness or even failure of the method.[7]

Due to the fact that LC-MS/MS is a type of off-line analytical technique that can only analyzed injected liquid samples, biological samples cannot be directly analyzed *in-situ* and, instead, must be acquired from the tested individual prior to the analysis. Solid brain tissue samples must be properly processed into a liquid state in order to be analyzed by LC-MS/MS instruments. Therefore, the general concept of sample preparation for bioanalysis of brain tissue

samples should include sample acquisition, pretreatment and the traditional concept of sample preparation. Traditional sample preparation in a narrow sense represents for one or a series of chemical or physical sample cleanup processes that may involve extraction, separation, derivatization, enrichment and many other techniques. In addition to obtain and prepare samples into an LC-MS/MS compatible form, sample preparation is of great important for brain tissue by removing impurities that may cause interference, ion suppression, column congestion and instrument contamination. In fact, the sensitivity, selectivity and reproducibility of an LC-MS/MS method are largely determined by the instrumentation, leaving the sample preparation even more critical in method development. Different techniques and strategies for sample preparation can be used by the analyst to gain more leverage in the optimization of the bioanalytical method.

Based on the significance and challenges faced in the bioanalysis of brain tissues, it is necessary to look into current techniques and strategies in sample preparation, which will be of great instructional value for future method development work. The goal of this review is to summarize published sample preparation methods in quantitation of small molecules in brain tissue by LC-MS/MS. All currently available methods and techniques for each step of sample preparation will be introduced and summarized in terms of their advantages and disadvantages.

2. Sample Collection

While samples from some biological fluids such as plasma, serum and CSF can sometimes be directly analyzed with LC-MS/MS, brain tissue samples, however, must be properly sampled and/or processed into a liquid form that can be analyzed by LC-MS/MS. In order to obtain a liquid sample from the brain tissue, homogenization, microdialysis, ultrafiltration and solid-phase micro extraction are the major techniques involved.

2.1 Homogenization

Since brain is a solid organ located in the skull of most tested animals, the most direct way of tissue sampling is to obtain the whole brain or a certain region of brain tissue by surgical disection after sacrifice, which is the most widely used approach in all published methods.[7-92] High throughput and low equipment cost are the most significant advantages of homogenization. The biggest disadvantage of homogenization is that the test animal has to be sacrificed to yield one data point, which means that multiple animals are need for experiments designed for multiple time points.

Several significant issues need to be highlighted in brain tissue homogenization. The excised brain tissue needs to be frozen at -80 °C or kept on ice in order to maintain a low temperature for the freshness of brain tissue as well as the stability of analytes. When the whole brain was excised from the tested animal, there is a small amount of blood left in the blood vessels in the brain. The analyte distributed in the circulatory system may exist at a high concentration in the dwelling blood in the brain tissue, which may raise the measured brain tissue concentration, especially when the analyte has poor blood-brain barrier (BBB) permeability and therefore a low brain tissue concentration. In order to remove the extraneous blood to achieve a more accurate measurement of the analyte concentration in the brain tissue, the tissue sample can be rinsed in cold physiological saline or PBS (phosphate buffered saline) buffer solutions.[28, 30, 40, 47, 56, 66, 78, 83] After the excess liquid is wiped off, the brain tissue sample can be weighed for quantitative purposes. However, when the analyte is very hydrophilic and water soluble, one should be extra careful with the rinsing step, because longer exposure to aqueous solutions may wash away analytes in the brain tissue and cause the measured concentrations to be lower than the actual values.

After being obtained from the test animal and the excess blood removed, solid brain samples will be homogenized into a liquid form. Homogenization is a process that uses mechanical power to disrupt the brain tissue and disperse it into a certain solvent or solution, so as to form a relatively stable suspension for further sample preparation. Also, spiked samples in the method validations are prepared by the addition of a known amount of analytes into blank matrices, so only liquid samples can be mixed well with the reference standards.

Based on the mechanism of action, tissue homogenization can be fulfilled by different types of techniques, including grinding, rotating blade, bead beating and ultrasonication.[93] Due to the softness of brain tissue, almost all the mechanical homogenization techniques mentioned here can be used for the preparation of brain tissue without extra treatment. Though it has a low throughput and high labor intensity, manual grinding with a Dounce homogenizer (a.k.a. Potter-type homogenizer) is still used in some methods, mainly due to its low cost and high availability.[17, 28, 44, 57, 60, 61, 66] Among all these homogenization methods, rotating blade is the most commonly used one for brain tissue samples, due to its low cost and easy operation.[19, 21, 22, 31, 36, 38, 40, 41, 43, 75, 77, 79, 81, 84-86, 90] However, extra attention needs to be paid to the cleanliness of the blades, otherwise contamination can be a possible issue with the traditional rotating blade homogenizer. Bead beating [15, 55] and ultrasonication [8, 24-26, 39] are both newer techniques that have the advantages of higher efficiency, higher throughput as well as lower chance of contamination when compared to the traditional grinding or rotating blade homogenization techniques, making these two new techniques more popular in recent studies. In addition, pulverization is another highly efficient homogenization method. In a paper published by Golovko et al. in 2008, brain samples were snap frozen in liquid nitrogen and then pulverized to a fine homogeneous powder.[17] The brain tissue powder could be extracted directly by organic solvents due to the

increased surface area of the brain tissue, making this homogenization protocol of great advantage with high efficiency. Though different techniques have been used for the homogenization of brain tissue samples, the purpose and the results are always to form a homogeneous suspension for further steps of sample preparation.

Another important factor affecting the outcome of homogenization is the media that a brain tissue is dispersed into, which can be pure water, organic acids, aqueous buffers, organic solvents or mixtures of more than one of these agents. Due to the simplicity of the method, water has been the most commonly used homogenization media.[13, 14, 19, 21, 22, 35, 40, 41, 43, 49, 50, 57, 70, 71, 74, 75, 77, 80, 81, 84-86, 88, 90] In these methods, to one unit weight of brain tissue, at least one to two volumes of ice-cold water is add for homogenization, forming a homogeneous suspension of diluted brain tissue. In some studies, more water is added to yield a more diluted brain homogenate, which can be treated similarly to plasma or even serum samples in sample preparation, making it possible to use the same method for multiple sample species.[71, 75] Other than the low cost and simplicity of preparation, another major advantage of using water as the homogenization media is the lysing effect resulting from the low osmotic pressure of pure water. When samples are lysed by water and homogenized by mechanical forces, it can be considered that the suspension is a uniform mixture of both the intracellular and extracellular contents of brain tissue.[40]

Instead of pure distilled water, there are also many methods using aqueous solutions as the homogenization media.[8, 16, 18, 20, 27, 29, 31, 33, 36, 44, 45, 47, 52, 54-56, 58, 59, 61, 63, 67, 69, 79, 82, 83, 87, 91, 92, 94] Organic acids, formic acid or acetic acid in most occasions, are common components of aqueous homogenization media, due to their ability to facilitate the breakdown of cell membranes as well as adjusting the pH of the resulting homogenate for the ionization/deionization or stability of the

analyte.[18, 36, 58, 61, 82, 91] Inorganic acids such as trichloroacetic acid (TCA)[54, 79] or perchloric acid (PCA)[52] solutions are also common aqueous homogenization media. In addition to lowering the pH, such strong acids can also irreversibly denature and therefore precipitate the proteins in the brain tissues samples. As a result, homogenization and protein precipitation of brain tissue samples can be combined together in one step, followed by centrifugation to finish the tissue sampling as well as the rough sample preparation. The supernatant can be neutralized and directly injected into the LC-MS/MS system or further processed by other sample preparation methods. Aqueous buffers made from salts and other additives are also common homogenization media used in brain tissue sample preparation.[8, 16, 20, 27, 29, 31, 33, 44, 45, 47, 55, 56, 59, 63, 67, 69, 83, 87, 92, 94] Buffered media are usually aqueous solutions of a weak acid and its conjugate base or a weak base and its conjugate acid, which can maintain the pH value of the solution at a certain level. By maintaining the osmolarity, ionic strength and pH value similar to the physiological conditions of biological samples, most buffer solutions used as homogenization media can dilute the tissue homogenate without changing the solution conditions or disrupting protein integrity, so that the distribution, charge state and solubility of the analytes are not drastically changed. Tris-HCl buffer is one of the most common homogenization buffers used in the current literature, with concentrations ranging from 5 mM to 100 mM.[8, 27, 33, 59, 87] The effective pH range of Tris-HCl buffer is between 7.07 and 9.07, so the physiological pH of 7.4 can be well maintained with the use of this buffering system, while a slightly basic pH can also be reached when needed. In a study about the determination of two endogenous isoprenoids, farnesyl-(FPP) and geranylgeranylpyrophosphate (GGPP), Tris-HCl buffer with pH 8.5 was used as the homogenization media together with phosphatase inhibitors, which was designed to neutralize the analytes for a hydrophobicity-based solid-phase extraction (SPE) process as well as to protect

the analytes from hydrolysis. Normal saline or phosphate bufferred saline (PBS) solutions are also common buffer solutions for brain tissue homogenization, because of their easy preparation, low cost, and, most importantly, being isotonic and non-toxic to cells.[20, 47, 56, 63, 67, 83, 95] However, non-volatile ions in Tris-HCl, saline, PBS and many other common buffers can cause a series of fatal issues for the mass spectrometry, including ion suppression, high base line levels and serious instrumental contamination. Therefore, if no further extraction is involved to separate the analytes from the ions, special attention needs to be paid to the LC separations of brain tissue homogenate made with such aqueous buffers, in order to make sure that the unwanted ions are identified, separated and diverted from the mass spectrometer. Considering this issue, aqueous buffer solutions containing volatile salts are widely used as tissue homogenization media, including ammonium formate and ammonium acetate as the most used ones.[16, 31, 92] These two buffer salts are composed of weak acid/base functional groups and, more importantly, are volatile under high temperature so that they can be evaporated, ionized and transported in the mass spectrometer without causing serious ion suppression or instrument contamination. Besides these common buffer systems used as homogenization media, there are many types of buffers used in brain tissue homogenization, which are more analyte specific.

In addition to pure water or aqueous solutions, organic solvents are also used in brain tissue homogenization in many studies.[9, 11, 15, 24-26, 28, 30, 32, 34, 38, 39, 42, 53, 68, 76] To be used as homogenization media, the organic solvent needs to be miscible with water and hydrophobic enough to compromise interactions between protein molecules. Due to their abilities to disrupt protein structures and therefore precipitate proteins, the use of organic solvents as homogenization media, which is similar to the use of TCA, can be combined with homogenization. Usually, at least two volumes of organic solvents are added to each unit weight

of brain tissue; while the more added, the more thorough the precipitation will be. Methanol (MeOH) and ethanol (EtOH) are the most commonly used organic solvents as the homogenization media of brain tissue, due to their low cost, and most importantly, their moderate hydrophobicity and precipitating strength.[11, 15, 34, 42, 76] When brain samples are homogenized with the addition of MeOH or EtOH, brain tissue will be dispersed into the media and form a suspension of flocculent structures, due to the protein precipitation. Acetonitrile (ACN) was another common organic solvent used as a homogenization media.[15, 34, 42, 76] It has a stronger precipitating ability and therefore can form precipitated pellets right after the homogenization. Differently from aqueous homogenization media that cannot precipitate brain tissue, brain homogenate prepared with precipitating organic solvents is unstable and thus hard to pipette with high volume accuracy. Therefore, the amount of brain tissue needs to be measured at the beginning of the experiment and the whole homogenate has to be used in all the following steps. Aliquots can only be taken after the homogenate is processed into a stable solution by centrifugation or extraction. In a study about the quantitation of dihydroetorphine in rat plasma and brain published by Ohmori in 2000, two volumes of MeOH was used in the homogenization of brain tissue weighed beforehand. After centrifugation, the supernatant was separated and used as the sample instead of the brain homogenate. In this case, the brain homogenate supernatant could be accurately transferred and further processed.

In order to utilize the properties of buffer systems as well as organic solvents, a mixture of more than one of these agents can be used as the homogenization media.[10, 23, 48, 60, 64, 66, 78] Instead of using pure organic solvents that have very strong precipitating abilities, a mixture of organic solvents (ACN or MeOH) and water (or aqueous solutions) was used as the homogenization media, so that the proteins in the brain tissue were not drastically precipitated

and, therefore, the resulting homogenate was more stable. Moreover, the presence of water in the homogenization media can make it possible to add ionic or polar additives, inorganic salts or acids for example, which otherwise are not soluble in pure organic solvents.

With the leverage of choosing proper solvents or solutions as the homogenization media, brain tissue can be uniformly dispersed to achieve ideal analyte recovery and method precision and accuracy. Nevertheless, low-concentration additives are also quite commonly used in the homogenization media to add unique effects to the homogenate. Since most extraction techniques are based on hydrophobic interactions or ion-exchange mechanisms, the charge states of the analytes are of great importance in the sample preparation process, which means that the pH value of the initial tissue homogenate needs to be well controlled in a small range to maintain the analytes in the intended charge states. As mentioned with the buffered homogenization media, acids and bases are common additives for adjusting the pH of the resulting homogenate. When hydrophobic extractions or reversed phase liquid chromatography (RPLC) are used in sample preparation, the analytes need to be neutralized; when ion-exchange extractions or hydrophilic interaction liquid chromatography (HILIC) are used, the analytes need to be positively or negatively charged, according to the properties of the analytes. To facilitate such chromatographic separations, the change of pH by the addition of acid or base can be used to alter the charge states of the analytes. In a study published by Wu and coworkers, an LC-MS/MS method was developed for the determination of rat plasma and tissue concentrations of melamine, a compound with multiple primary and tertiary amine groups.[79] The brain tissue was homogenized in 1% TCA, providing not only a mild protein precipitating environment, but also an acidic condition. Therefore the analyte was ionized by receiving protons and further separated by mixed-mode cation exchange (MCX) solid phase extraction (SPE). In addition to acid or base

used to adjust pH, there are also many other additives used in the homogenization media mainly for the stability of analytes. Enzyme inhibitors are common additives used to inhibit the activities of enzymes that can catalyze the decomposition or transformation of the analytes. Sodium fluoride (NaF) has been widely used as an esterase inhibitor. In a study for the determination of heroin in brain tissue, 4 mg/mL of NaF was added in the brain tissue homogenate to minimize the deacetylation of heroin, together with low pH and low temperature.[31] In another study for the determination of irinotecan and 7-ethyl-10-hydroxycamptothecin (SN-38), Goldwirt and coworkers added 100 mM zinc sulfate ($ZnSO_4$) to the brain tissue homogenate as a carboxylesterase inhibitor to preserve the analytes from degredation. In addition to enzymes, pH values and metal ions are also important for the stability of some analytes. In a study published by Najmanova et al., an LC-MS/MS method was developed for the determination of dopamine and serotonin in brain tissue, in which HCl and EDTA were added to increase the stability of the analytes. Dopamine and serotonin are unstable and oxidize rapidly, especially in a strong alkaline medium. Therefore the addition of HCl to the brain tissue led to the stabilization of dopamine and serotonin in their hydrochloride form. Furthermore, biogenic amines and their metabolites are sensitive to light. They are easily oxidized in the presence of transition-metal cations such as Fe^{2+}. Thus it was necessary to store the samples in the dark with the use of the chelating agent EDTA.

With the proper homogenizing instruments, media and additives, brain tissue can be processed into a relatively stable and homogeneous suspension, which should also fit the properties of the analytes as well as the following sample preparation methods. However, one of the biggest concerns with brain tissue homogenization was the difference between spiked samples and real biological samples, which may affect the credibility of the measured extraction

recovery. Real biological samples are body fluids or organ tissue samples directly obtained from the animal. If the analyte is a drug in its original form, its presence in the real biological is a refection of its natural absorption, distribution, metabolism and elimination (ADME); other analytes as drug metabolites or endogenous biomarkers are generated by the test animal and exist in the biological samples based on biosynthesis or metabolism. However, samples with known concentrations have to be used in the method development and validation experiments, so that recovery, stability and accuracy can be accessed against nominal concentration values. In this case, spiked samples are used as standards and quality control (QC) samples in method validations, which are made by adding a known amount of analytes to blank biological matrix to yield an artificial biological sample of a known concentration. When it comes to tissue samples, concerns arise that the recovery of spiked samples may not accurately reflect that of the real samples, since the distribution of spiked analytes could be different from that of the natural samples. Moreover, if the internal standards (IS) are added in the same approach to both spiked and real samples, the accuracy of the measurement may be affected, because the recovery of the IS may be different from that of the analytes. In order to prepare spiked samples as close to the real samples as possible, the homogenization step of the sample preparation needs to be thorough enough so that the resulting homogenate is a uniform system that has an even distribution of analytes. Meaning, the tissue cells need to be completely broken down, assuming that the analyte of interest can freely distribute in/out of tissue fragments and constituents in the tissue homogenate.[93] Due to the softness of brain tissue, this can be achieved with the use of a proper lysing buffer or organic solvent, together with powerful homogenizing equipment. By mixing analyte standards with a highly uniform brain homogenate, spiked samples are considered to be the same as real samples. Internal standards (IS) are usually added to the spiked samples or real

samples as the first step of sample preparation. With adequate vortexing, ISs are considered to be distributed in the same manner as the analytes. Nevertheless, IS can also be added before the homogenization. In a study published by Jantti et al. in 2010, ISs for the determination of steroids and their intact glucuronide conjugates were added to brain samples 1 h before homogenization to mimic the natural absorption of compounds into the brain matrix. In this case, the ISs are absorbed and distributed evenly in the brain tissue in a more similar way to the analytes. Even if the homogenization was not strong enough to yield a uniform homogenate, accurate recovery and concentration measurements can still be fulfilled, since the ISs can compensate for the loss of analytes due to incomplete homogenization or extraction.

When homogenization-based sample collection is finished, the brain tissue homogenate needs to be stored at -80 °C until use, so that the freshness of the homogenate and the stability of analytes can be protected. Though the storage of whole brain homogenate is the most common practice, there are methods using a different sample storage strategy with a simple sample pretreatment. After brain sample collection and the homogenization are completed, the homogenate is subject to one or two rounds of centrifugation to separate the cell residues. The resulting supernatant is then transferred to a new tube and stored frozen until use.[15, 37, 39, 55, 68, 77, 88] Instead of transferring brain homogenate that might stick to the pipette tips, one only needs to pipette the less viscous supernatant in all the following steps, which indirectly improves the precision and accuracy. One single concern about this method is non-specific binding. Only under the assumption that the analyte of interest is not largely bound to the cell residues can this method be considered effective without losing too much analyte in the centrifuged pellet.

2.2 Microdialysis

Microdialysis is a minimally-invasive sampling technique that can continuously collect free analytes directly from live animals. This technique originates from the principle of the "push-pull cannulae", which was first published by Gaddum in 1961.[96] The push-pull technique was largely improved when the "continuously perfused dialytrode" was introduced in 1972.[97] The actual technique that was most close to today's microdialysis was invented by Ungerstedt in 1974, when the dialytrode was modified with a tubular semipermeable membrane with a diameter of approximately 200 to 300 μm to fulfill minimally-invasive sampling.[98] Due to the advantages of continuous *in situ* sampling, this technique is now widely used in tissue sample bioanalysis.[95, 99-124]

The principle of microdialysis is based on passive diffusion of the analytes between the two sides of a semipermeable membrane. Generally, a microdialysis probe was inserted into the brain tissue, which was composed of a semipermeable membrane surrounding two flowing channels (inlet and outlet). The probe is continuously perfused with an isotonic solution (perfusate) at a constant flow rate, during which the small-molecule analyte in the tissue can freely pass through the semipermeable membrane and diffuse into the dialysate traveling to the outlet. The dialysate can be collected at multiple time points for analysis. At a low flow rate, the diffusion of the analyte of interest across the semipermeable is considered to be a dynamic equilibrium, so that the analyte concentration in the dialysate can be a reflection of that in the extracellular fluid of the tissue analyzed. In summary, the working mechanism of a microdialysis probe can be described as an artificial blood capillary manually inserted into the tissue of interest to sample small molecule analytes from a live animal.

To carry out microdialysis on the brain tissue, the first step starts with a minimally-invasive surgery on the test animal. Since microdialysis can be used to study the analyte concentration in the certain region of the brain, the cannulation site needs to be determined beforehand. After the test animal is immobilized and anesthetized, a small hole is drilled in the skull at the region of interest for cannulation. A cannula is stereotaxically implanted into the brain to a certain depth from the pre-drilled hole, according to the anatomy of the animal species. In most studies using rats, the stereotaxic atlas of Paxinos and Watson is used to determine the coordinates of cannulation.[125] At this point, a replaceable dummy probe is inserted into the cannula. Then the test animals are put back into the cages with free food and water access for at least 1 to 7 days to recover from the trauma of the surgery. At the beginning of the experiment, the dummy probe is replaced with a microdialysis probe, which is made of a 3-4 mm-long semipermeable membrane with a certain molecular weigh cut-off (MWCO) from 1 to 100 kDa. The inlet and the outlet of the microdialysis probe are connected to a microdialysis pump, by which the perfusate is pumped through at a constant flow rate of approximately 0.1-5 μL/min. The perfusate is usually an isotonic solution that can mimic the physiological environment of the cerebrospinal fluid (CSF). Ringer's solution is one of the most widely used perfusates in microdialysis, which is an aqueous solution containing 149 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 0.8 mM $MgCl_2$ with the pH adjusted to 7.4.[99, 100, 102, 103, 108, 109, 113, 115, 116, 118, 119, 121-123] Artificial cerebrospinal fluid (aCSF) is another commonly used perfusate in brain tissue microdialysis.[101, 104, 107, 110, 114, 117, 120, 124] aCSF may have different formulas in different studies, but in general it is an isotonic aqueous solution that is close to the composition and pH of physiological CSF. Other solutions used as perfusates in microdialysis include 2 mM ammonium acetate or Dulbecco's phosphate-buffered saline.[111, 112] Besides inorganic salts added in the

perfusate to maintain the osmotic pressure, additives can also be added for different purposes. Internals standards are commonly added in the perfusate as calibrators to calculate the microdialysis recovery.[103, 116] These internal standards are either structural analogues or stable isotope-labeled analogues of the analytes of interest. Small amounts of analyte stabilizer can also be added in the perfusate to protect the analyte of interest from degradation, as long the osmotic pressure and the pH of the perfusate is not drastically changed. In a study concerning the quantitation of remoxipride in brain tissue, an anti-oxidant was used in the microdialysate to prevent the oxidation of analytes.[95] After starting the perfusion, the test animal is put back into the cage for at least 1 to 24 hours to let the microdialysis system reach equilibrium, which also allows the animal to recover from the insertion of the probe. At this point, dialysate can be collected as blank samples to describe the baseline concentration of the analyte of interest. Then the experiment can begin by dosing the animal with the studied drug. If the study is of endogenous compounds in the brain, which do not require extra dosing of the animals, samples can be collected directly after the equilibrium. To collect the microdialysis samples, the dialysate coming out from the probe is collected either manually or, in most occasions, by an automatic fraction collector. Multiple samples can be collected at different time points to describe the change in analyte concentration over time.

If the microdialysis membrane and the perfusate are correctly chosen, the resulting samples should only contain the analytes of interest, inorganic salts, small-molecule endogenous compounds and minimal levels of small proteins or peptides. In this case, microdialysis samples are clean enough to be directly analyzed by LC-MS/MS. Since the microdialysate contains high concentrations of buffer salts, desalting is needed to protect the mass spectrometer from being affected by salt ions. The easiest approach to separate the analytes from the salts is online

desalting using a solvent delay.[104, 121] When a reversed phase LC column is used, the retention of the analytes is based on hydrophobic interactions. Therefore the salt ions would not be retained on the column and will elute at the dead time of the LC run. By using a divert valve to divert the LC eluent in the first several minutes to waste, the salts will not enter the inlet of the mass spectrometer. Meanwhile, the analytes of interest can be loaded into the mass spectrometer when the divert valve is switched back to the main pass position. Another way to fulfill online desalting is by using column-switching, which will be discussed in the LC chapter.[77, 102, 103, 108, 116, 123] When instrumentation is limited, desalting can also be fulfilled by offline techniques. In a study involving the quantitation of dopamine and its metabolites in brain microdialysates, Syslová et al. developed an offline desalting method using lyophilization.[117] After sample collection, microdialysis samples were freeze-dried and reconstituted in methanol. Since the inorganic salts were not soluble in pure organic solvent, centrifugation was used to separate the analyte dissolved in the supernatant from the precipitated salts. In most studies, the sample preparation methods for microdialysis samples are facile. Usually the samples only need to be mixed with internal standards and then can be directly analyzed by LC-MS/MS, which is the most common sample preparation method for microdialysis samples.[95, 99-104, 107-110, 112, 114, 115, 117, 121-124] In studies that require cleaner sample preparation, protein precipitation has also been used for microdialysis sample clean-up.[102, 113] In a study involving the determination of three opioidmimetics in rat brain dialysates, Igarashi and coworkers used protein precipitation as the sample preparation. To each 60 μL of microdialysate sample, 60 μL of methanol was added and mixed well. The supernatant obtained from the following centrifugal filtration was injected into the LC-MS/MS system for quantitative purposes.

Microdialysis is a technique that extracts analytes from the brain tissue of a live animal, yielding analyte concentrations that may not accurately reflect the actual concentration. To describe the extraction efficiency of microdialysis, one needs to consider the dialysis recovery, which is defined as the ratio of analyte concentrations between the dialysate and the CSF. All the factors that may affect the passive diffusion of analytes between the inside and outside of the semipermeable membrane can contribute to the final recovery and therefore, need to be carefully considered in microdialysis. These factors include the membrane MWCO and the flow rate. The MWCO of the semipermeable membrane has to be bigger than the molecular weight of the analyte but not so large as to allow impurities to pass through. The bigger the MWCO is, the easier it is for diffusion to reach equilibrium, resulting in a higher recovery and higher dialysate concentration. The flow rate of perfusion is usually 0.1-5 μL/min, which is considered to be enough for most small-molecule analytes to reach diffusion equilibrium between the two sides of the probe membrane. The lower the flow rate is, the more analytes in the brain tissue can diffuse into the perfusate, resulting in a higher recovery and therefore a higher dialysate concentration. With properly chosen membrane MWCO and flow rate, microdialysis can be conducted with high recovery and reproducibility.

Microdialysis can be considered as a semi-quantitative technique, since the real analyte concentration in the CSF can only be calculated with the measured concentration and the dialysis recovery. The dialysis recovery can be determined by several different techniques, among which retrodialysis is the most commonly used one, due to its simplicity of operations as well as high accuracy.[103, 107, 111, 115, 116, 118, 119, 121-124] To conduct *in vivo* retrodialysis, a probe that is the same as that used in the microdialysis is inserted into the brain of a live test animal. Instead of blank isotonic solutions, the probe is perfused with analyte solution of a known concentration at the

same flow rate as the microdialysis experiment. During this process, the analyte in the perfusate will pass through the semipermeable membrane and enter the CSF outside the probe. After the diffusion reaches the equilibrium, it is assumed that the distribution of analyte at both sides of the membrane is the same as the equilibrium reached by normal microdialysis. With the perfusate concentration (C_p) and dialysate concentration (C_d) measured by LC-MS/MS, the relative recovery of microdialysis (R) can be calculated using this equation: $R = (C_p - C_d) / C_d$. Therefore retrodialysis can be considered as the reverse process of microdialysis, yielding a relative recovery as the ratio of analyte passing through the membrane to that remaining on the original side. Instead of using real animals, retrodialysis can also be conducted *in vitro*. The calibrator used in retrodialysis can be the analyte itself, stable isotope-labeled (SIL) analogues or structural analogues. It is the most common practice to use the analytes as the calibrator, because of high accuracy and easy access.[107, 111, 115, 118, 119, 122-124] Retrodialysis with analyte calibrators must be conducted separately on a blank test animal, since the analyte calibrators will interfere with the analytes in real biological samples. SIL analogues can be a good choice as the retrodialysis calibrator, because they closely resemble both the physiochemical and the biological properties of the analyte.[116, 121] The most significant advantage of using SIL analogues as the retrodialysis calibrator is that they will not interfere with the analyte of interest, which means that the retrodialysis experiment can be conducted simultaneously with the microdialysis, simply by adding SIL analogues of the analyte in the perfusate. When SIL analogues are not available, structural analogues can also be used as the retrodialysis calibrator for simultaneous probe calibration.[103] Both SIL analogues and structural analogues can be used for the retrodialysis of either endogenous or exogenous analytes, while the analyte itself can only be used for exogenous analytes. In addition to retrodialysis, *in vitro* calibration is another method to

determine the microdialysis recovery.[109, 113, 118] By immersing the microdialysis probe into an isotonic solution containing the analyte at a known concentration (C_s) and perfusing the perfusate through the probe under the same conditions as the microdialysis experiment, this method can be considered to mimic microdialysis. Dialysate samples are collected at different time points and the steady-state concentration (C_d) is determined by LC-MS/MS. Assuming that the small amount of analyte entering the dialysate will not change the analyte concentration in the large volume of solution, the recovery (R) can be calculated by the equation: $R = C_d / C_s$. This method is a simple and direct way to measure microdialysis recovery. But the measured recovery may not sufficiently reflect the actual *in vivo* recovery, which can be affected by other factors, such as the possible interactions of tissue components with the analyte or the membrane materials.[118] After microdialysis recovery (R) is determined, the measured analyte concentration (C_m) can be converted to actual *in vivo* concentration (C_r) by the equation: $C_r = C_m / R$.

The microdialysis technique has multiple advantages over traditional homogenization-based sampling techniques. The most significant advantage of microdialysis is the capability of continuous sampling from a live animal. Unlike homogenization-based sampling methods that require the sacrifice of the animal for each sample, microdialysis can continue sampling at multiple time points from the live animal, which is of great convenience for pharmacokinetic (PK) studies. By sampling from the same animal, it can help improve the accuracy and credibility of the PK profile, since all the data points are obtained from the same animal and therefore inter-individual differences are avoided. Microdialysis also reduces the workload and cost by using fewer animals and less surgical operations. The throughput can also be greatly improved by the use of automated fraction collectors. Last but not least, microdialysis makes it possible to obtain samples from different functional regions of the brain simultaneously by

inserting multiple dialysis probes into the brain, which otherwise would be much more difficult to fulfill by homogenization.

However, microdialysis also has some limitations and disadvantages compared to homogenization. One of the major differences between homogenization and microdialysis is the analyte coverage. Since homogenization disperses everything in the brain tissue into the media, it does not require any specific properties of the analytes and can be considered a "lossless" sampling technique for most analytes. These characteristics make homogenization a widely compatible technique with nonspecific analyte coverage. Microdialysis, on the other hand, involves the diffusion of analytes from the brain tissue into the dialysate, and therefore limits its application to some analytes. Usually microdialysis can only be applied to small-molecule analytes, since it is hard for large-molecule analytes to diffuse into the dialysate. Another concern with microdialysis is the recovery, which is often much lower than that of traditional extractions following homogenization. Usually one would expect the microdialysis recovery to be in the range of 10 – 30%, which may compromise the sensitivity of the analytical method. In all microdialysis-based methods, the recovery needs to be determined to accurately measure the analyte concentrations in the targeted tissue, requiring extra experiments and labor. Unlike homogenization-based methods that can measure the total analyte concentrations in the targeted tissue, microdialysis can only measure the unbound analyte concentrations, since only unbound analytes can penetrate the semipermeable membrane. This will be a problem when total analyte concentrations are needed, especially for analytes with a high protein binding affinity. In addition, only analytes in the extracellular fluids of the targeted tissue can enter the dialysis probe, which means that microdialysis cannot measure analytes remaining in the tissue cells. In contrast, homogenization-based method can yield a total analyte concentration in the targeted

tissue, since all bound or unbound, intracellular or extracellular analytes are collected in the final extract. Finally, the temporal resolution of microdialysis is lower than that of homogenization. Unlike homogenization samples that can be obtained at an exact time point, it takes some time to collect microdialysis samples for each time point. Usually the middle point of the collecting period is used to plot the concentration curve over time. Therefore the temporal resolution of microdialysis is determined by the sample volume and the flow rate, which can be a potential issue for analytes with low stability or short half-lives.

2.3 Ultrafiltration

Ultrafiltration is another sampling technique that can be applied to the sample collection from brain tissue. It can be considered as an altered technique that is analogues to microdialysis. Similar to microdialysis, ultrafiltration is also an *in vivo* sampling technique that can directly extract samples from the brain tissue of live animals. Due its convenience and, more importantly, the capability of continuous sampling, ultrafiltration has gained more attention for the sample collection of brain tissue for quantitative LC-MS/MS analysis.

The principle the ultrafiltration sampling technique is the extraction of biological samples through a semi-permeable membrane under the driving force of negative pressure. Like microdialysis, *in vivo* ultrafiltration also relies on the use of an implanted semi-permeable membrane, which only allows molecules with the molecular weight below the molecular weight cut-off (MWCO) of the membrane material to pass through. By applying a negative pressure on the collecting side of the membrane, biological samples can be drawn from the tissue side to the collecting side. Due to the filtering property of the semi-permeable membrane based on molecular weight, tissue components, blood cells and other large-molecule impurities are blocked in the tissue side of the membrane, while small molecule analytes, together with the

extracellular fluid in the target tissue (CSF in the brain), will pass through the membrane enter the collecting side. Samples collected by ultrafiltration are real extracellular fluid from the target tissue, which can be further processed or directly analyzed by LC-MS/MS. In summary, *in vivo* ultrafiltration can be described as a sampling technique that can physically pull the biological samples from the target tissue and, in the mean time, filter the samples with a semi-permeable membrane.

To conduct ultrafiltration on the brain tissue sampling, the preparation of test animals is similar to that of the microdialysis experiment, which starts with the implantation of a sampling probe. After the site of cannulation is determined based on the need of the analysis, a small hole is drilled in the skull at the region of interest of the pre-immobilized and anesthetized test animal, according to the stereotaxic atlas of Paxinos and Watson.[125] Similar to the surgical operations in microdialysis, a replaceable dummy probe is inserted into the cannula and the test animal is allowed back to normal diet to recover from the trauma. At the beginning of the sampling, the dummy probe is replaced with an ultrafiltration probe, which is a hollow fiber made of a semi-permeable membrane. The semi-permeable membrane should have a specific MWCO (usually from 1 to 100 kDa) that is slightly great than that of the analyte of interest, so that the analyte and the CSF can pass through, while other impurities are excluded. To start the ultrafiltration sample collection, the sampling probe is connected to a vacutainer or a peristaltic pump, which applies a negative pressure to the probe and pulls the analyte-containing CSF through the probe membrane pores and into the collecting vial. The collected samples are ready for additional sample preparation or direct quantitative analysis.[126]

To develop an effective ultrafiltration method for the extraction of CSF samples from brain tissue, several factors need to be carefully considered. First, the membrane should be

chosen with the proper material, and more importantly, the proper MWCO. The most basic rule is that the MWCO of the membrane should be greater than that of the analyte of interest, so as to achieve satisfactory analyte recovery. Under this condition, lower MWCO can provide cleaner CSF samples, but will increase the pressure and require lower flow rate. Higher MWCO, on the other hand, is more technically favored but provide samples with more large-molecule impurities, so that further sample preparation will be needed. Usually, an ultrafiltration membrane with an MWCO of 3,000 will be suitable for most small-molecule sample collection from the brain tissue. Second, the size of the probe and the depth of the insertion should be considered, which contributes to the surface area of the tissue-membrane interface. The bigger the surface area is, the lower pressure difference is needed to pull the CSF samples out, which means that higher flow rate can be used. Meanwhile, bigger surface area may also lower the temporal resolution of the sampling site, especially when the CSF sample from a specific functional region of the brain is needed. Typically, the diameter of an ultrafiltration fiber ranges from 0.2 to 3 mm and the insertion is 0.5 to 5 mm into the brain tissue.[127] Third, the negative pressure needs to be controlled to maintain a proper flow rate. Higher flow can lead to higher throughput and therefore higher time point resolution. More importantly, higher flow rate can enhance the sweeping effect across the membrane surface and prevent deposition of impurities. However, high negative pressure and higher flow may lead to rapid loss of CSF and cause serious brain damage to the test animal. It also requires more expensive instrumentation including membrane, tubing and pump to achieve high flow rate. Compared to microdialysis, the sampling rate of ultrafiltration is slower (0.5 to 2 μL/hour/cm of membrane length) and can not exceed the rate at which the extracellular fluid is replaced by the blood vessels within the tissue

According to the basic principle and practical characteristics of ultrafiltration, ultrafiltration has several advantages over other sample collection techniques. Similar to microdialysis, ultrafiltration is capable of continuous *in vivo* sampling from the live animals, which allows for a better description of the analyte concentration change in the live animals over a certain period of time. Also, the CSF samples obtained by ultrafiltration are free of tissue components, blood cells or large-molecule impurities, which can be directly forwarded to LC-MS/MS analysis with none or minimum sample preparation in most occasions. In addition, ultrafiltration has a significant advantage over microdialysis, which is its high accuracy. Unlike microdialysis that only extracts sample solutes into the dialysate via diffusion, ultrafiltration pulls the real CSF from the brain tissue, which includes the analyte of interest, small-molecule solutes and the biological fluids. Therefore, the analyte concentrations in the samples obtained by ultrafiltration is a true reflection of the *in vivo* analyte concentrations in the brain tissue, which provides better accuracy than the semi-quantitative microdialysis technique. Finally, ultrafiltration can also be fully automated to achieve higher throughput and less labor intensity.

Ultrafiltration also has some drawbacks and limitations in the *in vivo* sampling from the brain tissue. The most significant issue with ultrafiltration is the membrane fouling. Since all the particles, cells and large-molecule impurities are blocked at the tissue side of the ultrafiltration membrane, they may accumulate over the sampling process and form a solid deposit on the membrane surface, leading to the clogging of the membrane. Optimization of membrane pore size and flow rate is needed when membrane fouling is affecting the ultrafiltration. Another big limitation of the ultrafiltration on the brain sample collection is the limited quantity of CSF samples. Only a very limited volume of CSF can be continuously drawn from the test animal before causing serious damage. Therefore the flow rate of microdialysis and the total volume of

samples are strictly limited for *in vivo* ultrafiltration on the brain tissue. Another possible issue with ultrafiltration is the nonspecific binding of the analytes on the membrane, which may lead to lower recovery. At last, the cost of instrumentation and consumable materials is relatively high for ultrafiltration, especially when high pressure, high flow rate or some specific conditions are needed.

2.4 Solid-phase Microextraction

Solid-phase microextraction (SPME) is another sampling technique that can be applied to the sample collection from the brain tissue. In terms of instrumentation and principle, SPME is also analogous to microdialysis and ultrafiltration. Generally, SPME can be described as a sample collection and preparation technique that involves the use of a fiber coated with an extracting phase, which is capable of extracting the analyte of interest from the live animals or biological samples.[128]

The principle of SPME is similar to that of solid-phase extraction (SPE). By immersing a porous fiber that is coated with a specific extracting stationary phase into the target tissue of a live animal or collected biological samples, sample collection, preparation and enrichment can be fulfilled at the sample time.[129] Different coating materials can be chosen for the sampling probe, which is based on reversed-phase or hydrophobic interactions between the analytes and the hydrocarbon stationary phase (C4, C8, C18 or HLB) in most occasions.[130-132] After the analytes of interest are adsorbed onto the surface of the coating material, the sample probe is pulled out and washed with a strong solvent, resulting in a sample solution that can be analyzed with LC-MS/MS with none or minimum sample preparation. This technique can be applied to collected biological fluids or tissue homogenate as a sample preparation technique.[132] However, what attractions more attention to the SPME technique is its capability of *in vivo* sampling from a live

animal.[133] By implanting a sampling probe in the brain or other organs of the test animal, sample collection can be fulfilled from the live animal over a certain period of time, which is especially suitable for the use of pharmacokinetic studies or other studies that require samples from the same animal at multiple time points.

To conduct *in vivo* SPME, the procedure includes animal pretreatment, surgical implantation of the sampling probe and sample collection, which is similar to the procedure of microdialysis and ultrafiltration. What is different in SPME is that the sample collection process is not assisted with any liquid or gas, which is simply based on the free movement of analytes in the target organ and the unassisted adsorption onto the sampling probe. After a fixed period of time, the probe is removed from the target tissue. Unlike microdialysis and ultrafiltration that yield a liquid sample after the collection process, SPME only extracts solutes from the target tissue or biological samples, which include the analyte of interest together with other impurities that can be adsorbed under the same conditions. Therefore, the analytes need to be washed from the probe with a strong solvent (e.g. MeOH, ACN), resulting in a liquid sample that can be forwarded to LC-MS/MS analysis or further sample preparation. Since samples containing high concentrations of organic solvents will not run well in LC-MS/MS, a round of evaporation and reconstitution may be needed after SPME.[133]

To effectively conduct an SPME-based sample collection method, several important factors need to be optimized. First, the coating material on the sampling probe needs to be properly selected based on the properties of the analytes. In addition to the hydrocarbon materials that can provide affinity to non-polar analytes based on hydrophobic interactions, ion exchange interactions can also be utilized when ionic analytes are measured.[134, 135] By selecting the proper sorbent, SPME-based sample collection method can provide very high recovery and

specificity, yielding clean samples with high analyte concentrations. Second, all the parameters determining the amount of extracted analytes need to be well controlled. The amount of analytes adsorbed onto the sampling probe is proportional to the radius of the sampling probe, depth of the insertion into the tissue and the length of the sampling time, which need to optimized and then fixed in the whole experiments to reach satisfactory sensitivity, precision and accuracy. However, one should be careful with maximizing these parameters, which may lead to the adsorption of large amounts of unwanted impurities.

Based on the principle and practical properties of SPME, this sampling technology has several significant advantages that made it increasingly popular for LC-MS/MS analysis. First, SPME combined the sample collection, sample preparation and sample enrichment all in one procedure, which is very convenient and greatly improves the throughput. Second, SPME can provide very high specificity towards the analytes of interest By using the proper sorbents coated on the sampling probe, SPME can selectively extract analyte molecules without picking up other impurities, which not only provides a clean sample, but also improves the sensitivity. Samples collected by SPME usually only require minimum preparation before LC-MS/MS analysis. Third, SPME can provide high sensitivity. Since SPME is a solvent-free sample collection method, there is no dilution of analytes due to any addition of solvents. Moreover, the sampling process is also a sample enrichment process, so that the final concentration of analytes can be much higher than that in the biological samples, which is a big advantage for methods measuring trace-level analytes. Finally, SPME can also be fully automated to reduce labor intensity.

SPME also has some disadvantages and limitations that one need to consider before choosing this technique. First, the accuracy of SPME greatly relies on the similarity between the calibration standards and the real tissue, when online *in vivo* SPME is used. Because the sample

concentration measured with SPME is only a proportional reflection of the original concentrations in the tissue, the exact concentration values cannot be measured directly. Calibration curves are built by calibration standards (spiked samples with known analyte concentrations), which have to be as close to the original tissue as possible. Second, when measuring analytes with very high *in vivo* concentrations, SPME probe may be saturated, yielding nonlinear recovery and therefore low precision and accuracy. Third, as adsorption of analytes on the probe is the primary mechanism of SPME, nonspecific binding of unwanted impurities can also be potential problem. Fourth, SPME only extracts and measures the fraction of free analytes in the target tissue, leaving the intracellular and protein-bound analytes hard to assess. Fifth, it takes from several to 30 minutes to finish the sample collection of each data point by SPME, which limits the density of data points in a continuous sampling experiment. Finally, the cost of SPME can be higher than other traditional sample collection techniques.

3. Sample preparation

After brain tissue samples are obtained from test animals, there are large quantities of impurities remaining in the samples, especially for brain tissue homogenates that maintain all of the tissue components. Impurities such as lipids, proteins and salts can cause a series of problems for LC-MS/MS analysis, including but not limited to matrix effects, peak shape distortion, column congestion and instrument contamination. Therefore, to achieve satisfactory sensitivity, and selectivity of the analytical methods as well as to maintain the performance of the instruments, further sample preparations of brain tissue samples are needed before the injection into the LC-MS/MS system. As mentioned in the last section, microdialysis samples are usually clean enough for direct LC-MS/MS analysis. Sample preparations discussed in this section are mainly focused on brain tissue homogenate. Due to their low cost and ease of development, the

most common sample preparation methods are protein precipitation (PPT), liquid-liquid extraction (LLE), or solid phase extraction (SPE). There are also other minor sample preparation methods available for the clean-up of brain tissue homogenate, including online SPE and enzymatic digestion. One can choose the proper technique or combinations of multiple techniques for the preparation of brain homogenate samples, on the basis of the analytical goal, sensitivity, cost or efficiency.

3.1 Protein Precipitation-based Sample Preparation

Protein is one of the major components of brain tissue homogenate, which may cause matrix effects and column congestion. It is important to remove proteins from the biological samples before LC-MS/MS analysis or further finer sample preparation operations. Since insoluble proteins can be easily removed by filtration or centrifugation, the major targets of sample preparation are soluble proteins. In order to remove the soluble proteins from the brain tissue homogenate, the proteins dissolved in the buffer or water need to first be precipitated. Protein precipitation is a traditional sample preparation technique designed for such purposes. It is capable of simple and fast treatment of biological samples, and can be considered as the most widely used sample preparation method. [9, 10, 13, 15, 16, 19-22, 24, 25, 29-31, 34-39, 41, 42, 46, 48, 49, 51-53, 55, 57, 58, 60-63, 66-68, 72, 74, 76-80, 87, 88, 90, 92, 94]

Proteins are large biological molecules that are composed of amino acids linked by peptide bonds. Under physiological conditions, a soluble protein has one or multiple peptide chains assembled into a folded conformation, with most hydrophobic amino acid residues facing the inside and charged or hydrophilic amino acid residues exposed on the surface. On the inside of the protein, the peptide chains are folded together mainly by hydrophobic interactions between hydrophobic amino acid residues; while other interactions including hydrogen bonds, salt bridges

or disulfide bonds are also contributors to the folded structure. On the outside of the protein, charged or polar surface residues can interact with the environment and increase the solubility of a protein. In a brain tissue homogenate, repulsive electrostatic forces exist among soluble proteins to prevent aggregation and facilitate dissolution. When a soluble protein is dissolved in water or an electrolyte buffer during tissue homogenization, water forms a solvation layer surrounding the protein and establishes a concentration gradient with the highest concentration at the protein surface. This weakens the ionic interactions between proteins and decreases the likelihood of aggregation. Therefore, to precipitate or induce the accumulation of proteins from the solution or suspension, proper agents can be added to reduce the hydration layer.

Protein precipitation usually involves three steps: addition of precipitants, mixing and removal of the precipitated proteins. First, a proper amount of precipitating agents are added to the brain homogenate. Organic solvents and inorganic acids are the most often used protein precipitants for small-molecule analytes in brain tissue homogenate, though other agents like neutral salts, non-ionic hydrophilic polymers and polyelectrolytes have also been used for protein precipitation. When miscible organic solvents are added to the brain tissue homogenate, the solvation layer around the protein will decrease as the organic solvent molecules displace water molecules from the protein surface. With a smaller solvation layer, proteins can get closer and form interactions with each other via attractive electrostatic or dipole interactions, leading to the aggregation of proteins. Commonly used organic solvents for protein precipitation are acetonitrile (ACN) and methanol (MeOH). ACN is the most common organic precipitant for the pretreatment of brain tissue samples, due to its strong precipitating ability.[13, 15, 19-21, 34, 37, 42, 49, 55, 57, 58, 62, 63, 67, 72, 77, 80, 87, 88] Unlike ACN that can yield more rigid protein pellets, MeOH is a milder organic precipitant, yielding flocculent protein sediments. It is also widely used in brain tissue

sample preparation, due to its lower cost.[9, 14, 22, 24, 39, 51, 53, 61, 68, 90, 136] To each unit weight of brain tissue, at least two volumes of organic solvents are needed for efficient protein precipitation; while the more organic added, the more thorough the precipitation will be. Though pure organic solvents can perfectly fit the purpose of precipitating proteins, other additives or solutes are commonly added for other purposes. It is a common practice to dissolve internal standards (IS) in the organic protein precipitants, which allows for the addition of the IS at the same time as protein precipitation.[20, 42, 51, 55, 61] This practice not only improves the throughput by combining two steps together, but also enhances the precision by pipetting larger volumes of IS solutions instead of a small-volume spike. Organic acids, formic acid and acetic acid for example, are also often added in the organic protein precipitants to lower the pH for the adjustment of analyte charge states.[15, 19, 24, 62, 72] The protein precipitation can also be facilitated by the addition of such acids, which can help with the reduction of the hydration layer around proteins. Other than the composition and pH of organic protein precipitants, the temperature is another factor to consider during protein precipitation. Usually ice-cold organic solvents are used to guarantee the stability of analytes, especially for those that are temperature sensitive.[15, 37, 39, 55, 68, 77, 88]

Similar to the choice of homogenization media, inorganic acids can also be used as the protein precipitant for the preparation of brain homogenate. Trichloroacetic acid (TCA) and perchloric acid (PCA) are the most commonly used acids for protein precipitation.[25, 41, 52, 54, 66, 79] Though the actual mechanism is not well know, it has been believed that the precipitation by TCA and PCA involves the denaturing of proteins.[137] Upon addition of these acids, the pH of the solution is greatly lowered and the protein conformations are drastically changed, leading to the exposure of hydrophobic amino acid residues resulting in the aggregation of proteins.[138, 139] The ideal concentration of TCA for protein precipitation is approximately 15%, while either lower or

higher concentrations generally result in lower efficiency. Similar to the organic protein precipitants, low temperature and the addition of IS can also be featured when inorganic acids are used as precipitants. In a study published by Onorato et al. in 2010, pre-chilled 10% TCA with supplemented IS was used for the protein precipitation of pulverized brain tissue samples.[54] Another important feature is that the lowered pH by the addition of acids can also be utilized for further sample preparations. In a study about the quantitation of cotinine and metabolites published by Li et al. in 2012, TCA is used for the protein precipitation of brain tissue homogenate.[41] Since all the analytes have a tertiary amine structure, they were ionized at lower pH caused by the addition of TCA, which facilitated the following sample preparation by solid-phase extraction (SPE). The retention of the protonated analytes on the mixed-mode cation exchange (MCX) SPE cartridges was enhanced at the lower pH.

Protein precipitants with more than one component are also used in multiple published studies.[10, 16, 30, 31, 38, 48, 76, 78] In a recent study about the quantitation of loxapine, amoxapine and their hydroxylated metabolites, combinations of MeOH and PCA at different relative ratios were tested in the sample preparation of brain tissue homogenate, suggesting that one volume of MeOH and 7 volumes of PCA yielded the best analyte recovery.[78] In another method published by Karinen et al. in 2009, a mixture of ACN and MeOH was used for the protein precipitation of brain homogenate.[31] Advantages of different agents can be taken at the same time when multiple protein precipitants are used in a single protocol.

Besides the choice of correct protein precipitants, the time of the addition of precipitants can also be different. As mentioned in the section of homogenization-based sample collection, protein precipitation can be combined with the step of homogenization, by using protein precipitants as the homogenization media.[9, 11, 15, 24-26, 28, 30, 32, 34, 38, 39, 42, 52-54, 68, 76, 79] If added after

the homogenization, the addition of precipitant can be stepwise, which can yield higher precipitation efficiency by allowing for more complete interactions between the solvent molecules and the proteins. In a paper published by Hatziieremia et al. in 2007, 2 × 300 μL of ACN was added to each 100 μL 20% brain homogenate to achieve complete protein precipitation.[21] In another study about the disposition of cannabichromene, cannabidiol, and Δ^9-tetrahydrocannabinol and its metabolites in mouse brain, Poklis and coworkers developed a protein precipitation method for mouse brain homogenate by adding ice-cold ACN drop by drop while vortex mixing, which maximized the ACN-protein interactions and therefore fully utilized the precipitating effects of added ACN.[57]

After the addition of protein precipitants, the mixture of brain tissue homogenate and the protein precipitants needs to be fully mixed by vortexing. Usually at least 1 to 10 min of vortexing is needed, depending on the volume of samples. During vortexing, the precipitant molecules are evenly distributed across the fluid eddies and interact with proteins. The solvation layers around the protein molecules are greatly reduced by precipitants, making it possible for proteins to get closer to each other. Protein molecules will collide into each other and form submicroscopic sized protein aggregates, under the influence of attractive electrostatic forces. These protein aggregates keep growing by diffusive additions of other protein molecules and eventually reach a critical size for precipitation, forming protein sediments or flocculus. In all the common protein precipitants, ACN and TCA have the strongest precipitating abilities and can form very solid protein precipitates of larger particle sizes. Being a weaker protein precipitant, MeOH forms looser flocculent precipitates. When proteins are precipitated by the addition of precipitants, small-molecule analytes will still remain in the solution. Meanwhile, analytes bound

to proteins will be released into the solution, since the protein conformations are changed by the precipitants and therefore cannot hold on to the analytes any more.

When adequate vortexing has been applied to the mixture, most proteins in the homogenate have already been precipitated. The next step of protein precipitation-based sample preparation is a physical process to separate precipitated proteins from the supernatant, in which the small-molecule analytes remain dissolved. Usually a round of centrifugation at 3,000 to 12,000 × *g* is sufficient to settle the protein precipitates and form a solid pellet at the bottom of the container. The higher speed of centrifugations is used, the more solid the pellet will be, making it easier to obtain the supernatant without disturbing the integrity of the pellet. Multiple rounds of centrifugations can be used for better results.[60]

Theoretically the supernatant obtain from protein precipitation is free of most proteins and can be directly analyzed by LC-MS/MS. However, additional processes are involved before LC-MS/MS analysis in many studies. Considering centrifugation is not efficient enough to remove insoluble impurities, especially those with lower densities than that of the supernatant, centrifugal filtration has been widely used to further purify the supernatant.[15, 21, 48, 52, 61, 92] By loading the supernatant onto a filter tube with submicrometer-sized (0.2 or 0.45 μm in most applications) membrane, small, low-density and insoluble impurities can be removed under centrifugation. Since at least two volumes of organic solvents or 15% TCA are needed for protein precipitation, the resulting supernatant usually has a high organic composition or very low pH, neither of which is favorable for LC-MS/MS analysis or further sample preparation. Therefore, either dilution or evaporation can be used to lower the concentrations of organic solvents or acids. By diluting the supernatant with aqueous solutions, the relative organic or acid concentrations can be effectively lowered. Since the dilution method also lowers the analyte

concentrations inevitably, it is not used as much as evaporation in current methods. By evaporating the supernatant to complete dryness and reconstituting in aqueous solutions with proper pH values, organic or acid concentrations can be greatly reduced for further extractions or direct LC-MS/MS analysis.[9, 20, 21, 24, 25, 31, 58, 60, 68, 72, 87, 88] Moreover, sample enrichment can be achieved by reconstituting the residue in a reduced volume relative to the original sample volume, which improves the method sensitivity at the same time. Due to the fact that protein precipitation is a rough sample preparation method that may not be enough to remove interfering impurities in some situations, it is often coupled with other sample preparation methods, including liquid-liquid extraction and solid-phase extraction.[10, 13, 16, 24, 25, 29, 30, 36, 39, 41, 53, 58, 60, 68, 72, 74, 76, 78-80]

In addition to the traditional protein precipitation by the addition of chemical agents, microwave fixation is another alternative sample preparation technique that can denature the proteins in the brain samples, which has been used in some studies for their specific purposes.[140-143] By applying a rapid high-energy microwave fixation to the brain samples collected by surgical dislocation, proteins in the brain tissue can be denatured. Unlike other traditional protein precipitation techniques that are mostly focused on the removal of undesired protein, microwave fixation is focused on the denaturing of the enzymes in the brain tissue. Brain concentrations of some analytes, including endogenous neurotransmitters, prostanoids and lipid-mediators, can be changed in response to conditions like ischemia or post-mortem delay, which are similar to the effects of decapitation. Therefore, after the brain tissue is removed from the decapitated animal, concentrations of these analytes in the brain tissue may be changed, leading to inaccurate concentration measurements. Accordingly, a rapid, head-focused and high-energy microwave radiation can be introduced to the brain sample right after the decapitation, so that all the

enzymes can be denatured and the analyte concentrations are "frozen" at the original levels. In a study published by Bazinet *et al.* in 2005, an LC-MS/MS method was developed for the determination of anandamide (N-arachidonoylethanolamine, AEA) in rat brain. Due to that brain AEA concentrations were reported to increase in response to ischemia and decapitation, brain samples were subject to a head-focused microwave irradiation (5.5 kW, 3.4 s) following decapitation to stop the brain metabolism. Similarly, such technique can be applied to studies that involve endogenous analytes in the brain that can be altered by enzymatic metabolism associated with decapitation.

Protein precipitation is a fast and low-cost sample preparation method that has been widely used for the pretreatment of brain tissue homogenate samples for LC-MS/MS studies. The easy three-step operation is simple to conduct, leaving less chance for error resulting from inter-batch or inter-operator differences. Cost of reagents and equipment for protein precipitation are very low, since only common precipitants and centrifuges are needed in most occasions. Protein precipitation is capable of removing most proteins and cell residue from samples, yielding a relatively clean solution for further sample preparation or direct LC-MS/MS analysis. With the option of evaporation and reconstitution in different solutions, samples prepared by protein precipitation are compatible with most other sample preparation techniques. Another significant advantage of protein precipitation is its high recovery. Since only proteins are hypothetically removed by this sample preparation method, small-molecule analytes should remain in the solution and yield a theoretical recovery of 100%. Such advantages of protein precipitation have made it very popular in bioanalytical applications.

However, there are also some disadvantages of protein precipitation, among which low selectivity is the most significant one. Without specific selectivity for the analytes of interest,

protein precipitation is just a general sample cleanup technique to remove proteins from biological samples. The types and quantities of impurities removed are mostly determined by the types of precipitants chosen for the protein precipitation, leaving less leverage for the analyst to have analyte-specific sample preparation to prepare cleaner samples and achieve higher selectivity. Another significant disadvantage of protein precipitation is nonspecific binding. Analytes can be adsorbed on the brain tissue residue or even the surface of the containers. In a study published by Zhou *et al.* in 2010, an LC-MS/MS method was developed for the quantification of sunitinib in mouse plasma, brain tumor and normal brain. When protein precipitation was used for sample preparation, peak shape and recovery were not satisfactory, due to the non-specific binding of sunitinib to the brain tissue. This issue is more common with lipophilic analytes, which have higher affinity with the fats, lipids and proteins in brain tissue. In some other occasions, protein precipitation is not efficient enough to yield samples that are clean enough for direct LC-MS/MS analysis, especially for brain tissue homogenate samples with large amounts of proteins and lipids. Direct analysis of tissue samples prepared by protein precipitation will cause a series of issues, including matrix effects, column congestion and instrument contamination. Therefore additional sample preparation techniques are often needed following protein precipitation.

3.2 Liquid-liquid Extraction-based Sample Preparation

Liquid-liquid extraction (LLE) is a common sample preparation technique that has been widely used in the bioanalysis of brain tissue samples by LC-MS/MS. It involves the extraction of solutes from one liquid solution to another immiscible liquid, usually biological samples and organic solvents are the scenario for bioanalytical sample preparation. The mechanism of LLE is based on the simple principle “like dissolves like”, suggesting that a solute will dissolve best in a

solvent that has a similar polarity to itself. Due to hydrophobic interactions between solutes and organic solvents, nonpolar compounds have higher solubility in organic solvents. Ionic or polar compounds, in contrast, have higher solubility in aqueous solutions, which is facilitated by multiple interactions including ion-dipole interactions, ion-induced dipole interactions and hydrogen bonding. When more than one immiscible liquid phase is present, solutes will prefer to be dissolve in the phase with similar polarity. If one solute is currently dissolved in the solution with less similar polarity, it will diffuse across the liquid-liquid interphase and enter the liquid phase with more similar polarity, which is a thermodynamically driven process. In the case of LLE-based sample preparation for brain tissue samples, nonpolar analytes or impurities in the aqueous phase can be extracted when an immiscible organic solvent is added, so that the separation of multiple solutes (analytes and impurities) is fulfilled by the physical separation of these two immiscible phases.

Based on the mechanism of LLE, two basic strategies have been widely used in the preparation of brain homogenate samples, "forward extraction" and "backward extraction". The "forward extraction" here refers to the direct extraction of the analytes of interest. By applying the proper organic solvents and pH conditions, the analytes of interest are extracted into the organic phase. Then the aqueous phase is discarded and the organic phase is forwarded to further preparation and finally LC-MS/MS analysis. This is the most direct practice of LLE and has been widely used in many studies, especially for highly hydrophobic analytes.[11, 13, 17, 28, 33, 39, 40, 47, 50, 71, 81, 83-86, 89, 91, 95, 136] However, large amounts of hydrophobic lipids and proteins present in the brain tissue can also be extracted by organic solvents, which may cause serious interference in LC-MS/MS analysis. In this case, the opposite strategy "backward extraction" can be employed to avoid the interference of hydrophobic impurities. The "backward extraction" refers to the

practice of extracting unwanted hydrophobic impurities by organic solvents and leaving the analytes of interest in the aqueous phase. After the organic phase is removed, the aqueous phase containing the analytes can either be further processed or directly analyzed by LC-MS/MS.[8, 17, 28, 54, 68] This strategy is more suitable for ionic and polar analytes, which are hard to extract by organic solvents without extracting other interfering impurities. In a study concerning the determination of 5-hydroxytryptamine, norepinephrine, dopamine and their metabolites, Su and coworkers developed a "backward extraction" method, extracting hydrophobic impurities from the brain tissue sample by using the mixture of chloroform and isopropanol (10:3, v/v).[68] Since all the analytes are highly polar compounds, they were not extracted by the organic solvent and remained in the aqueous phase for direct LC-MS/MS analysis. In situations that require higher selectivity for the analytes, both strategies can be utilized to yield cleaner samples, which is referred to as "double extraction" or "back-and-forth extraction". In a study published by Hou et al., a double LLE method was used to prepare brain tissue homogenate samples for the quantitative determination of a novel anti-Parkinson's disease candidate drug FLZ. Endogenous hydrophobic impurities were first extracted by *n*-hexane, while the analyte was not extracted. With the organic phase removed, the aqueous phase was further extracted by ethyl acetate, yielding a clean sample with high purity and low matrix effects.[28] However, recovery and throughput might be compromised by using such complex and selective methods.

Similar to protein precipitation, LLE-based sample preparation involves three major steps: addition of organic solvents, mixing and separation. To achieve efficient LLE as the sample preparation of brain tissue homogenate samples, several important factors need to be considered for the choice of organic solvents. First, based on the fundamental mechanism of LLE, the organic solvent chosen must have a proper polarity based on the solute of interest. The

analyte or impurity to be extracted must have higher solubility in the solvent than in the biological samples. Second, the organic solvent chosen for LLE has to be immiscible with the brain tissue homogenate, so that a sharp interface can form between the organic solvent and the aqueous phase. Only when the two phases are physically separated can one of them be removed to achieve separation of certain solutes from the samples. Though not being mandatory, volatility is another important property for LLE solvents. In "forward extractions" when organic phases including analytes are obtained, the samples need to be evaporated and reconstituted in LC friendly solvents. Volatile organic solvents are usually preferred in LLE, due to the fact that they can shorten the time needed for evaporation. The last property of an LLE solvent that needs to be considered is the density. One needs to know the density of the solvent compared to that of the water, so that the organic phase can be recognized after extraction.

Different organic solvents have been used in LLE-based sample preparations for brain tissue homogenate, including chloroform [8, 17], methyl *tert*-butyl ether (MTBE) [13, 40, 71, 95], *n*-hexane [17, 28], dichloromethane (DCM) [13, 91], ethyl acetate (EA) [13, 28, 33, 47, 81, 83, 136] and diisopropyl ether (DIPE) [84, 85]. Their physical properties are listed in Table 2.1. All the solvents have a positive partition coefficient (LogP) values, meaning that they are all nonpolar and immiscible with water (or have very low solubility in water). According to the "like dissolves like" principle, solvents with a higher LogP value are more capable of extracting more hydrophobic solutes. Generally, chloroform and hexane are usually used for the extraction of highly nonpolar analytes or phospholipids in the brain tissue homogenate. In a study published by Golovko and Murphy in 2008, chloroform was used to extract prostanoids, a series of highly nonpolar analytes, from the brain tissue samples.[17] DCM and DIPE are moderately nonpolar solvents that have a wider compatibility with most analytes. In a study concerning the quantitation of several

different drug analytes, olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone, DIPE was used to extract all the analytes simultaneously from brain tissue homogenate.[84] EA and MTBE are relatively polar solvents that are commonly used for the extraction of slightly hydrophobic analytes. In a study for the determination of the brain concentration of remoxipride (LogP 2.1), MTBE was used in the LLE of the analyte from brain homogenate. To achieve finer extraction aiming at a specific analyte, multiple solvents can be combined in LLE.[11, 39, 50, 54, 68, 86, 89] The mixture of chloroform and methanol (2:1, v/v) has been used by multiple studies, due to its moderate polarity and high extraction efficiency. A study involving the quantitation of cocaine and its metabolites in the brain tissue successfully used this combination of LLE solvents.[11]

Similar to homogenization media and protein precipitants, additives are also common in LLE for different purposes. One of the most important factors affecting LLE recovery is the pH, which can be adjusted by the addition of acids, bases or salt buffers.[11, 17, 33, 50, 54, 71, 84-86, 91, 95] The basic principle is that charged analytes have higher solubility in the aqueous phase and will not be extracted by organic solvents. If the analyte of interest is the target of LLE, the pH of the sample needs to be adjusted to maintain analyte molecules in their uncharged state to the largest extent, so that a high extraction recovery can be achieved. For example, Zimmer and coworkers published a study for the quantitation of metrifonate, an acidic compound that is charged at medium to high pH. In this study, orthophosphoric acid was added to the brain homogenate to lower the pH, so that the analyte was uncharged and therefore able to be extracted by DCM.[91] Sometimes the pH needs to be very finely adjusted to achieve satisfactory recovery of the analytes. In a paper published by Zhang and coworkers, an LLE-based sample preparation method required the pH to be adjusted to exactly 10.69 by disodium phosphate (Na_2HPO_4), so

that the analytes olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone could be neutralized and maximumly extracted by DIPE.[84] If the extraction is aimed at the hydrophobic impurities, the analyte of interest needs to be maintained in its charged states to minimize the amount extracted by the solvent, which will be discarded in the separation step. In the study published by Bystrowska and coworkers in 2012, TCA was used for protein precipitation, which lowered the pH and put a positive charge to cocaine and its metabolites as the analytes. The following LLE by the organic solvent only extracted unwanted hydrophobic impurities, leaving the analytes in the aqueous phase, which will be further processed and injected in the LC-MS/MS system.[11] Internal standards (ISs) should also be added with the solvents at the beginning of LLE, since they are needed to compensate for the extraction recovery. ISs can also be dissolved in the organic solvents and added to the samples in one step. This practice allows for higher throughput and precision, because ISs are added in a large volume of solvents in a single step.[81, 136] Last but not least, additives that stabilize the analytes can be dissolved in the organic solvents and added at the same time. In the study published by Golovko and Murphy, 0.005% butylated hydroxytoluene (BHT) was dissolved in the extraction solvent chloroform and added to the samples to prevent the oxidation of a series of air sensitive prostanoids.[17]

Brain tissue homogenate samples can be processed by LLE either directly or after some additional pretreatment, depending on the sample collection and other sample preparation processes. Brain tissue homogenate prepared with aqueous homogenization media can be directly processed by LLE, since the aqueous homogenate is immiscible with organic solvents. This has been the most widely used practice, due to convenience and high throughput.[13, 33, 40, 47, 50, 71, 81, 83-86, 89, 91, 95, 136] Brain tissue samples pretreated by PPT using inorganic acids can also be

treated in this manner, since the samples after PPT are still aqueous solutions. In a study concerning the quantitation of malonyl-CoA in rat brain tissue, Onorato *et al.* added the LLE solvent chloroform/methanol (2:1, v/v) directly to the supernatant obtained from PPT by TCA.[54] One needs to make sure that the analytes of interest are not charged under such low pH conditions, otherwise addition of base is needed to raise the pH before LLE. However, brain tissue samples homogenized or precipitated by organic solvents usually need some extra processing, since the organic-based samples are miscible with LLE solvents.[8, 11, 17, 28, 39, 68] One can change the solution composition of organic-based brain homogenate by evaporation of the organic solvents and reconstitution in aqueous solutions. In the study published by Su et al., brain tissue samples homogenized and precipitated by methanol were evaporated to dryness by vacuum freeze-drying. The residue was then reconstituted in water, which will be further extracted by the mixture of chloroform and isopropanol (10:3, v/v).[68] The reconstituted biological samples were purely aqueous and immiscible with the organic solvents. Another approach to facilitate the phase separation between the organic-based brain homogenate samples and organic LLE solvents is by dilution with water. By adding water to the system to adjust the organic/aqueous ratio, phase separation can also be achieved. In the study published by Hou *et al.*, brain tissue samples dissolved in methanol were diluted by a 4-fold volume of water before the extraction by *n*-hexane.[28] Similar approaches can also involve the LLE system composed of chloroform, methanol and water, with the volume ratio subject to adjustment to reach sharp phase separation.[8, 11, 39]

With properly pretreated brain homogenate samples and correctly chosen organic solvents, LLE can be initiated by the addition of solvents into the samples. In the "forward extraction", the extraction efficiency is primarily determined by the partition coefficient of the

analyte of interest between the aqueous and organic phase, which is defined as the concentration ratio between the two phases when partition equilibrium has been reached. Therefore, when such conditions as pH and temperature are fixed, the amount of analytes extracted into the organic phase is determined by the volume ratio between the organic solvent and the biological sample. In the LLE of analytes from brain tissue homogenate, the more organic solvent that is used, the higher the extraction efficiency that can be reached. Usually, at least 1 to 2 volumes of organic solvent are added to each unit volume of brain homogenate sample.[33] When the intended extraction efficiency cannot be achieved, one can increase the volume of organic solvent to extract more analytes. However, with more organic solvent added, more hydrophobic impurities are also extracted, which may cause matrix effects or instrument contamination in the later LC-MS/MS analysis. Therefore the volume of organic solvents needs to be optimized to reach maximum recovery with the least matrix effects. Similar principles can also be applied to "backward extraction" techniques. With more organic solvent added, more hydrophobic impurities can be extracted to yield a cleaner sample, which also increases the chance of compromising recovery by extracting more analytes. The addition of organic solvents can be finished in one step or multiple steps. Multiple-step extraction has been a commonly used strategy to increase extraction recovery.[39, 71, 84, 85, 91, 136] In a study for the determination of 5-fluorouracil and methotrexate in mouse brain, the LLE of analytes by 10-fold volume of fresh EA was conducted twice, with the resulting supernatant combined for further processing.[136] Compared to adding 20-fold volume of fresh EA at once, extraction with 10-fold volume of EA twice can further improve the recovery by extracting more analytes.

After the addition of organic solvents, the extraction system needs to be well mixed. LLE is a thermokinetic process that involves the diffusion of analytes (or impurities) from the aqueous

phase to the organic phase, which takes a certain period of time. Therefore, the extraction efficiency is highest after the distribution equilibrium of the analyte (or impurity) of interest has been reached, when the amount of analyte (or impurity) crossing the interphase will not increase any more. To facilitate the diffusion of the analyte (or impurity) of interest to reach its equilibrium, vortexing is usually used as the mixing technique. Since the aqueous biological samples are immiscible with the organic LLE solvents, turbulence from the vortexing will create small droplets of both phases and mix them together at a high speed. The formation of droplets can largely increase the interface area between the aqueous phase and the organic phase, facilitating the diffusion of solutes and shortening the time needed to reach equilibrium. Considering the requirement of time and mixing, 10 min of vortexing is typically needed for efficient LLE of biological samples by organic solvents.

After the distribution of the analyte or impurities of interest has reached equilibrium, separation of the organic and aqueous phases is needed to isolate the analyte of interest from unwanted impurities. Centrifugation is the most commonly used technique to fulfill the phase separation. Usually, at least 10 min of centrifugation above 2,000× *g* is needed to achieve sharp phase separation. If the brain homogenate has not been pretreated by PPT or the precipitates from PPT have not been removed, extended centrifugation at higher speed might be needed to turn the insoluble impurities into solid pellets. Then the phase separation can be easily fulfilled by transferring the wanted phase or removing the unwanted phase, while attention needs to be paid to the pipetting operations not to disturb the pellets. The relative positions of organic and aqueous phases are determined by the densities of both phases. Most common organic solvents used in LLE, n-hexane, DIPE, MTBE and EA for example, are lower than that of water, meaning that they will be the supernatant after the phase separation, which is easy to transfer or remove.

Chloroform and DCM, however, have densities higher than that of water and are usually the bottom layer in LLE. If they are used in "backward extraction", which means the aqueous phase is the one to be retained, phase separation is easier to fulfill by transferring the analyte-containing aqueous supernatant into a new tube.[8, 54, 68] If chloroform or DCM is used in "forward extraction" applications, the bottom organic layer needs to be transferred into a new tube, which might be hard to achieve, especially when PPT residues are still in the system. Different strategies have been employed to facilitate the phase separation without losing sensitivity or selectivity. One approach addressing this issue is multi-step transfer. In a study concerning the determination of metrifonate enantiomers in brain samples, Zimmer and coworkers developed an LLE method involving multiple liquid transferring steps. After vortexing and centrifugation following the addition of DCM, the organic layer was transferred to a centrifuge tube (A) and afterwards transferred in a second step to another centrifuge tube (B). The intermediate transfer of the extract to tube A was to remove small amounts of aqueous phase transferred together with the organic extract in the first step.[91] Another approach commonly used to facilitate phase separation is freezing, which utilizes the fact that the melting points of organic solvents are usually lower than that of aqueous solutions. In the study published by Golovko *et al.* concerning the quantitation of brain prostanoids, brain homogenate samples were extracted by chloroform. After being transferred to a new tube, the organic phase was cooled at -20 °C for at least 2 h. This step allowed the separation of any residual upper phase, which was frozen and easily removed before analysis.[17]

With the organic and aqueous phases separated from each other, the extraction of the analytes of interest or unwanted impurities can be considered finished. However, some additional steps may be needed before LC-MS/MS analysis, depending on the extraction strategies

involved. If the last step of extraction is "forward extraction", the analyte of interest will be present in the organic phase. Since pure organic solvents are strong eluents in reversed phase liquid chromatography (RPLC) and will largely weaken the analyte retention in the column, the analyte-containing organic extract cannot be directly injected into a reversed phase column. Also, large volumes of organic solvents are usually used in LLE, which means that analyte concentrations in the final extract are lower than those in the original samples. Direct analysis of such low-concentration extracts will greatly lower the method sensitivity. Considering both the LC separation and method sensitivity, evaporation and reconstitution are commonly used following LLE to prepare samples for LC-MS/MS conditions.[11, 13, 17, 28, 33, 39, 40, 47, 50, 71, 81, 83-86, 89, 91, 95, 136] After being separated from the aqueous sample residues, the organic extract is evaporated to complete dryness by nitrogen flow, vacuum centrifugation or lyophilization. The evaporated samples will be reconstituted using the initial mobile phase from the LC separation, which can be facilitated by vortexing or ultrasonication. Sample enrichment can be achieved by decreasing the volume of mobile phase added in this step, which can improve the method sensitivity. If there are any insoluble residues left after the evaporation, an extra centrifugation step can be added after the reconstitution to yield clean samples for LC-MS/MS analysis.[13, 28, 33, 39, 40, 54, 83-86, 95] If the last step of extraction is "backward extraction", the analyte-containing aqueous phase can be directly forwarded to using RPLC-MS/MS analysis, since water is a weak eluent and will not interfere the retention of analytes.[8, 68] However, evaporation and reconstitution can also be applied to the aqueous samples to achieve sample enrichment or the removal of insoluble impurities. In the study published by Onorato *et al.*, 1 mL of brain homogenate was used in the sample preparation by LLE. After the "backward extraction" by 10 mL of chloroform/methanol (2:1. v/v), the aqueous samples were blow-dried, reconstituted into

150 μL of 0.1% formic acid in 10 mM ammonium acetate/methanol (80:20, v/v) and centrifuged before LC-MS/MS analysis. These post-extraction steps not only removed insoluble impurities, but also improved the method sensitivity by approximately 7-fold.

Conventional LLE is widely used in the sample preparation of brain homogenate samples for quantitative LC-MS/MS studies, due to several significant advantages. First, it has higher specificity and selectivity compared to PPT. Based on the difference in hydrophobicity, analytes of interest can be separated from endogenous impurities in the biological samples. With the leverage of altering extraction solvents, pH and the choice of "backward" or "forward" extraction, the selectivity of LLE can be even improved. Theoretically, the only impurities that will remain in the extracts are compounds that have very similar hydrophobicity as that of the analytes at the specific pH used in LLE. Second, the cost of LLE is relatively low, since only common agents and instrumentations are needed. Third, the throughput of LLE can be increased when multiple samples are processed at the same time.

However, LLE also has several disadvantages preventing it from being used in all studies. First, the selectivity of LLE can only be considered as moderate, compared to PPT and SPE. If high recovery and throughput are pursued, the selectivity of LLE has to be compromised. Usually the final samples injected into the LC-MS/MS system still contain large amounts of impurities from the biological matrices. Second, LLE is a relatively labor-intensive sample preparation technique with repeated manual operations, including liquid transfer, capping, centrifugation and so on. Third, LLE often involves the addition of large volumes of organic solvents, leading to the dilution of analyte concentration. If the organic solvent is compatible with the LC-MS/MS method, the sensitivity of the method is compromised when directly analyzed by LC-MS/MS; if evaporation and reconstitution are employed, extra labor is needed

and the throughput is lowered. Fourth, the separation of two liquid phases can be difficult when an emulsion was formed during the vortexing process. Solutions such as protein precipitation pretreatment, choice of different solvents or the addition of emulsion inhibitors can be used here to avoid this issue. Considering all the advantages and disadvantages, LLE can be considered as fast sample preparation method with moderate recovery, selectivity and throughput.

Based on the essential principles of LLE, there are also different improved LLE-based techniques used in quantitative LC-MS/MS analysis for brain homogenate samples. Due to the fact that LLE is a relatively labor-intensive sample preparation technique, automated LLE has been developed to reduce the human labor and improve throughput. By the adoption of 96-well formatted microliter plates and, more importantly, robotic liquid-handling systems, LLE can be conducted with minimum manual operations. Up to 96 samples can be processed simultaneously, which largely increases the method throughput. In a study for the quantitation of reboxetine enantiomers in rat plasma and brain by LC-MS/MS, Turnpenny and Fraier used the automated LLE technique for sample preparation.[71] Pressurized liquid extraction (PLE) is another improved LLE technique that has been used in current studies. PLE is a new LLE-based sample preparation technique that employs elevated temperatures and pressures during extractions. The elevated temperature allows the sample to become more soluble and achieve a higher diffusion rate while the elevated pressure keeps the solvent below its boiling point. Therefore, the new technique PLE has the advantages of high extraction efficiency, less solvent usage and shorter extraction time. In the method published by Zhou *et al.* in 2012, PLE was conducted at 140 °C and 10.34 MPa to achieve high extraction efficiency.[89] They also used a dispersion agent in the PLE, which enhanced solute exchange and reduced the solvent volume. However, PLE also has its significant disadvantages. Since the extraction of each sample has to be done individually in a

pressurized extractor, the overall throughput of PLE is actually much lower than conventional LLE, though the extraction time for each sample is slightly shorter. Solid-supported liquid-liquid extraction or matrix-assisted liquid-liquid extraction has been reported as a new sample preparation technique based on LLE, which can be described as LLE with the assistance of inert sorbents.[59] Solid sorbents provide a surface for the aqueous sample to interact with the organic solvents, improving the extraction efficiency. More importantly, better recoveries and precision can be achieved by reducing the emulsions that are often formed in conventional LLE.

3.3 Solid-phase Extraction-based Sample Preparation

Solid-phase extraction (SPE) is a powerful sample preparation technique that can be used for the enrichment and purification of biological samples before LC-MS/MS analysis. The mechanism of SPE is similar to that of column liquid chromatography, which is based on the affinity between solutes dissolved in a liquid (mobile phase) and solid materials (stationary phase). Due to different physical and chemical properties, different components in the liquid sample have different affinities with the stationary phase in the SPE devices. Therefore, by passing the liquid sample through the stationary phase, either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase, so that separation of desired and undesired components can be achieved. The general strategy of SPE is based on the retention and elution of analytes of interest. If the mobile phase passing through the stationary phase contains the analytes of interest, it is collected for further preparation or LC-MS/MS analysis. If the mobile phase passing through the stationary phase contains only undesired impurities, which means the analytes of interest are retained on the column, the mobile phase passing through the stationary phase is discarded. Then the analytes can be eluted from the stationary phase when rinsed with a proper eluent. By separating the analytes of interest from

undesired impurities in different column eluents, sample preparation can be achieved with high selectivity.

Though different devices, both the stationary phase and mobile phase can be optimized in SPE to achieve different selectivity. The general procedures of SPE for brain homogenate samples are similar in most studies. Since brain homogenate samples contain large amounts of insoluble cell debris that may cause congestion of the SPE stationary phase, centrifugation is commonly applied to brain homogenate before SPE.[16, 36, 45, 56, 60, 65, 70, 74, 82] Endogenous proteins can cause serious issues including stationary phase congestion, nonspecific binding and matrix effects. Therefore, PPT is also used frequently as a sample pretreatment before SPE.[23, 24, 26, 30, 41, 53, 58, 64, 78-80] Depending on the different SPE stationary phases and the option of evaporation/reconstitution, either organic solvents or inorganic acids can be used as the PPT agents. After the centrifugation (evaporation and reconstitution in some studies), the supernatant can be forwarded to SPE. In most occasions, the SPE stationary phase needs to be properly prepared before the biological samples are loaded. The SPE stationary phase first needs to be rinsed with pure organic solvents, ACN and MeOH for example, as the activation step. Due to the low surface tension, organic solvents can quickly soak the stationary phase and pass through, dissolving and removing impurities trapped in the stationary phase during manufacturing. This step is also important for removing air in the packing material to maximize the extraction efficiency of SPE. Then the SPE stationary phase is subjected to the equilibrium step, during which a weak eluting agent is flushed through to prepare the stationary phase into proper conditions for the retention of analytes. After the stationary phase is activated and conditioned, biological samples are loaded and allowed to pass through the stationary phase slowly by gravity. Typically in conventional SPE, the analytes of interest need to be retained on the

stationary phase, which is then subjected to one or two rounds of washing steps. Weak eluting agents are applied to remove unretained impurities from the stationary phase, while the retention of the analytes of interest should be minimally affected. During all the activation, condition, sample loading and washing steps, the flow rate of liquid passing through the stationary phase should be maintained dropwise. The flow must be stopped when the liquid level reaches the surface of the packing material to prevent the stationary phase from drying, except for the last washing step after which the stationary phase is completely dried. Finally, the analytes are eluted by applying a strong eluting agent, which is optimized to maximally elute the analytes of interest but minimally elute hydrophobic impurities retained on the stationary phase. If the SPE protocol is properly optimized, the analytes of interest are retained on the stationary phase as a short plug. By eluting the analytes with small amounts of eluting agents, sample enrichment can be achieved to improve sample sensitivity and fulfill trace level detection. Multiple eluting steps with small amounts of eluting agents can be used to achieve higher analyte recovery. If the solution condition is compatible with the LC method, the eluent containing the analytes of interest can be directly analyzed by LC-MS/MS.[64, 80] In most occasions, however, a round of evaporation and reconstitution is employed.[16, 24, 26, 27, 30, 36, 41, 43, 45, 53, 56, 58, 60, 65, 70, 74, 78, 79, 82, 117] Since the eluent condition is optimized to minimize the retention of analytes, direct injections of such solutions into the LC-MS/MS system may result in poor separation, if the retention mechanism of the SPE method is similar to that of the LC method. By evaporating the eluent and reconstituting in an LC-compatible solution, better LC separation can be achieved. In addition, the adjustment of reconstituting volume can be utilized to improve the method sensitivity, especially when the eluent volume is bigger than the original samples. An extra centrifugation step can be added here to further eliminate insoluble impurities for the protection of the LC column and instruments.[78, 82]

Many types of SPE have been developed based on different chemistry. Physical and chemical properties of the analytes of interest, including hydrophobicity, hydrogen bonding and pKa, should be considered during method development when using SPE-based sample preparation. The type of chemistry and all the solutions or solvents used in SPE can be optimized to achieve high recovery and selectivity. Normal phase, reversed phase and ion exchange are commonly used SPE chemistries.

Due to the fact that most drugs, metabolites or endogenous compounds found in brain tissue are hydrophobic compounds, reversed phase is the most commonly used SPE chemistry in the sample preparation of brain homogenate samples.[16, 23, 24, 26, 36, 45, 58, 70, 80, 82, 117] Reversed phase SPE separates analytes and impurities based on their polarities, providing selectivity for nonpolar analytes. Nonpolar analytes or impurities will be retained on the stationary phase, while polar impurities will pass through. The affinity between the analytes and the stationary phase is based on pi-pi bonding and hydrophobic interactions. The stationary phase of reversed phase SPE is usually made of a silica or polymeric sorbent derivatized with varying length hydrocarbon chains. Similar to LC columns, C4, C8 and C18 are common stationary phases for reversed phase SPE. Longer hydrocarbon chains can provider better selectivity for more nonpolar analytes. Based on the mechanism of reversed phase SPE, water is the weak solvent, which should be used in the conditioning, sample and washing solutions to achieve better analyte retention on the stationary phase. Brain tissue samples homogenized or precipitated by organic solvents often need to be centrifuged, evaporated and reconstituted in an aqueous solution.[58, 80] Dilution with water or aqueous solutions can also be used to lower the percentage of organic solvents.[26] In contrast, organic solvents, ACN and MeOH in most applications, are the strong eluting solvents. Therefore, organic solvents are the strong solvents and should be used in the

final eluting solutions, which can maximize the amounts of analytes eluted from the stationary phase and maximize recovery. In a study published by Liu *et al.* in 2011, a reversed SPE-based sample preparation method was developed. Since the analyte hydralazine was a hydrophobic compound under neutral pH, an ODS-C18 SPE cartridge was chosen. After the cartridge was activated with 3 mL of MeOH and balanced with 3 mL of water, the biological sample in an aqueous solution was loaded. Following a washing step with 1 mL of 10% MeOH, the analyte was eluted with 1 mL of 80% MeOH. In this method, pure water and low percentage organic solvents were used to maintain the retention of the analyte. Unretained hydrophilic impurities were washed off by the weak wash (10% MeOH), while the strong wash (80% MeOH) was used for the elution of analytes.[45] Besides the polarity of solvents, pH is another important condition to adjust in reversed phase SPE. The general principle is that uncharged analytes (or impurities) can be retained on the reversed stationary phase, while charged analytes (or impurities) have high polarity and therefore cannot be retained. Accordingly, retention of the analytes of interest or undesired impurities can be adjusted by altering the charge states under different pH conditions. In a study publish by Goldwirt *et al.* in 2012, a reversed phase SPE sample preparation method was used to prepare brain homogenate samples for LC-MS/MS analysis. Both analytes, irinotecan and 7-ethyl-10-hydroxycamptothecin (SN-38), are hydrophobic compounds that are uncharged at low pH. Besides the selectivity provided by the adjustment of solvent polarity, ammonium acetate buffer (10mM, pH 3.5) was used for both conditioning and washing, in which the analytes are uncharged and well retained on the stationary phase.[16] Reversed phase SPE has been widely used in the sample preparation of biological samples due to its multiple advantages. Its selectivity for nonpolar analytes is suitable for most drugs, metabolites and endogenous compounds in brain tissue. Because hydrophobic interactions are

the major driving force in reversed phase SPE, method development is relatively simple, which is primarily fulfilled by use of different types and percentages of organic solvents. However, it also has some significant disadvantages, which need to be considered during the method development. Brain tissue has large amounts of hydrophobic lipids, proteins and other endogenous compounds that may co-elute with the analytes, causing matrix effects and instrument contamination in LC-MS/MS analysis. These impurities sometimes cannot be fully removed by method optimization. Another issue with reversed phase SPE is its incompatibility with organic PPT and reversed phase LC, which requires additional evaporation and reconstitution steps before and after the SPE. Throughput of the method will be compromised when such extra operations are added.

In addition to the conventional reversed phase SPE, hydrophilic-lipophilic-balanced (HLB) SPE is a newer technique based on reversed phase SPE. By using sorbents that have both hydrophobic and hydrophilic function groups, such SPE stationary phases can be used for the separation of a wide range of acids, bases and neutrals. In a study published by Hooff and colleagues in 2010, SPE using HLB cartridges was used as the sample preparation of brain homogenate for the quantitative LC-MS/MS analysis of farnesyl-(FPP) and geranylgeranylpyrophosphate (GGPP). The extract was finally eluted with an ammonium hydroxide/propanol/n-hexane mixture (1:7:12, v/v/v), which lowered the pH and increased the organic solvent composition for the maximum elution of analytes.[27] HLB SPE is a powerful sample preparation technique, due to its dual selectivity and wide compatibility. High recovery and sensitivity can be achieved by using HLB SPE, especially when simultaneous measurement of multiple analytes with different properties is needed.

Normal phase SPE can provide selectivity for polar compounds, which is opposite to that of reversed phase SPE. A stationary phase of underivatized silica or functionally bonded silica with short carbons chains is commonly used in normal phase SPE. The retention of analytes on the stationary phase is on the basis of dipole-dipole interactions and hydrogen bonding, creating affinity between polar analytes and the stationary phase. In typical normal phase SPE, polar analytes and impurities are retained on the stationary phase, while nonpolar impurities can pass through with little or zero retention. In contrast with reversed phase SPE, nonpolar organic solvents are the weak solvents in normal phase SPE, which can be used for conditioning, sample loading and washing; water and polar solvents are the strong solvents, which are used for the elution of analytes in the final step. In a study published by Richardson *et al.* in 2007, a normal phase SPE method was used to prepare brain homogenate samples for LC-MS/MS analysis.[60] The analytes, endocannabinoids and related compounds, are a series of nonpolar compounds that have only weak retention in normal phase SPE, so that silica SPE cartridges were used for the cleanup of polar impurities. After samples were loaded onto the cartridges in chloroform, 3 × 1 mL of chloroform was used to wash unretained nonpolar lipids. Then the analytes were eluted in three steps: 2% methanol in chloroform (2 × 1 mL), 2% methanol and 0.2% TEA in chloroform (4 × 1 mL), and 2% methanol and 0.05% TFA in chloroform (4 × 1 mL), which altered the solvent polarity and pH to achieve maximum analyte recovery.

Ion exchange SPE is another commonly used SPE technique that provides selectivity for charged analytes. The separation of the analytes of interest from the undesired impurities is based on electrostatic interactions between the solute ions and the charged sorbents. At a pH where the stationary phase and the analytes of interest are oppositely charged, the analyte ions are retained on the stationary phase via ion exchange mechanisms, while uncharged impurities

will pass through the stationary phase with little or zero retention. To elute the analytes of interest from either the sorbents, the stationary phase is washed with a solvent that neutralizes the charge of the analyte, the stationary phase, or both. If charged functional groups are mixed with hydrophobic carbon chains and hydrophilic function groups of the sorbent, hydrophobic selectivity can also be added to the stationary phase. This results in mixed-mode ion exchange stationary phases, which are now widely used in the majority of ion exchange SPE techniques. Therefore, both pH and percentage of organic solvents in the mobile phase can be adjusted to achieve optimal sample preparation performance. Based on the different selectivity, ion exchange SPE can be divided into two major categories, anion exchange SPE and cation exchange SPE. Anion exchange SPE uses a positively charged stationary phase, which can provide selectivity for negatively charged ions, such as acids. Strong anion exchange sorbents contains permanently charged quaternary ammonium groups, providing affinity for moderate to weak bases; weak anion exchange sorbents have amine groups which are charged at pH below 9, providing affinity for strong acids. The washing and eluting conditions are summarized in Table 2.2. In a study published by Shah *et al.* in 2008, mixed-mode strong anion exchange SPE was used for the preparation of brain slice superfusion samples. Because all the analytes, N-acetylaspartate, N-acetylaspartylglutamate and glutamate, were weak acids, mixed-mode strong anion exchange was chosen as the SPE chemistry. After the stationary phase was balanced with 1M phosphate buffer (pH 6.5), superfusion samples in 0.1 M ammonium hydroxide were loaded, followed by a two-step wash with 2 mM phosphate buffer (pH 6.0) and deionized water. The high pH of the sample solution and the slightly acidic pH in the washing solutions were both well above the pKa of the analytes, meaning that the analytes were negatively charged and therefore retained on the stationary phase. The bound analytes were eluted from the SPE column

using a mixture of 0.3 M HCl and methanol (80:20, v/v).[64] The pH of the eluting solution was low enough to change the analytes into their uncharged state, disrupting the ionic exchange interactions. Together with the increase of organic solvent composition, the analytes of interest were eluted from the stationary phase to the largest extent. In contrast to anion exchange SPE, cation exchange SPE uses stationary phases bearing negatively charged functional groups, which can provide specificity for positively charged analytes, such as bases and amines. When loaded on the stationary phase, positively charged analytes and impurities can be retained via electrostatic interactions, while uncharged impurities will pass through with little or no retention. Strong cation exchange sorbents contain aliphatic sulfonic acid groups that are always negatively charged, providing affinity with moderate to weak bases. The retention or elution is adjusted by the change of charge states of the analytes. In contrast, weak cation exchange sorbents contain aliphatic carboxylic acids that are charged when the pH is above 5, which is more specific for strong bases or quaternary amines. The retention or elution is adjusted by the change of charge states of the stationary phase, shown in Table 2.2. In a study concerning the determination of dihydroetorphine in rat plasma and brain samples, Ohmori and colleagues develop an SPE-based method to prepare biological sample for LC-MS/MS analysis. Since the analyte of interest has a tertiary amine group and is positively charged at low pH, mixed-mode strong cation exchange SPE was used. Considering organic solvents can weaken the retention of analytes on the mixed-mode SPE sorbent, brain homogenate samples pretreated by MeOH PPT were diluted with 6-fold volume of 50 mM phosphate buffer (pH 6.0). After the SPE cartridges were washed sequentially with 3 mL of methanol and 3 mL of 50 mM phosphate buffer (pH 6.0), the diluted samples were loaded. Then the sorbent was washed with 3 mL of 100 mM acetic acid, which removed neutral, negatively charged and hydrophilic impurities that were not retained. The low pH maintained the

analyte in its positively charged state and did affect its retention. This was followed by another wash with 2 mL of methanol, which removed neutral or negatively charged impurities that were retained solely by hydrophobic interactions. Finally, the analyte was eluted using 4 mL of 2% (v/v) ammonium hydroxide in ethyl acetate.[53] At high pH, the positive charge on the analyte was neutralized and the ion exchange interactions no longer existed. Together with the hydrophobic interactions compromised by the organic solvent, complete elution of the analyte was achieved for high extraction recovery. Ion exchange SPE has been widely used to prepare brain homogenate samples for the LC-MS/MS analysis of charged analytes. Especially for strong acids and bases, which cannot be well extracted by reversed phase or normal phase SPE, ion exchange SPE can provide good selectivity as well as high analyte recovery. Moreover, the additional selectivity based on hydrophobicity makes mixed-mode ion exchange SPE more powerful in terms of removing undesired impurities.

SPE can also be integrated with other sample preparation techniques. In a study for the determination of phosphodiesterase-5 inhibitors and their main metabolites in rat brain tissue, Unceta *et al.* used HybridSPE-PPT cartridges (Supelco, Bellefonte, USA) in the sample preparation.[72] After adding brain homogenate and protein precipitant into the cartridges, the sample preparation was finished by vortexing and vacuum eluting. The SPE sorbent worked as a filter, removing precipitated proteins. It also worked in a manner of reversed phase SPE, strongly binding and removing hydrophobic lipids from the brain homogenate. By integrating PPT and SPE in one step, endogenous proteins and lipids can be effectively removed with high throughput.

Besides the different chemistries that can be used to achieve analyte-specific sample preparation, there are also different formats of SPE that can be chosen for the consideration of

cost, throughput and performance. Cartridges and manifolds are the most commonly used SPE devices.[16, 24, 26, 27, 30, 36, 41, 43, 45, 53, 56, 58, 60, 65, 70, 74, 78-80, 82] The sorbents are packed in syringe-shaped cartridges that are available in different sizes and chemistry. These disposable cartridges can be mounted on the ports of a manifold, which can be connected to vacuum via a control valve to facilitate the flow of mobile phase. Alternatively, positive pressure can also be used for the same purpose.[60] Waste and samples are collected by test tubes located in the manifold. The biggest advantage of such configurations is the low cost, due to the low prices of cartridges and manifolds. However, the disadvantage of low throughput is also very significant. Multiple steps of liquid transfer and replacement of collecting test tubes require intensive labor. In addition, the size of the manifold, commonly 24-port at most, largely limit the number samples that can be simultaneously processed and therefore decreases throughput. To improve the throughput of SPE, 96-well plate and automatic liquid handling robots have been introduced in recent years. However, the high cost of such configurations prevents it from being widely used. SPE spin-columns are another format of SPE devices, which are made by packing sorbents into small columns with a collection chamber. By applying centrifugation instead of vacuum, the SPE processing time can be reduced. Simultaneous processing of multiple samples also contributes to its high throughput. Moreover, smaller amounts of solvents are needed for SPE spin-columns, which can enrich the analytes of interest and therefore improve the method sensitivity.[64] Similar improvements have also been made by the invention of SPE tips, which put SPE sorbents in the pipette tips. SPE in a smaller scale with higher sensitivity can be achieved using SPE tips. Throughput, however, is lowered, since each sample has to be processed individually. [117] Online SPE can be considered as the most advanced SPE technique, due to its capability of complete automation. The column balancing, sample loading, washing and analyte eluting procedures are

all completed automatically with the use of an online SPE column, and more importantly, a column switching system equipped with multiple solvent pumps. In a study published by Heinig and Bucheli in 2008, an online SPE system was used for the sample preparation of biological samples, which is directly coupled with the LC-MS/MS system.[23] Manual operations are minimized in online SPE, greatly improving the method throughput. Nevertheless, the method development can be very difficult, which involves not only condition optimization but also instrument engineering. Moreover, high cost of instrumentation is another significant limitation of online SPE.

SPE is a commonly used technique for the sample preparation of brain homogenate for quantitative LC-MS/MS analysis. Several advantages have made it a very powerful sample preparation technique. First, SPE is compatible with a very wide range of analytes by using different SPE chemistries. Specific and efficient analyte extraction can be achieved by SPE to purify most compounds, whether the analytes of interest are polar, nonpolar, basic or acidic. Second, SPE can be optimized to be very specific for the analytes of interest. By optimizing proper washing and eluting conditions, the analytes of interest can be separated from the biological sample with minimal amounts of co-eluting impurities. Such issues as matrix effects, LC peak distortion, column congestion and instrument contamination can be minimized when employing a specific SPE method, which is of great importance for complex biological samples like brain homogenate. Third, high recovery can be achieved by SPE, thanks to its wide compatibility and high specificity. Fourth, sample enrichment can be fulfilled by SPE, which is hard to perform by PPT or LLE. Fifth, SPE can be automated to largely decrease manual operations. However, SPE also has some significant disadvantages. The most crucial disadvantage of SPE is its low throughput, compared to PPT and LLE. Unless automated SPE

systems are used, multiple steps of liquid transfer, evaporation and reconstitution are often involved, making conventional SPE a labor-intensive and low-throughput sample preparation technique. Another disadvantage of SPE is its complexity. Since there is no universal SPE protocol available, large amounts of extraction conditions and parameters need to be optimized during the method development, which can be very costly and time-consuming. Last but not least, SPE has a much higher cost than that of PPT or LLE, due to the cost of the consumable materials and equipment of SPE. With all the advantages and disadvantages, SPE can be considered as a highly specific but more complex technique in sample preparation method development. Due to its high performance in recovery, specificity and compatibility, SPE can be used when a satisfactory sample preparation cannot be achieved by simpler techniques like PPT and LLE. SPE can be an ideal sample preparation technique in situations that require simultaneous measurement of multiple very different analytes, thorough cleanup of highly complex biological samples or fully automatic sample analysis.

4. Summary

Research on the ADME and mechanism of action of small-molecule drugs, metabolites and biomarkers in the CNS are largely dependent on the distribution and dynamics of such chemicals in the brain tissue. Methods to accomplish this must be capable of quantitating of concentrations of one or a series of analytes. Among all the current techniques, LC-MS/MS has been widely used for the concentration measurements of small-molecule analytes in brain tissue samples. In order to effectively measure analytes in brain tissue using LC-MS/MS, brain tissue samples need to be obtained from the test individual and prepared into an LC-MS/MS compatible status, making sample collection and sample preparation two essential steps in bioanalysis.

Traditional sample collection involves sacrifice of the test individual, surgical removal of the brain tissue and physical homogenization in a proper media, generating a homogeneous brain tissue suspension. This is a low-cost, convenient but rough sample collection technique, yielding a brain homogenate that is extremely complex. In contrast, microdialysis, ultrafiltration and SPME are more advanced sample collection techniques, which involve the *in situ* extraction of analytes from the brain of a live test animal. Though these techniques can yield very clean samples, they all have some significant disadvantages limiting them from being widely used in all studies. Therefore, it is suggested that homogenization is the primary sample collection technique to be considered in most occasions. Only when the large quantities of interferences introduced by brain homogenate severely affect the method performance, one should consider microdialysis, ultrafiltration or SPME for sample collection.

Sample preparation is of crucial importance for brain sample analysis by LC-MS/MS, not only due to its capability of preparing the tissue sample into an injectable liquid that is LC-MS/MS compatible, but also because of its capacity of removing impurities that may cause column congestion, matrix effects, signal interference and instrument contamination. Protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are common sample preparation techniques, which are listed in their order of ascending selectivity. However, cost, labor and processing time of these three techniques also increase in the same order. When a new sample preparation method needs to be developed for the extraction of a certain analyte from the brain tissue samples, one needs to consider the choice of a sample preparation technique or combinations of multiple techniques based on the properties of the analytes, biological matrices and the expectations of the method. All the advantages and disadvantages of these available sample preparation techniques need to be taken into account. Different materials and

conditions can be adjusted to achieve the optimal balance between the analyte recovery and the selectivity. A proper sample preparation method should maximally remove the impurities under the prerequisite condition of losing minimal amounts of the analytes of interest.

Different sample collection and sample preparation techniques have different characteristics, allowing the analysts to choose or combine the optimal techniques in the method development. Disadvantages and disadvantages of all the sample collection and preparation techniques discussed in this paper are summarized in Table 2.3. By using proper sample collection and sample preparation techniques, clean, enriched and representative liquid samples can be obtained for LC-MS/MS analysis, contributing to a sensitive, selective and reproducible method for the quantitation of small-molecule analytes in the brain tissue. With no universal technique that can fit every bioanalytical scenario, one should always carefully choose the most proper sample preparation strategies for the analytes of interest, by comprehensively considering analyte properties, recovery, selectivity, throughput, cost and complexity during method development.

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Table 2.1 Partition coefficient (LogP3), density and boiling point values of common LLE solvents at 25 °C, 1 atm.

	LogP3	Density (g/mL)	Boiling point (°C)
Ethyl acetate	0.7	0.897	77.1
Methyl *tert*-butyl ether	0.9	0.735	55.2
Dichloromethane	1.5	1.326	39.8
Diisopropyl ether	1.5	0.725	68.5
Chloroform	2.3	1.483	61.2
n-Hexane	3.9	0.655	68.7

Table 2.2 Summary of four commonly used ion exchange SPE techniques.

Chemistry	Stationary Phase	Analyte Selectivity	Washing Conditions	Eluting Conditions
Strong Anion Exchange	Quaternary Ammonium	Acids (Weak)	High pH Low Organic	Low pH High Organic
Strong Cation Exchange	Aliphatic Sulfonic Acid	Bases (Weak)	Low pH Low Organic	High pH High Organic
Weak Anion Exchange	Amine	Strong Acids	Low pH Low Organic	High pH High Organic
Weak Cation Exchange	Aliphatic Carboxylic Acids	Strong Bases Quaternary Amines	High pH Low Organic	Low pH High Organic

Table 2.3 Summary of advantages and disadvantages of all the sample collection (A) and sample preparation (B) methods discussed in this paper.

A.

	Advantages	Disadvantages
Homogenization	Wide compatibility; Low material and instrument cost; Loss-less sampling technique; High precision and accuracy.	Higher chance of contamination; Spiked samples different from real samples; Single data point from one test animal; Extensive sample preparation needed;
Microdialysis	Direct *in vivo* sampling; Continuous sampling from live animals; None or minimum sample preparation; Fewer animals and less surgical operations; Automation capability.	Small-molecule analytes only; Semi-quantitative technique; Only measures free drug fraction; Low precision and accuracy; Low temporal resolution. High cost.
Ultrafiltration	High precision and accuracy; Direct *in vivo* sampling; Continuous sampling from live animals; None or minimum sample preparation; Fewer animals and less surgical operations; Automation capability.	More suitable for small-molecule analytes; Membrane fouling; Only measures free drug fraction; Limited sample volume and data points; Nonspecific binding; Low temporal resolution. High cost.

Solid-phase microextraction	Direct *in vivo* sampling; Sample enrichment; Continuous sampling from live animals; None or minimum sample preparation; Fewer animals and less surgical operations; Automation capability.	Semi-quantitative technique; Saturation; Nonspecific binding; Only measures free drug fraction; Limited data point density;; High cost.

B.

	Advantages	Disadvantages
Protein precipitation	Low cost; High throughput and low labor intensity; High precision and accuracy; High recovery due to minimum loss of analytes.	Further sample preparation often needed; Sample dilution due to addition of agents; Non-specific adsorption; Matrix effects caused by remaining impurities.
Liquid-liquid extraction	Higher selectivity over protein precipitation; Low cost; Multiple adjustable factors to achieve specificity; Automation capability.	Cannot remove all the impurities; Labor-intensive; Evaporation and reconstitution often needed; Emulsion; Matrix effects caused by remaining impurities.
Solid-phase extraction	Wide analyte coverage; High specificity; High recovery achievable with proper conditions; Automation capability.	High cost; Low throughput; Labor-intensive if not automated;. Extensive method development needed

CHAPTER 3

QUANTITATION OF COTININE AND ITS METABOLITES IN RAT PLASMA AND BRAIN TISSUE BY HYDROPHILIC INTERACTION CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (HILIC-MS/MS)

Pei Li, Wayne D. Beck, Patrick M. Callahan, Alvin V. Terry, Jr., Michael G. Bartlett. 2012. *Journal of Chromatography B*. 907: 117-125.
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Abstract

In this work, we developed a sensitive method to quantify cotinine (COT), norcotinine (NCOT), *trans*-3'-hydroxycotinine (OHCOT) and cotinine-N-oxide (COTNO) in rat plasma and brain tissue, using solid phase extraction (SPE), hydrophilic interaction liquid chromatography (HILIC) and tandem mass spectrometry (MS/MS). The linear range was 1–100 ng/ml for each analyte in rat plasma and brain homogenate (3-300 ng/g brain tissue). The method was validated with precision within 15% relative standard deviation (RSD) and accuracy within 15% relative error (RE). Stable isotope-labeled internal standards (IS) were used for all the analytes to achieve good reproducibility, minimizing the influence of recovery and matrix effects. This method can be used in future studies to simultaneously determine the concentrations of COT and three major metabolites in rat plasma and brain tissue.

Key words

Cotinine; norcotinine; *trans*-3'-hydroxycotinine; cotinine-N-oxide; plasma; brain; hydrophilic interaction chromatography; tandem mass spectrometry.

1. Introduction

Cotinine (COT), the primary metabolite of nicotine (NIC) in humans and other mammalian species, is currently being evaluated as a prototypical therapeutic agent for Alzheimer's disease (AD) and related neurodegenerative disorders. Like nicotine, cotinine been observed to have positive effects on attention, working memory, and other domains of cognition in animal models [1-3]. In addition, both *in vitro* and *in vivo* studies suggest that COT might have disease-modifying effects (i.e., neuroprotective effects and the ability to delay disease progression) in conditions like AD. For example, COT protects against toxic insults in PC12 cells in culture with potency similar to that of nicotine [1, 3] and it was found (when administered chronically) to prevent memory loss in transgenic (Tg) 6799 AD mice as well as to stimulate the Akt/GSK3β pathway and reduce Aβ aggregation in their brains [4]. As a potential therapeutic agent, COT also appears to have several advantages over nicotine. For example, COT has a longer biological half-life (15-19 hours) and lower toxicity (mouse oral LD50 = 1604 mg/kg) than nicotine (half-life = 2-3 hours, mouse oral LD50 = 50 mg/kg) as well as less addictive potential [3].

COT can be further metabolized into several downstream metabolites, among which norcotinine (NCOT), *trans*-3'-hydroxycotinine (OHCOT) and cotinine-N-oxide (COTNO) are of interest for similar pharmacological activities and therapeutic potential in AD. In addition, determination of these compounds can also provide distribution and metabolism information for COT.

In order to facilitate further investigations into the effects of COT and its metabolites on the central nervous system (CNS), a sensitive method that can simultaneously quantify these compounds in both plasma and brain tissue is needed. With the determination of the actual

concentrations in plasma and brain, blood-brain barrier (BBB) permeabilities, efficacies and toxicities of COT and the metabolites can be assessed in animal studies.

As COT can be used as a biomarker of tobacco exposure, numerous liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been reported for the quantification of COT and its metabolites in a variety of biological fluids, i.e. plasma [5-8], serum [9-11], urine [5, 12-14], saliva [15, 16], whole blood [17] and breast milk [18]. Plasma is the most widely used species in animal tests, due to the high drug concentrations and easy accessibility. Because of the great differences in polarity and pKa of COT, NCOT, OHCOT and COTNO (shown in Figure 3.1), very few current methods have simultaneous determination of all four analytes with good sensitivity [5]. Moreover, some of the LC-MS/MS methods for plasma COT require a large sample volume (1 mL) [5, 7] or complicated sample preparation [5] to achieve high sensitivity.

Though plasma concentration can provide information about drug exposure, brain tissue concentrations are also of great importance for such drugs as COT and its metabolites, whose targeting site is the brain. However, there are very limited current quantitation methods for brain tissue. Several gas chromatography-mass spectrometry (GC-MS) methods were reported for NIC and COT quantitation in brain tissue [19-21], the lowest limit of detection (LOD) of COT among which was 10 ng/g [21]. The first LC-MS/MS methods for COT and metabolites quantitation in human brain was reported by Shakleya and Huestis [22], with the linear range 25 – 5,000 ng/g for COT and 50 – 5000 ng/g for OHCOT. Recently Vieira-Brock and coworkers reported an LC-MS/MS method of simultaneous quantification of NIC and all the metabolites, including COT, NCOT, OHCOT and COTNO, in rat brain, with the linearity of 25-7,500 ng/g [23]. However,

cotinine metabolites, NCOT, OHCOT and COTNO, in real samples were not detected in these studies, due to their sensitivities.

Hydrophilic interaction chromatography (HILIC) is a type of partition chromatography first introduced by Alpert in 1990 [24]. Its specificity for polar compounds, high organic mobile phase, low buffer concentrations and early elution of hydrophobic impurities make it a good choice for LC-MS/MS quantitation of polar analytes in biological samples [25]. HILIC-MS/MS has been reported for its application for quantitation of NIC, COT and metabolites in biological fluids, due to the high polarities of NIC and COT [9, 26, 27]. HILIC can also be applied with other chromatographic techniques, like capillary LC, to achieve higher sensitivities for the quantitation of COT and metabolites [28]. However, there have not been any HILIC-MS/MS methods for the simultaneous determination of COT and all its major metabolites in plasma or brain.

In this study, we developed and validated a HILIC-MS/MS method for the simultaneous quantitation of COT, NCOT, OHCOT and COTNO in rat plasma and brain tissue. This method was used to quantify COT and its metabolites in preclinical studies on rats, to study the distributions and activities of these compounds for AD therapy.

2. Experimental

2.1 Chemicals and reagents

(-)-Cotinine (COT) was purchase from Sigma-Aldrich (St. Louis, MO). (*R,S*)-norcotinine (NCOT), *trans*-3'-hydroxcotinine (OHCOT) and cotinine (*S*)-cotinine-N-oxide were from Toronto Research Chemicals (Toronto, Canada). Chemical structures of analytes are shown in Figure 3.1. Stable isotope labeled internal standard (IS) (±)-Cotinine-D3 solution (1mg/mL in methanol) was obtained from Cerilliant (Round Rock, Texas). (*R,S*)-norcotinine-d4 (NCOT-d4),

trans-3'-hydroxycotinine-d3 (OHCOT-d3) and (*R,S*)-cotinine-N-oxide-d3 (COTNO-d3) were purchased from Toronto Research Chemical (Toronto, Canada). Trichloroacetic acid and ammonium acetate were bought from Baker (Phillipsburg, NJ). Formic acid was from Sigma (St. Louis, MO). Acetonitrile, methanol and water were from Fisher (Pittsburgh, PA) as HPLC/ACS grade.

2.2 Instrumentation

LC-MS/MS analysis was performed by using an Agilent 1100 binary pump HPLC system (Santa Clara, CA) interfaced to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer with an ESI(+) source (Milford, MA). Instrument control was carried out with Masslynx 4.0 software by Waters (Beverly, MA).

2.3 LC-MS/MS conditions

The analytes were separated on a Phenomenex Kinetex™ HILIC column (50 × 2.1 mm ID, 2.6 μm) coupled with a SecurityGuard™ ULTRA HILIC guard column for HILIC UHPLC, sub-2 μm and core-shell columns with 2.1mm internal diameters (ID). Mobile phase A was 10mM ammonium formate aqueous buffer with 0.1% formic acid and mobile phase B was acetonitrile (ACN). After an injection of 10 μL for each sample into the column, analytes were separated with the following gradient (time/minute, % mobile phase B): (0, 95), (8, 50), (8.1, 95), (15, 95). Flow rate was set at 0.3 mL/min and column temperature was 25 °C. The LC system was interfaced by a six-port divert valve to the mass spectrometer, introducing eluents from 1.0 to 6.0 min to the ion source. The autosampler injection needle was washed with methanol after each injection.

The mass spectrometer was operated in positive ion ESI mode. Nitrogen was used as the desolvation gas at a flow rate of 500 L/h and a temperature of 500 °C. The cone gas flow was set

to 20 L/h. Argon was the collision gas and the collision cell pressure was 3.5×10^{-3} mbar. The source temperature and capillary voltage were set at 120 °C and 3.5 kV, respectively. Multiple reaction monitoring (MRM) functions were used for the quantification of analytes. The cone voltage was 20 V and collision energy was 20 eV. Ion transitions monitored for analytes were 177-80 for COT, 163-80 for NCOT, 193-80 for OHCOT and 193-96 for COTNO. Ion transitions for IS were 180-80 for COT-d3, 147-84 for NCOT-d4, 196-80 for d3-OHCOT and 196-96 for d3-COTNO.

2.4 Solutions and standards

Individual stock solutions of all the analytes and IS were prepared by dissolving 1.0 mg of compounds in 1.0 mL of methanol to obtain drug concentrations of 1.0 mg/mL, except for COT-d3, which came in as 1.0 mg/mL methanol solution. Combined working solutions were obtained by serial dilution with 90% ACN/water (v/v 9/1). Standard working solutions containing COT, NCOT, OHCOT and COTNO were prepared at concentrations of 10.0, 20.0, 50.0, 100.0, 200.0, 500.0 and 1000.0 ng/mL. Quality control (QC) working solutions were 10.0, 30.0, 300.0 and 750.0 ng/mL. IS working solutions containing COT-d3, NCOT-d4, OHCOT-d3 and COTNO-d3 were prepared at a single concentration of 500.0 ng/mL in the same solvent. Stock solutions were kept at -20 °C when not in use.

2.5 Spiked samples and real samples

Blank rat plasma with sodium EDTA was purchased from Bioreclamation (Westbury, NY). Blank brains were obtained from drug-free control rats and homogenized with two volumes of water to obtain blank brain homogenate. 10 μL of standard or QC working solution was spiked into 100 μL of plasma or brain homogenate to generate corresponding standard or QC

samples. The final concentrations of calibration standards were 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL in plasma or brain homogenate. The QC samples were 3.0, 30.0 and 70.0 ng/mL.

Real samples were obtained from 1 mg/kg subcutaneously dosed rats after 30 minutes of pretreatment. Plasma was collected via cardiac puncture and transferred to EDTA vacutainers. Brain samples were homogenized in the same manner as blank brain.

All biological samples were stored at -20 °C before use. Fresh standards and QC samples were prepared for each day of validation.

2.6 Sample preparation

Sample preparation was carried out by protein precipitation and solid phase extraction (SPE). Each 100 μL of plasma or brain homogenate was added to 10 μL of IS working solution (500.0 ng/mL), 800 μL of water and 100 μL of 25% (w/v) TCA. The mixture was vortexed for 10 min and then centrifuged at 4500 × g for 10 min to remove the proteins.

An Oasis MCX SPE cartridge from Waters (Milford, MA) was conditioned with 1 mL of methanol and equilibrated with 1 mL of water. The supernatant from protein precipitation was loaded onto the cartridges and allowed to flow by gravity. Then the cartridge was washed twice by 1 mL of 5% methanol, 5% formic acid in water (v/v), followed by vacuum drying for 5 min. Analytes were eluted by 1 mL of fresh 20% methanol, 5% ammonia in water (v/v). The eluent was evaporated to complete dryness in a centrifuge evaporator at 50 °C. The sample was reconstituted by 100 μL of 95% ACN/water (v/v 9/1) with 2% formic acid and ready for injection.

2.7 Method validation

Linearity was tested by spiked standard as well as blank biological samples, since endogenous COT was observed in blank matrices. Calibration curves were made from peak area

ratios between analytes and IS, using 1/x weighted linear regression. The intra-day (n = 5) and inter-day (n = 15) precision and accuracy were assessed by QC samples at the lower limit of quantitation (LLOQ), 3.0, 30.0 and 70.0 ng/mL.

Autosampler stability (25 °C, 12 hours), bench-top stability (25 °C, 8 hours) and freeze-thaw stability (3 freeze-thaw cycles, -20 °C, 72 hours) in plasma and brain homogenate were tested for all the analytes at both low (3 ng/mL) and high (75 ng/mL) concentrations (n = 3), by comparing freshly spiked samples and samples subject to stability tests.

Matrix effects, relative recovery and absolute recovery for both plasma and brain homogenate were calculated from peak areas of spiked samples, post-preparation spiked samples and neat standard solutions of concentrations at 3.0, 30.0 and 70.0 ng/mL (n = 3).

Dilution validation was conducted to accommodate real samples with analyte concentrations over the upper limit of quantitation (ULOQ). After diluting spiked samples from 1500 ng/mL into the concentration at ULOQ (100 ng/mL) with corresponding matrices (plasma or brain homogenate), precision and accuracy (n = 5) were tested.

3. Results and Discussion

3.1 LC-MS/MS method development

In order to develop a sensitive and selective method for simultaneous quantification of COT and its metabolites, optimizations of different factors and parameters were made in tandem mass spectrometry and liquid chromatography.

To achieve higher sensitivity, the triple quadrupole mass spectrometer was set to unit resolution mode. For instrument tuning, general parameters for desolvation and ionization were obtained by a constant infusion at 10 μL/min of a 1 μg/mL COT solution. The detection of analytes and ISs were conducted using MRM functions, providing high sensitivity and

selectivity. A product ion mass spectrum was obtained by collision activated dissociation (CAD) for each analyte and IS, and the most abundant product ions were used in the MRM ion transitions. Collision energy and cone voltages were optimized with injections of 10 μL of 100 ng/mL individual standards for each analyte and IS.

The separation of analytes was carried out by HILIC. During the development of the LC method, both reversed phase liquid chromatography (RPLC) and HILIC were tried for the separation of analytes in both neat samples and spiked samples. The retention of analytes, especially for COTNO with high polarity, on the reversed phase column (Agilent ZORBAX XDB-C18 column) was weak and a high aqueous percentage was required in the eluting mobile phase, which would lower the ionization efficiency when using electrospray. However, all analytes had better retention on the HILIC column (Phenomenex Kinetex™ HILIC column). High organic percentage was used in the mobile phase, which provided better compatibility with the ESI ion source. Moreover, early elution of hydrophobic impurities, especially for brain samples, on the HILIC method contributed to lower possibility of ion source contamination by lipids.

3.2 Sample preparation method development

Before LC-MS/MS analysis, sample preparation was required for biological samples, especially for brain homogenate, which contained more proteins and lipids. In method development, common sample preparation approaches such as, protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE), were all tested for plasma and brain homogenate. Samples prepared only by protein precipitation still contained impurities, which became more significant when the samples were evaporated and reconstituted at higher concentrations. Based on this, further sample clean-up, either LLE or SPE, was needed after

protein precipitation. LLE was first tried with different extractants, isopropanol, chloroform, ethyl acetate and methylene chloride, among which ethyl acetate provided the highest recovery for COT (73% in brain homogenate, 62% in plasma). Nevertheless, the recovery of the most polar analyte, COTNO, was almost zero. Considering the wide range of polarities among analytes, SPE was used as an alternative for better selectivity. Two types of SPE cartridges, Waters Oasis HLB (hydrophilic-lipophilic balance) and MCX (mixed mode cation exchange) were tested. Since the extraction mechanism of HLB was similar to that of LLE, the recovery for extremely polar analytes was also very low. However, MCX cartridges provided acceptable recoveries, as all analytes were protonated in acidic solution and bound to cartridges via cation exchange interactions. Different levels of matrix effects were observed for the four analytes, which could be reduced by increasing the strength of the washing agent or decreasing the strength of the eluting agent. However, recoveries of the analytes were reduced when the matrix effects were reduced by such approaches. To balance the recovery and matrix effects for all analytes, the strongest washing agent and weakest eluting agent were optimized to provide acceptable recoveries for all of the analytes.

3.3 Linearity and sensitivity

Calibration curves made for COT, NCOT, OHCOT and COTNO in plasma and brain homogenate are shown in Table 3.1. Good linearity ($R^2 > 0.99$) was observed for all of the analytes over the range from 1 – 100 ng/mL in plasma and brain homogenate (3 – 300 ng/g in brain tissue). A 1/x-weighted linear regression was used to generate all calibration curves. Slopes, intercepts and R^2 values are shown in Table 3.1. A Student t-test was conducted for all the intercept values to determine the statistical significance of the difference from theoretical zero value, which could suggest the endogenous levels of analytes. COT in blank plama is very

significantly different from theoretical zero, based on the 0.01 level; while endogenous plasma NCOT, brain COT, brain NCOT and bran OHCOT were significantly different from zero on the 0.05 level. Considering errors caused by signal saturation and linear regression, low endogenous levels of analytes (small intercept values), NCOT and OHCOT, can be negligible even with significant non-zero intercepts. The mean values and statistical differences from theoretical zero suggested COT had significant endogenous levels in blank rat plasma and brain. The sensitivity of the method was defined by the lower limit of quantitation (LLOQ), which was the lowest concentration within 20% precision and accuracy. LLOQs for all the analytes in were 1 ng/mL in plasma or brain homogenate (3 ng/g in brain tissue). Signal-to-noise ratio (S/N), another parameter to assess sensitivity, was greater than 10 at the LLOQ for each analyte in both matrices.

3.4 Precision and accuracy

Precision and accuracy were calculated for LLOQ and QC samples of all four analytes in both matrices, shown in Table 3.2. Precision, defined as the closeness of measurements of the same concentration, was assessed by the coefficient of variation (CV) or relative standard deviation (RSD) among measured concentrations. Accuracy, defined as the closeness between measured and true values, was assessed by the relative error (RE) between measured concentrations and nominal concentrations. Both intra-day (n = 5) and inter-day (n = 15) precision and accuracy were tested. RSD and RE values for COT, NCOT, OHCOT and COTNO in plasma and brain homogenate are shown in Table 3.2, which met the FDA requirements of less than 15% for QCs and less than 20% for LLOQs.

3.5 Recovery and matrix effect

Recovery and matrix effect were tested for all the analytes at the three QC concentrations (n = 3) in both matrices, shown in Table 3.3.

For each concentration of analytes in either matrix, three spiked samples and three neat solutions were prepared. Besides, three "post-preparation spiked" samples were made by spiking standard working solutions into blank matrices processed by the same sample preparation. The absolute recoveries were calculated by the peak area ratio between spiked samples and neat standards. Relative recoveries were calculated by the peak area ratio between "post-preparation spiked" samples and spiked samples, quantitating the loss due to sample preparation. Matrix effects were calculated by the peak area ratio between "post-preparation spiked" samples and neat standards, providing the influence of the matrix on the signal response. In addition, types of matrix effects (enhancement or suppression) are shown in Table 3.3.

As mentioned in the method development section, the sample preparation had been optimized to achieve both acceptable recovery and matrix effects for all the analytes. Since stable isotope-labeled ISs were used in this method, matrix effects became less prominent, because they only slightly affected the sensitivity but not the precision or accuracy. Recovery, which is more directly related to the sensitivity of the method, became more important. Due to the great differences in polarity and pKa among analytes (Figure 3.1), selectivity of sample preparation had to be compromised to yield satisfactory recoveries for all of the analytes, which would increase the matrix effects at the same time. TCA was used for both protein precipitation and protonating analytes for SPE based cation exchange. In the SPE, the strongest washing agent, which was still very weak, was used for lowest analyte loss; while the weakest eluting

agent was used to minimize co-eluting lipid-based impurities as well as providing acceptable recoveries for all the analytes.

All of the matrix effects observed were from ion suppression. Considering the very weak eluting conditions in SPE, lipid-based or protein-based impurities were unlikely to co-elute with the analytes. Therefore, we considered the ion suppression effects to result from salts or positively charged ions introduced by matrices or sample preparation, which could compete with the analytes during ESI and reduce analyte signal response.

3.6 Specificity

Representative chromatograms obtained from blank biological matrices and spiked with LLOQ standard (1 ng/mL for plasma and brain homogenate) are shown in Figure 3.2 and Figure 3.3. No interference from cross-talk was observed among the MRM channels. However, endogenous COT was observed in the blank plasma, as well as, brain homogenate. With matched retention time and ion transitions, the signal in blank matrices was confirmed to result from the same compound. After eliminating the possibility of contamination during sample preparation, the blank matrices were confirmed to contain endogenous COT, the level of which was observed to be stable among individuals. Considering the common contamination of COT in water and air due to smoking, this was thought to be acceptable as long as the endogenous level was consistent and did not affect method robustness. Adjustments were made for calibration curves, including blank matrices as calibration points for all the analytes and matrices.

3.7 Stability

After an intra-day validation, QC samples at 3.0 and 70.0 ng/mL in plasma and brain homogenate (n = 5) were left in the autosampler for 12 hours and reanalyzed for autosampler stability. Spiked plasma and brain homogenate at two concentrations, 3.0 and 70.0 ng/mL were

prepared for all the analytes. One set of samples (n = 3) was prepared and analyzed right afterwards, which was used as a time zero control group. At the same time, another two sets of samples (n = 3) spiked together with the first group were subject to bench-top stability and freeze-thaw stability tests. One of the sets was left on the bench-top (25 °C) for 8 hours and then prepared and analyzed. The other set was stored at -20 °C for 24 hours and then completely thawed at 25 °C on the bench-top without assistance. After another two freeze-thaw cycles, the samples were prepared and analyzed. For all the stability tests, response factors (IS concentration times peak ratio between analyte and IS) were obtained for analyzed samples. Stabilities were calculated by the response factor ratio between samples after and before stability tests, shown in Table 3.4. All the analytes, COT, NCOT, OHCOT and COTNO, at all the concentrations in both plasma and brain homogenate were confirmed to be stable in terms of autosampler, bench-top and freeze-thaw stability, with the deviation from the time zero control of less than 10%.

3.8 Dilution validation

The sensitive method was developed for simultaneous quantification of COT and metabolites at low concentrations. However, these analytes might have different concentrations in the biological samples, especially for COT that usually has much higher concentrations than the others. In order to adjust the method for samples with higher analyte concentrations, the dilution validation was conducted by diluting spiked samples (n = 5) from 1500 ng/mL into the concentration at ULOQ (100 ng/mL) with corresponding blank matrices. Precision and accuracy of these samples were calculated, which are shown in Table 3.5. The precision and accuracy for all the analytes in both plasma and brain homogenate were within the acceptance of 15%, suggesting sample dilution within 15 fold was validated and applicable to real samples.

3.9 Application

Plasma and brain samples from rats (n = 3) subcutaneously dosed with 1 mg/kg of COT were obtained 30 min after dosing. Paralleled experiments either with or without a 15-fold dilution were conducted for each individual. The same sample preparation and quantitation method were applied to these samples, giving out the result shown in Table 3.6. The representative chromatograms of these samples are shown in Figure 3.4 and Figure 3.5.

All the analytes could be detected in both plasma and brain. COT concentrations in plasma and brain were largely above the ULOQ, which could still be well quantified after dilution. Concentrations of OHCOT and COTNO were within the linear range in plasma, but below the LLOQ in the brain. NCOT concentrations were below LLOQ in both plasma and brain. All those concentrations below the LLOQ were calculated with extrapolated calibration curves, giving out results with less credibility. Assuming 1 g of brain tissue is equivalent to 1 mL plasma, COT showed great BBB permeability with very high brain-to-plasma concentration ratio 0.7, making COT more promising as an anti-AD drug targeting at the brain. NCOT might also have high BBB permeability, but the credibility of the brain-to-plasma concentration ratio was low. OHCOT and COTNO showed low BBB permeability, due to their high polarity and water solubility. These results provided important information for further investigation of distributions and activities of these drugs in AD therapies.

4. Conclusions

A selective and sensitive LC–MS/MS quantitation method for the simultaneous determination of COT, NCOT, OHCOT and COTNO in rat plasma and brain tissue was developed and validated. This method provided good precision and accuracy for the quantitation of analytes within the linear range of 1 – 100 ng/mL for all the analytes in plasma and brain

homogenate (3 – 300 ng/g in brain tissue), with the LLOQ of 1 ng/mL in plasma and 3 ng/g in brain tissue. A low sample volume, 100 μL of rat plasma or brain homogenate, was needed for this method. Protein precipitation and solid-phase extraction was used as sample preparation, yielding acceptable recovery and matrix effect. This method has been successfully applied to preclinical studies of COT, NCOT, OHCOT and COTNO on rats for their anti-AD activity research.

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Table 3.1 Calibration curves for COT, NCOT, OHCOT and COTNO in plasma and brain homogenate. (n=3)

Analyte	Plasma			Brain		
	Intercept	**Slope**	**R^2**	**Intercept**	**Slope**	**R^2**
COT	0.8731± 0.0126**	0.8317± 0.0244	0.9979±0.0008	0.7621± 0.1593*	0.8634± 0.0440	0.9983±0.0014
NCOT	0.1312± 0.0493*	0.7453± 0.0111	0.9977±0.0014	0.1099± 0.0412*	0.7122± 0.0101	0.9989±0.0001
OHCOT	0.1370± 0.0831	1.0830± 0.0119	0.9988±0.0007	0.0834± 0.0198*	1.0089± 0.0502	0.9993±0.0003
COTNO	0.4582± 0.1902	1.0717± 0.0403	0.9973±0.0005	0.2483± 0.1124	1.1244± 0.0842	0.9947±0.0048

*P < 0.05, Student *t*-test.

**P < 0.01, Student *t*-test.

Table 3.2 The intra-day (n = 5) and inter-day (n = 15) precision (RSD) and accuracy (RE) of the LC–MS/MS method used to quantitate COT, NCOT, OHCOT and COTNO in rat plasma and brain homogenate.

			Intra-day			Inter-day		
	Analyte	**Nominal Conc. (ng/mL)**	**Measured Conc. (ng/mL)**	**RSD (%)**	**RE (%)**	**Measured Conc. (ng/mL)**	**RSD (%)**	**RE (%)**
Plasma	**COT**	1.0	0.92±0.11	12.30	-8.00	0.91±0.12	13.44	-9.27
		3.0	3.08±0.31	10.03	2.67	2.87±0.26	9.12	-4.42
		30.0	30.05±0.89	2.97	0.15	28.63±1.25	4.37	-4.56
		75.0	74.79±1.73	2.31	-0.28	71.86±2.58	3.59	-4.18
	NCOT	1.0	0.92±0.03	3.44	-8.00	0.92±0.06	6.13	-8.33
		3.0	2.99±0.05	1.71	-0.40	2.89±0.11	3.85	-3.76
		30.0	29.49±0.44	1.48	-1.71	28.22±1.05	3.71	-5.92
		75.0	72.05±1.06	1.47	-3.93	69.93±2.31	3.30	-6.76
	OHCOT	1.0	0.87±0.02	2.21	-12.80	0.91±0.07	7.49	-8.80
		3.0	2.83±0.08	2.93	-5.53	2.81±0.09	3.22	-6.40
		30.0	28.36±0.64	2.26	-5.47	28.38±0.49	1.71	-5.39
		75.0	70.79±1.12	1.59	-5.61	70.70±1.20	1.70	-5.73
	COTNO	1.0	0.904±0.06	6.71	-9.60	0.90±0.17	18.85	-10.20
		3.0	2.886±0.20	6.84	-3.80	2.87±0.24	8.41	-4.36
		30.0	27.234±1.18	4.34	-9.22	27.16±0.85	3.12	-9.48
		75.0	66.53±1.26	1.89	-11.29	67.77±3.22	4.75	-9.63

			Intra-day			Inter-day		
	Analyte	**Nominal Conc. (ng/mL)**	**Measured Conc. (ng/mL)**	**RSD (%)**	**RE (%)**	**Measured Conc. (ng/mL)**	**RSD (%)**	**RE (%)**
Brain	**COT**	1.0	0.94±0.08	9.00	-6.00	0.90±0.10	10.72	-9.53
		30	2.82±0.04	1.55	-6.07	2.82±0.07	2.52	-6.02
		30.0	27.12±0.52	1.91	-9.59	27.94±0.83	2.98	-6.86
		75.0	70.14±1.47	2.09	-6.48	72.05±2.12	2.94	-3.93
	NCOT	1.0	0.94±0.03	2.81	-6.00	0.94±0.04	4.55	-6.27
		3.0	2.90±0.05	1.60	-3.33	2.94±0.08	2.59	-2.00
		30.0	27.99±0.88	3.15	-6.71	28.52±1.11	3.89	-4.94
		75.0	71.66±3.62	5.05	-4.45	72.71±3.23	4.45	-3.05
	OHCOT	1.0	0.92±0.03	3.64	-7.60	0.90±0.04	4.35	-10.13
		3.0	2.92±0.08	2.66	-2.53	2.92±0.06	2.21	-2.53
		30.0	28.63±0.72	2.51	-4.57	28.67±0.53	1.85	-4.44
		75.0	72.64±3.40	4.68	-3.15	72.89±2.08	2.86	-2.82
	COTNO	1.0	1.01±0.05	4.73	0.80	1.03±0.15	14.73	2.60
		3.0	3.00±0.11	3.77	-0.07	2.97±0.14	4.57	-0.84
		30.0	29.61±0.22	0.75	-1.31	27.86±1.44	5.17	-7.14
		75.0	75.65±4.57	6.04	0.87	72.08±4.01	5.56	-3.89

Table 3.3 Absolute recovery (%AR), relative recovery (%RR) and matrix effect (%ME) of the method. Mean ± SD values are shown for all the metabolites at 3.0, 30.0 and 75.0 ng/mL concentrations in plasma and brain homogenate. Types of matrix effect are shown in percentage of enhancement or suppression.

Matrix	Analyte	Conc. (ng/ml)	AR (%)	RR (%)	ME (%)	Type
Plasma	COT	3.0	37.26±1.20	52.94±1.71	70.38	29.62% suppression
		30.0	23.82±8.41	40.54±14.31	58.75	41.25% suppression
		75.0	30.79±7.42	41.97±10.12	73.36	26.64% suppression
	NCOT	3.0	62.42±5.66	76.80±6.97	81.28	18.72% suppression
		30.0	63.41±9.13	83.90±12.09	75.57	24.43% suppression
		75.0	66.51±8.04	73.55±8.89	90.43	9.57% suppression
	OHCOT	3.0	36.04±2.32	89.51±5.75	40.26	59.74% suppression
		30.0	37.09±12.42	73.61±24.66	50.38	49.62% suppression
		75.0	41.56±3.49	76.77±6.45	54.14	45.86% suppression
	COTNO	3.0	33.54±9.73	41.81±12.14	80.22	19.78% suppression
		30.0	45.83±2.21	56.99±2.75	80.41	19.59% suppression
		75.0	34.56±4.00	45.06±5.21	76.69	23.31% suppression
Brain	COT	3.0	41.27±4.39	58.75±6.24	70.25	29.75% suppression
		30.0	30.88±2.73	37.60±3.33	82.12	17.88% suppression
		75.0	39.15±3.38	63.01±5.43	62.12	37.88% suppression
	NCOT	3.0	60.35±1.79	75.38±2.23	80.07	19.93% suppression
		30.0	65.68±1.35	96.80±2.00	67.85	32.15% suppression
		75.0	71.84±4.98	97.27±6.75	73.86	26.14% suppression
	OHCOT	3.0	27.50±1.82	78.61±5.19	34.98	65.02% suppression
		30.0	41.30±2.91	82.95±5.85	49.79	50.21% suppression
		75.0	36.45±1.23	100.03±3.36	36.44	63.56% suppression
	COTNO	3.0	19.96±5.08	31.88±8.11	62.61	37.39% suppression
		30.0	39.24±6.20	68.00±10.74	57.71	42.29% suppression
		75.0	25.30±7.23	39.69±11.35	63.74	36.26% suppression

Table 3.4 Autosampler stability (n = 5), bench-top stability (n = 3) and freeze-thaw stability (n = 3) of COT, NCOT, OHCOT and COTNO at 3.0 and 75.0 ng/mL concentrations in plasma and brain homogenate. Stabilities are shown in forms of percentage of relative concentration to time zero control (mean ± SD).

Matrix	Analyte	Conc. (ng/mL)	Autosampler Stability (%)	Bench-top Stability (%)	Freeze-Thaw Stability (%)
Plasma	COT	3.0	92.36±4.57	97.43±8.31	104.10±4.59
		75.0	98.08±3.28	100.15±4.88	102.29±1.63
	NCOT	3.0	98.65±3.63	102.65±5.47	99.56±2.55
		75.0	96.94±3.03	99.76±3.57	102.49±5.04
	OHCOT	3.0	100.20±2.39	100.82±3.47	97.26±1.79
		75.0	101.39±1.75	99.28±4.76	102.46±0.88
	COTNO	3.0	99.87±4.88	105.72±5.02	98.32±1.15
		75.0	100.54±3.19	101.17±4.75	100.31±1.58
Brain	COT	3.0	101.60±1.80	95.26±3.94	95.61±10.62
		75.0	96.95±2.38	102.39±6.13	101.88±2.98
	NCOT	3.0	101.53±3.28	100.49±3.65	97.09±9.24
		75.0	98.93±2.69	100.64±4.54	97.88±0.29
	OHCOT	3.0	101.74±3.81	100.78±5.88	93.70±7.34
		75.0	101.33±3.12	101.48±3.08	99.14±2.35
	COTNO	3.0	99.44±3.73	99.68±4.99	103.13±6.72
		75.0	98.27±2.89	106.59±3.08	95.69±2.89

Table 3.5 Precision (RSD) and accuracy (RE) of spiked samples (n = 5) with 1500.0 ng/mL analyte concentration in plasma and brain homogenate diluted 15 folds into ULOQ (100 ng/mL) concentration.

		Plasma			Brain		
Analyte	**Nominal Conc. (ng/mL)**	**Measured Conc. (ng/mL)**	**RSD (%)**	**RE (%)**	**Measured Conc. (ng/mL)**	**RSD (%)**	**RE (%)**
COT	1500.0	1420.92 ± 29.02	2.04	-5.27	1530.24 ± 26.03	1.70	2.02
NCOT		1362.81 ± 30.01	2.20	-9.15	1613.43 ± 15.44	0.96	7.56
OHCOT		1370.49 ± 36.11	2.63	-8.63	1656.96 ± 30.67	1.85	10.46
COTNO		1275.69 ± 30.65	2.40	-14.95	1583.43 ± 23.89	1.51	5.56

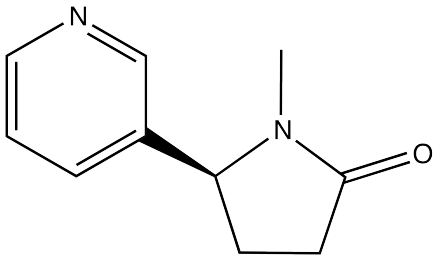
Table 3.6 Plasma and brain concentrations (mean ± SD) of COT, NCOT, OHCOT and COTNO in biological samples obtained from rats (n = 3) subcutaneously dosed by 1 mg/kg COT.

Analyte	Plasma Conc. (ng/ml)	Brain Conc. (ng/g)	Brain-to-Plasma Ratio***
COT	1364.35±61.58*	959.4±73.84*	0.70
NCOT	0.89±0.09**	0.48±0.06**	0.54
OHCOT	5.31±1.13	0.91±0.17**	0.17
COTNO	7.13±1.95	0.18±0.16**	0.03

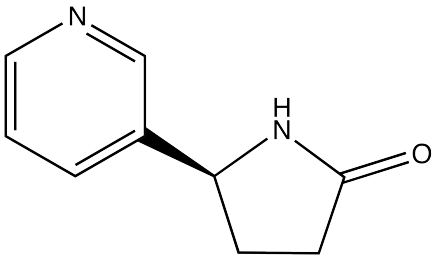
* Samples over the ULOQ were diluted 15 folds and analyzed with the method.

** Concentrations below the LLOQ but still detectable were calculated with extrapolated calibration curves.

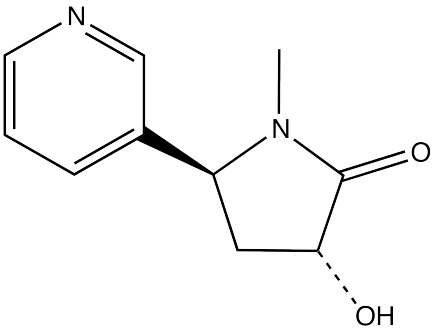
*** Brain-to-plasma ratios were calculated with the assumption that 1 g of brain tissue was equivalent to 1 mL of plasma.



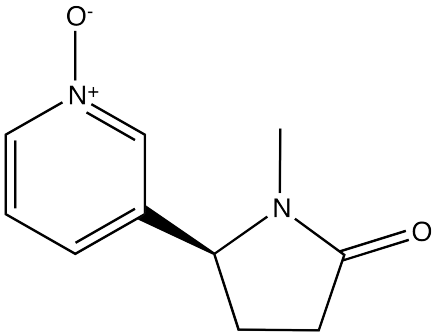
Cotinine (COT)
pKa = 4.8, XLogP3 = -0.3



Norcotinine (NCOT)
pKa = 7.8, XLogP3 = 0.1



Trans-3'-hydroxycotinine (OHCOT)
pKa = 4.3, XLogP3 = -1.0



Cotinine-N-oxide (COTNO)
pKa = 3.4, XLogP3 = -1.3

Figure 3.1 Chemical structures, pKa and XLogP values of COT, NCOT, OHCOT and COTNO. Structures were generated by ChemBioDraw Ultra 12.0 software. pKa and XLogP3 values were obtained from PubChem database.

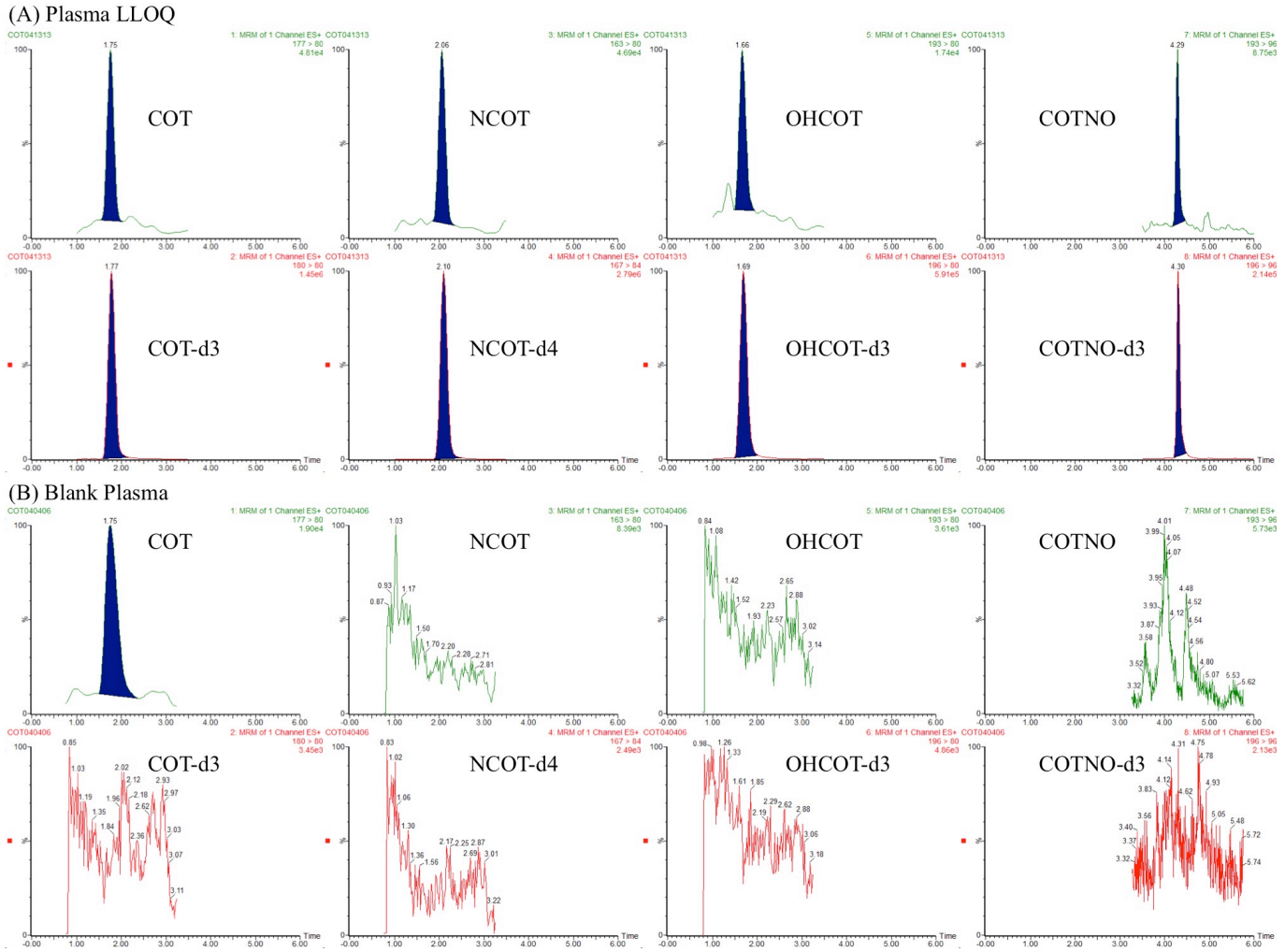


Figure 3.2 Representative chromatograms of plasma samples. For each analyte, chromatograms of the analyte and IS were shown for both a spiked standard at LLOQ (1 ng/mL) (A) and a blank sample (B). The concentrations of IS were all 50 ng/mL.

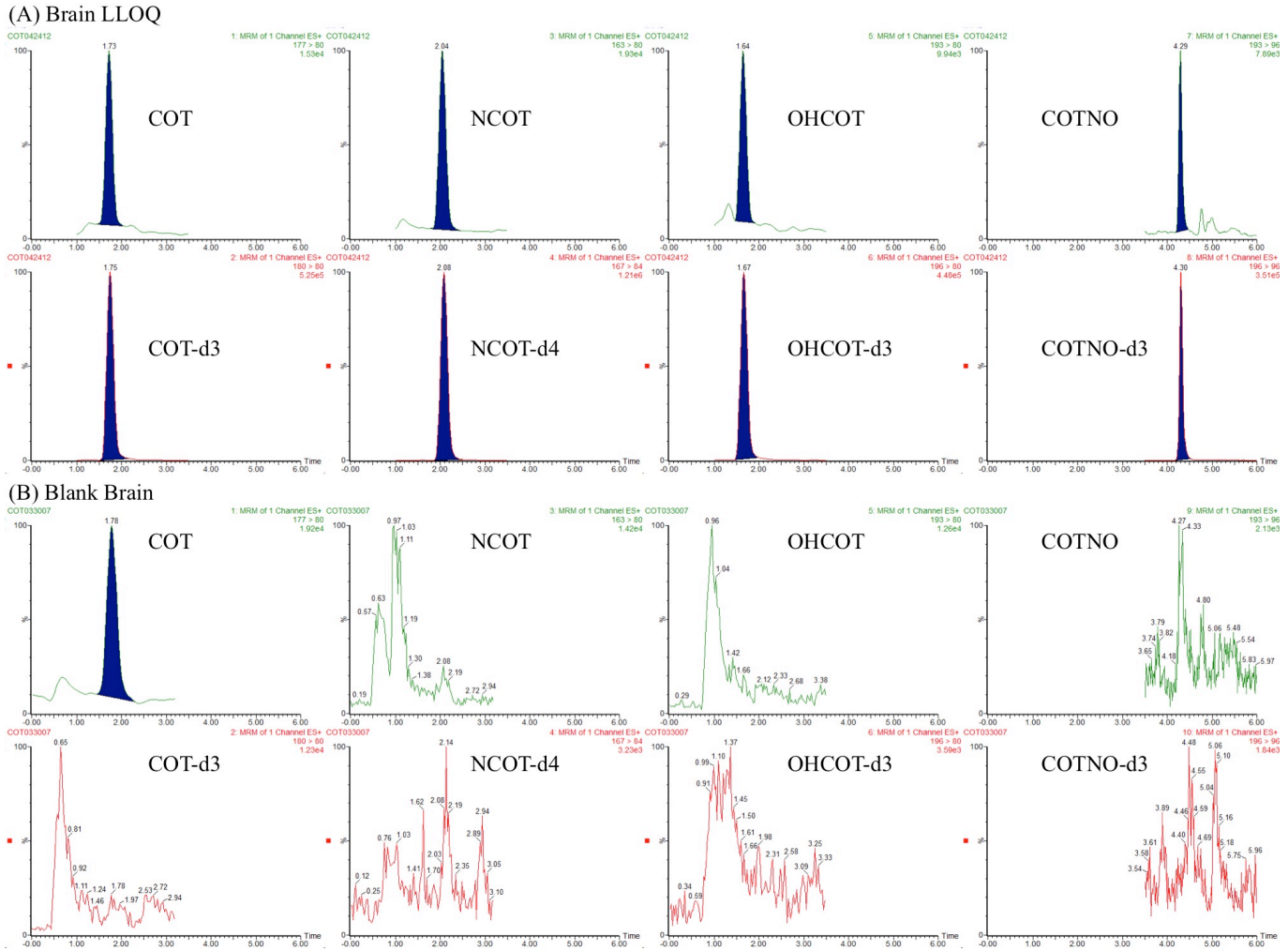


Figure 3.3 Representative chromatograms of brain homogenate samples. For each analyte, chromatograms of the analyte and IS were shown for both a spiked standard at LLOQ (1 ng/mL) (A) and a blank sample (B). The concentrations of IS were all 50 ng/mL.

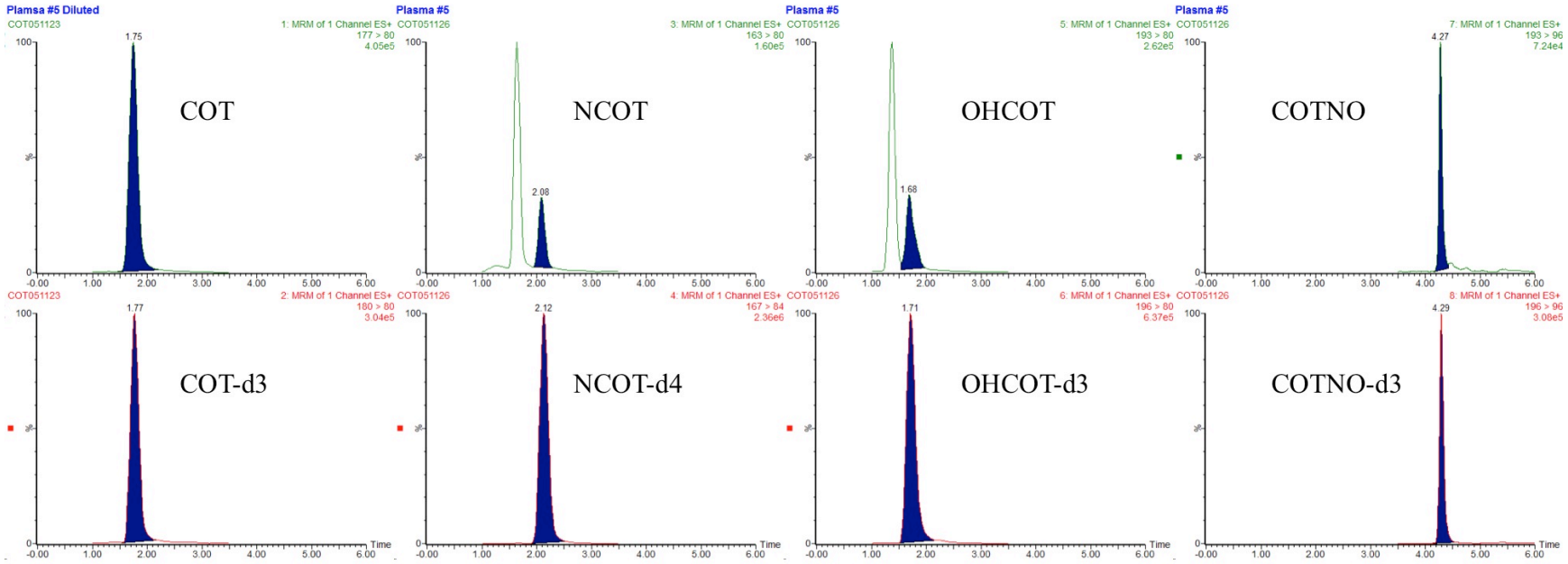


Figure 3.4 Representative chromatograms of plasma samples from rats subcutaneously dosed by 1 mg/kg COT: (A) chromatograms of COT and IS in samples that were diluted 15 folds with blank plasma, with the original COT concentration of ng/mL; (B) chromatograms of NCOT and IS, with NCOT concentration below LLOQ; (C) chromatograms of OHCOT and IS, with OHCOT concentration of ng/mL; (D) chromatograms of COTNO and IS, with COTNO concentration of ng/mL.

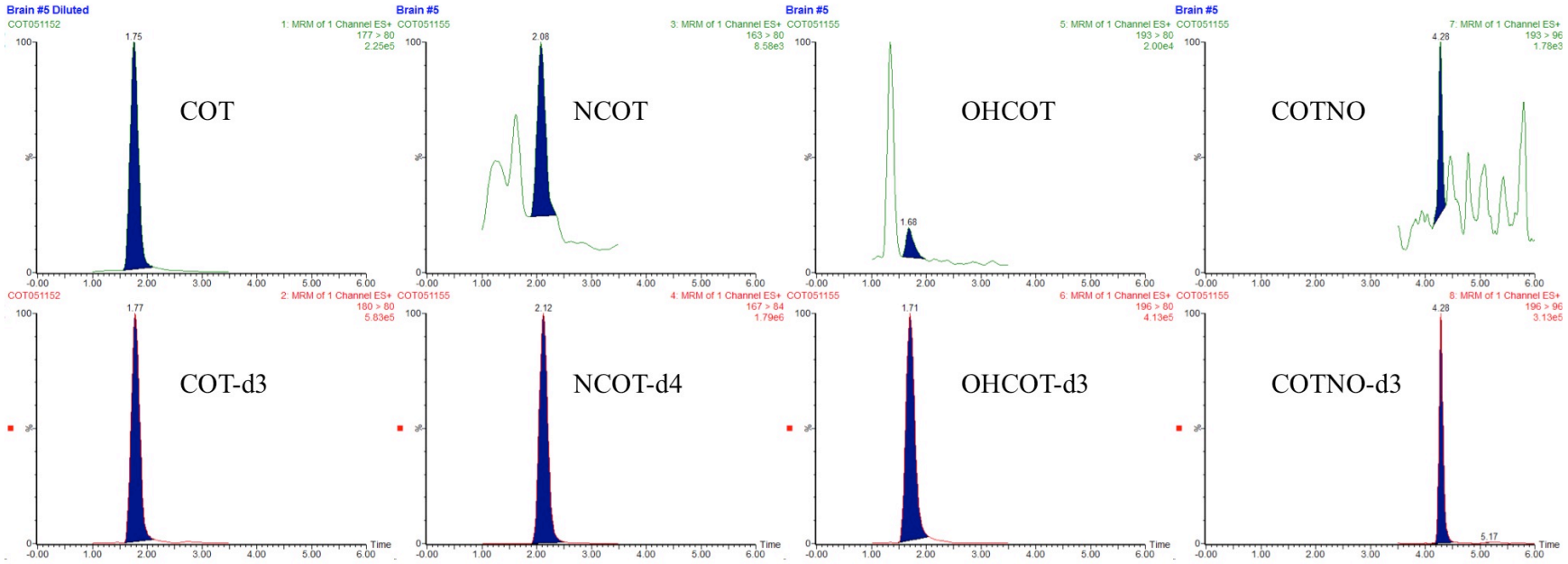


Figure 3.5 Representative chromatograms of brain samples from rats subcutaneously dosed by 1 mg/kg COT: (A) chromatograms of COT and IS in samples that were diluted 15 folds with blank brain homogenate, with the original COT concentration of ng/g; (B) chromatograms of NCOT and IS, with NCOT concentration below LLOQ; (C) chromatograms of OHCOT and IS, with OHCOT concentration below LLOQ; (D) chromatograms of COTNO and IS, with COTNO concentration below LLOQ.

CHAPTER 4

PHARMACOKINETICS OF COTININE: A POTENTIAL THERAPEUTIC FOR IMPROVING COGNITIVE FUNCTION

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Abstract

Attention has been paid to cotinine (COT), one of the major metabolites of nicotine (NIC), for its pro-cognitive effects and potential therapeutic activities against Alzheimer's Disease (AD) and other types of cognitive impairment. In order to facilitate pharmacological and toxicological studies on COT for its pro-cognitive activities, we conducted a pharmacokinetic (PK) study of COT in rats, using a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) bioanalytical method. In this study, plasma samples were obtained, prepared and analyzed up to 48 hours after COT was administered to rats orally and intravenously (IV) at a dose of 3 mg/kg. The data were fitted into a one-compartment model and a two-compartment model for the oral and IV groups, respectively, providing important PK information for COT including PK profiles, half-life, clearance and bioavailability. The results suggested fast absorption, slow elimination and high bioavailability of COT in rats. Information about the pharmacokinetics of COT in rats revealed in this study is of great importance for the future studies on COT or potential analogues as potential therapeutic agents for improving cognition.

Key words

Cotinine; hydrophilic interaction chromatography; tandem mass spectrometry; pharmacokinetics; cognition.

Introduction

As a result of the unprecedented growth of elderly populations, one of the most significant results of global population aging is the rise in the number of people suffering from age-related forms of dementia. Among these, Alzheimer's disease (AD) accounts for more than 75% of dementia cases.[1, 2] Currently there are more than 26.6 million people worldwide suffering from AD, which is predicted to affect over a billion people globally by 2030.[3, 4] AD is a major cause of physical disability, institutionalization, decreased quality of life and shortened life expectancy of the elderly. It also causes a significant burden to the economy, society and health care systems.[1] In addition, other cognitive deficits, including schizophrenia and mild cognitive impairment (MCI) also contribute to cognition-related health problem.[5]

AD is a progressive, severe and incurable neurodegenerative disorder first described by Alois Alzheimer in 1906.[6] The actual cause and pathological mechanism of AD are still not clearly known.[7] However, different theories have been published as the possible mechanisms of AD. The most accepted one is related to the aggregation of amyloid-β (Aβ) peptides, amyloid angiopathy, and neurofibrillary tangles of phosphorylated tau protein in the brain.[8-10]

Different strategies have been developed for the treatment of AD. Memantine is an FDA-approved drug for the alleviation of AD symptoms, due to its antagonistic activities on glutamatergic (NMDA receptor), serotonergic (5-HT3 receptor), cholinergic (nicotinic acetylcholine receptor) and dopaminergic (D2 receptor) pathways.[11] Galantamine is another drug demonstrated to be effective on AD, as it can inhibit acetylcholinesterase (AchE).[12] In addition, xanomeline, a muscarinic receptor agonist, has also been demonstrated to have positive effects on the cognitive impairment caused by AD.[13, 14] However, all of the current

therapeutic agents can only alleviate the symptoms of AD and may also come with severe side effects.

The pro-cognitive effects of tobacco have been of great interest to researchers.[15-17] Nicotine (NIC) has been demonstrated to have pro-cognitive effects on the central nervous system by acting as an agonist for nicotinic acetylcholine receptors (nAChRs).[18, 19] However, due to the short half-life (2 – 6 hours), high toxicity (mouse oral LD50 = 50 mg/kg) and high addictive potential of NIC, it is unlikely to be developed as an effective and safe therapeutic agent. The major metabolite of NIC, cotinine (COT), has shown very weak nAChR agonist activity but significant pro-cognitive effects.[20-22] COT was also reported to reduce amyloid-β aggregation and improve memory in AD animal models.[23] Moreover, the longer biological half-life (15–19 h) and lower toxicity (mouse oral LD50 = 1604 mg/kg) of COT make it a more practical prototype drug candidate for the treatment of AD and other mild to severe forms of dementia.

Several studies have been conducted to reveal the pharmacokinetic (PK) properties of NIC.[24-27] NIC was reported to have a low oral bioavailability (about 20%) and short half-life (2 – 6 hours). A few PK properties of COT were revealed from some PK studies focused on NIC, providing limited information about the half-life and clearance of COT as a metabolite of NIC.[25, 28] Two studies about the PK of COT in humans were published in 1987 and 1990, respectively, revealing important PK parameters of COT in non-smoking healthy volunteers.[29, 30] However, no PK studies have been conducted on rodents, which are frequently used as animal models in non-clinical and pre-clinical drug research and development. In humans, COT is primarily metabolized by cytochrome P450 2A6 (CYP2A6), which does not exist in rodents including mice and rats.[31] Therefore, COT may display different metabolism, disposition and

PK in rodent species. A PK study of COT in rodents is needed to obtain important PK parameters for future non-clinical and pre-clinical studies on the pharmacology, toxicology and drug delivery of COT.

In this study, we used a sensitive, precise and accurate LC-MS/MS method for the quantification of COT and three other major metabolites in rat plasma.[32] Test rats were dosed with a single dose of COT at 3 mg/kg both orally and intravenously, which was the therapeutic dosage level for pro-cognitive effects. Important PK information of COT including PK profiles, half-life, clearance and bioavailability were revealed in this study, which suggested fast absorption, slow elimination and high bioavailability of COT in rats. Moreover, three major metabolites of COT, norcotinine (NCOT), *trans*-3'-hydroxcotinine (OHCOT) and cotinine (*S*)-cotinine-N-oxide were also analyzed simultaneously, providing more information about the bio-transformation of COT. These results about the PK of COT in rats are of great importance for future studies on COT and its pro-cognitive effects.

Experimental

1. Chemicals and reagents

(-)-Cotinine (COT) was purchase from Sigma-Aldrich (St. Louis, MO). Stable isotope labeled internal standard (IS) (±)-Cotinine-D3 solution (1mg/mL in methanol) was obtained from Cerilliant (Round Rock, Texas). (*R,S*)-norcotinine (NCOT), *trans*-3'-hydroxcotinine (OHCOT) and cotinine (*S*)-cotinine-N-oxide, (*R,S*)-norcotinine-d4 (NCOT-d4), *trans*-3'-hydroxycotinine-d3 (OHCOT-d3) and (*R,S*)-cotinine-N-oxide-d3 (COTNO-d3) were purchased from Toronto Research Chemical (Toronto, Canada). Chemical structures of COT, NCOT, OHCOT and COTNO are shown in Figure 4.1. Trichloroacetic acid and ammonium acetate were obtained

from Baker (Phillipsburg, NJ). LC-MS grade formic acid, acetonitrile (ACN), methanol and water were from Sigma (St. Louis, MO).

2. Solutions and standards

Individual stock solutions of all the analytes and IS were prepared as 1.0 mg/mL methanol solutions. Combined working solutions were obtained by serial dilution with 90% ACN/water (v/v 9/1). IS working solutions containing COT-d3, NCOT-d4, OHCOT-d3 and COTNO-d3 were prepared at a single concentration of 500.0 ng/mL in the same solvent. Stock solutions were kept at -20 °C when not in use.

Blank rat plasma with sodium EDTA was purchased from Bioreclamation (Westbury, NY). Blank brains were obtained from drug-free control rats and homogenized with two volumes of water to obtain blank brain homogenate. 10 μL of standard or QC working solution was spiked into 100 μL of plasma to generate corresponding standard or QC samples. The final concentrations of calibration standards were 20, 50, 100, 200, 500, 1000, 5000 and 10000 ng/mL in plasma while the QC samples were 30, 750 and 7500 ng/mL. Fresh standards and QC samples were prepared on the day of experiments.

3. Dosing and sample collection

Pre-canulated albino Wistar rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) approximately 2 months old were housed in pairs in a temperature controlled room (25 °C), maintained on a 12:12 h normal light-dark cycle (lights on at 6AM) with free access to water and food until used for PK studies. All procedures employed during this study were reviewed and approved by the Georgia Regents University Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain and discomfort

in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

To four rats used in the oral group and three in the IV group, a single dose of 3 mg/kg (1.5 mg/mL for oral, 3 mg/mL for IV) COT in saline was administered via intravenous bolus injection through the jugular vein cannula or by oral gavage. Serial blood samples (200 μL) each were collected from the jugular vein cannulae at 0, 15, 30 min and 1, 2, 4, 6, 8, 12, 24 and 48 hours following oral administration of COT. Similarly, blood samples were obtained at 0, 15, 30, 60, 90 min and 2, 3, 4, 5, 6 and 24 hours from the animals in the IV group. The blood samples were placed in centrifuge tubes pretreated with potassium EDTA, followed by centrifugation for 15 min at 2500 × *g* at 4-5 °C. The separated plasma was frozen at -80 °C until analyzed.

4. Sample preparation

Sample preparation was carried out by a combined method of protein precipitation (PPT) and solid-phase extraction (SPE). To each 50 μL of plasma, 10 μL of IS working solution (500.0 ng/mL), 90 μL of 25% (w/v) TCA and 850 μL of water were sequentially added. The mixture was vortexed for 10 min and then centrifuged at 4500 × *g* for 10 min for PPT.

An Oasis MCX SPE cartridge from Waters (Milford, MA) was conditioned with 1 mL of methanol and equilibrated with 1 mL of water. The supernatant obtained from PPT was loaded onto the cartridges and allowed to flow without assistance. Then the cartridge was washed twice by 1 mL of 5% methanol and 1 mL of 5% formic acid in water (v/v), respectively. After drying under vacuum for 5 min, the analytes were eluted using 1 mL of fresh 20% methanol, 5% ammonia aqueous solution (v/v). The eluent was evaporated to dryness in a centrifuge evaporator at 50 °C. The residue was reconstituted by 100 μL of 95% ACN/water (v/v 9/1) with 2% formic acid and forwarded for LC-MS/MS analysis.

5. LC-MS/MS assay

LC-MS/MS analysis was performed using an Agilent 1100 binary pump HPLC system (Santa Clara, CA) interfaced to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer with an ESI(+) source (Milford, MA). Data acquisition and processing were carried out using Masslynx 4.0 software by Waters (Beverly, MA).

The analytes were separated on a Phenomenex Kinetex™ HILIC column (50 × 2.1 mm ID, 2.6 μm) coupled with a SecurityGuard™ ULTRA HILIC guard column (sub-2 μm, 2.1mm ID). Mobile phases were A) 10mM ammonium formate aqueous buffer with 0.1% formic acid and B) ACN. After an injection of 5 μL for each sample into the column, analytes were separated with the following gradient (min, %B): (0, 95), (8, 50), (8.1, 95), (15, 95). Flow rate was set at 0.3 mL/min and column temperature was 25 °C. The LC system was interfaced by a six-port divert valve to the mass spectrometer, introducing LC eluents from 1.0 to 6.0 min to the ion source.

The mass spectrometer was operated in positive ion ESI (ESI +) mode. Desolvation temperature and source temperature were set at 500 °C and 120 °C, respectively. Nitrogen was used as the desolvation gas at a flow rate of 500 L/h and the cone gas flow was set to 20 L/h. Argon was the collision gas and the collision cell pressure was 3.5 × 10-3 mbar. The capillary voltage was set to 3.5 kV. Multiple reaction monitoring (MRM) functions were used for the quantification of analytes. The cone voltage was 20 V and collision energy was 20 eV. Ion transitions monitored for analytes were 177> 80 for COT, 163> 80 for NCOT, 193> 80 for OHCOT and 193> 96 for COTNO. Ion transitions for IS were 180> 80 for COT-d3, 147> 84 for NCOT-d4, 196> 80 for d3-OHCOT and 196> 96 for d3-COTNO.

6. PK analysis

COT plasma concentration versus time data were analyzed by WinNonlin 5.3 software (Pharsight, Mountain View, CA). COT plasma concentrations and corresponding time points were plotted in a semi-logarithmic scale coordinate to obtain the plasma concentration-time curves (PK profiles). To obtain more specific PK parameters, oral and IV data from each test animal were fitted into different compartmental PK models. The area under the plasma concentration-time curves (*AUC*) ratio between the oral and IV groups were used to determine the absolute bioavailability (*F*) for orally dosed COT in rats. By comparing the fittings of different PK models, optimal models were chosen to provide the peak plasma concentrations (C_{max}), time to reach peak concentration (t_{max}), half-life ($t_{1/2}$), clearance (*CL*) and other PK parameters.

Results and Discussion

1. Sample preparation

Before LC-MS/MS analysis, sample preparation was required to prepare plasma samples into a cleaner injectable sample. In this method development protein precipitation (PPT) and solid-phase extraction (SPE) were combined to achieve sufficient sample clean-up as well as satisfactory analyte recovery. Samples prepared only by PPT still contained impurities, which became more significant when the samples were evaporated and reconstituted at higher concentrations. Based on this, SPE was used following PPT to more effectively remove impurities with high selectivity. MCX (mixed mode cation exchange) SPE provided acceptable recoveries, as all analytes were protonated in acidic solution and bound to cartridges via cation exchange interactions. Cleaner samples and lower matrix effects could be achieved with more specific SPE conditions, although this lowered the analyte recovery. To balance the recovery and

matrix effects for all analytes, the strongest washing agent and weakest eluting agent were optimized to provide acceptable recoveries for all of the analytes. Since stable isotope-labeled internal standards were used for all the analytes, matrix effects were compensated for without affecting the method precision and accuracy.

2. LC-MS/MS analysis

The LC-MS/MS method was originally validated for COT, NCOT, OHCOT and COTNO from 1 to 100 ng/mL in plasma and rat brain tissue homogenate, with intra-day (n = 5) and inter-day (n = 15) precision within 20% for the lower limit of quantitation and 15% for three different QC concentration levels.[32] In order to fit the higher analyte concentrations in this PK study, the linear ranges of COT, NCOT, OHCOT and COTNO were adjusted to be from 20 to 10000 ng/mL in plasma. Accordingly, some minor changes were made to the sample preparation and LC-MS/MS method to avoid saturation of the detector. In sample preparation, 50 μL of plasma was used instead of 100 μL, while the volume of reconstitution remained the same. Also, the injection volume was decreased from 10 μL to 5 μL in the LC-MS/MS method. Therefore, the on-column concentrations of analytes were four times lower than samples analyzed by the original method. Good linearity ($R^2 > 0.99$) was observed with the elevated range for each analyte. In order to ensure the precision and accuracy of the modified method, a partial method qualification experiment was conducted by analyzing spiked samples (n = 3) at the LLOQ (20 ng/mL) and three QC levels (30, 750 and 7500 ng/mL) together with real PK samples, giving out satisfactory precision (coefficient variation) and accuracy (relative error from the nominal values) within 15%.

3. Intravenous PK of COT

To obtain basic PK parameters, a single dose of 3 mg/kg COT was given to rats (n = 3) via IV administration. Plasma samples obtained up to 24 hours from the administration were analyzed by the LC-MS/MS method described above. All the COT concentrations measured were within the linear range of the analytical method, only except one data point that was slightly over the upper limit of quantitation (ULOQ) by 0.46%. Plasma COT concentrations were plotted in a regular scale coordinate, as shown in Figure 4.2. Great agreement was observed among the three tested animals, including the absolute concentrations as well as the trend of concentration changes.

According to the trend of plasma COT concentration changes over the time, the concentration-time curves with a sharp defection point suggested two different first order kinetic phases, the distribution phase and the elimination phase. Therefore, the concentration versus time data were fitted into a two-compartment IV-bolus PK model with first-order elimination, with the schematic diagram and equation shown in Figure 4.3. By running this PK model with concentration-time data from three tested animals, three individual PK profiles were obtained, as shown in Figure 4.4. All three PK profiles had good fit with the PK model and great similarity between each other. Different models were tested. However, the two-compartment IV-bolus PK model showed the best fit with the lowest AIC values.

PK parameters for intravenously dosed COT were generated using the two-compartment IV-bolus model, shown in Table 4.1. After entering the central compartment via an IV bolus injection, COT was rapidly distributed into the peripheral compartment with a high distribution rate constant (k_{12} = 6.84 h^{-1}) and a very short distribution half-life ($t_{1/2,\alpha}$ = 0.07 h). During the distribution phase, COT plasma concentrations experienced a rapid drop, due to the distribution

into the peripheral compartment (tissues) as well as the elimination from the central compartment (circulatory system). A pseudo-equilibrium was reached in a short time (3 to 5 $t_{1/2,\alpha}$) and the PK of COT entered the elimination phase, where COT was cleared from the central compartment (k_{10} = 2.34 h^{-1}) while slowly redistributing from the peripheral compartment (k_{21} = 0.53 h^{-1}). This combination resulted in COT showing very slow elimination from the body with a long elimination half-life ($t_{1/2,\beta}$ = 5.49 h). Though COT had rapid elimination from the central compartment (k_{10} half-life = 0.31 h), it still showed a low clearance of 116.67 mL/hr/kg, due to the small volume of the central compartment (V_1 = 53.09 mL/kg). Among a large steady state volume of distribution of 712.01 mL/kg, the peripheral compartment accounted for the predominant portion (V_2 = 658.92 mL/kg).

All of the PK parameters for IV dosed COT obtained in this study showed good agreement with previously published PK studies for COT in humans. COT was reported to have a long elimination half-life of 12.2 h and 15.5 h by two studies conducted on humans.[29, 30] COT intravenously dosed to rats showed a shorter elimination half-life of 5.49 h in our study, which was a reasonable result, due to the fact that rats usually have faster drug clearance than humans. An elimination half-life of 5.49 h can still be considered to be long for rats, which showed consistence with the fact that COT had a long half-life in humans.

Based on the PK parameters obtained from the IV experiments, COT showed PK properties that agreed with its chemical and physical properties. As COT is a small and polar compound that can easily pass through cellular membranes, the distribution of COT from the circulatory system into the peripheral tissues was fast and thorough. The short distribution phase also suggests that COT tended to enter rapidly equilibrating tissues including red blood cells, liver and kidney. Since COT is a highly polar small-molecule drug, the elimination from the

circulatory system is expected to be fast, due to the high water solubility and membrane permeability. However, COT showed a long apparent half-life in PK studies, which was caused by this extensive presence in the peripheral tissues and the sustained redistribution from the peripheral tissues back into the circulatory system. The long half-life of COT explains the observation of the lasting pro-cognitive effects of nicotine, which has a short half-life. The PK properties of COT revealed in these experiments, including the fast distribution into peripheral tissues, lasting redistribution back into the circulatory and long half-life, make COT of great potential to be developed as a therapeutic agent for the treatment of AD and other types of dementia.

4. Oral PK of COT

To obtain bioavailability and other additional PK parameters, a single dose of 3 mg/kg COT was orally administered to rats (n = 4). Due to the longer absorption and elimination phases, plasma samples were collected up to 48 hours from the administration and analyzed using LC-MS/MS. All the COT concentrations measured were within the linear range of the analytical method. Plasma COT concentrations versus time were plotted in a regular scale coordinate in Figure 4.5, in which the four tested animals showed similar concentration changes over time.

After different models were tested, a one-compartment oral PK model was chosen as the optimal model, due to the lowest AIC values. The schematic diagram and equation are shown in Figure 4.6, and the four individual PK profiles are shown in Figure 4.7. PK profiles from the four tested subjects showed good fit with the PK model. The PK parameters were obtained by fitting the concentration-time data into the one-compartment oral PK model, as shown in Table 4.2.

According to the PK model, orally dosed COT would be rapidly absorbed by the (gastrointestinal) GI tract with a high absorption constant (k_{01} = 2.33 h^{-1}) and a short absorption half-life (reach peak plasma concentrations (C_{max}) at 2476.86 ng/mL in less than two hours (T_{max} = 1.34 h). As demonstrated by the PK of IV dosed COT, the elimination of COT followed a two-compartment model with first-order elimination. However, orally dosed COT only demonstrated an absorption phase and a one-compartment elimination phase, without any significant distribution phase. This was due to the rapid distribution of COT, which was even faster than the absorption ($k_{12} > k_{01}$). After orally dosed COT was absorbed by the GI tract, it was rapidly distributed from the central compartment into the peripheral compartment. With the short distribution phase overlapped with the absorption phase, the two-compartment model appeared as a one-compartment model, because the concentration changes resulting from the distribution were not as significant and were covered by the absorption. Instead of having multiple PK parameters in a two-compartment model, orally dosed COT demonstrated a low elimination rate constant (k_{10} = 0.12 h^{-1}) and a long apparent elimination half-life of 5.75 h, even though the theoretical elimination of COT from the central compartment was supposed to be fast (k_{10} half-life = 0.31 h). The clearance of COT obtained in the oral experiment was 125.25 mL/hr/kg, which was consistent with the clearance obtained in the IV experiment.

By integrating the area under the concentration-time curve (*AUC*), the total exposure of orally dosed 3 mg/kg COT was represented by the *AUC* of 24124.74 hr*ng/mL. Similarly, the total exposure from IV dosed 3 mg/kg COT was also represented by the *AUC* of 25900.49 hr*ng/mL. By comparing the oral *AUC* with the IV *AUC* at the same dosage level, the bioavailability of orally dosed COT was obtain by the *AUC* ratio of 93.14 ± 9.39%, suggesting that almost all orally dosed COT was absorbed via the GI tract. Results for oral PK of COT

obtained in this section also showed good agreement with previously published PK studies of COT in humans. According to De Schepper and coworkers, COT showed that oral bioavailability of COT ranged between 0.84 and 1.11 following 10 mg and between 0.97 and 1.03 following the 20 mg dose in humans.[29]

The PK properties revealed in these experiments, including fast absorption and high bioavailability, were highly consistent with the fact that COT was a small, polar and higher water-soluble chemical. Slow clearance and long apparent half-life also agreed with the results from the IV PK experiments.

5. Metabolism of COT

Plasma concentrations of NCOT, OHCOT and COTNO were measured simultaneously when COT concentrations from intravenously dosed rats were analyzed by LC-MS/MS. After concentration-time data were obtained for each of these COT metabolites, *AUC*s were calculated by fitting the data into non-compartment models. NCOT, OHCOT and COTNO demonstrated *AUC*s of 42.77, 465.78 and 408.84 hr*ng/mL, respectively. By comparing the metabolite *AUC*s with that of COT, the percentage of COT metabolized into each metabolite was calculated as 0.16% for NCOT, 1.80% for OHCOT and 1.58% for COTNO. These results suggested that only a small fraction of dosed COT was metabolized into these major metabolites. The major fraction of COT was excreted from the body unchanged. According to the previously published studies, only 10 to 12% of COT was excreted unchanged from the body when intravenously dosed to humans.[29, 30] This number was much higher in our study, which might result from the absence of CYP 2A6, the primary enzyme for the metabolism of COT, in rats. The percentage of metabolized COT was estimated based on the concentrations of three major metabolites of COT,

which was not based on a direct measurement of metabolized or excreted COT. Urine collection and renal clearance measurements are still needed for more accurate results.

Conclusions

A PK study of orally and intravenously dosed COT was conducted, using a selective and sensitive LC–MS/MS quantitation method for the simultaneous determination of COT, NCOT, OHCOT and COTNO in rat plasma. Results from this study revealed important PK parameters of COT in rats, including high bioavailability, rapid absorption, fast tissue distribution and a long half-life of COT. All PK parameters obtained in this study were important for COT to effectively enter the tissue and demonstrate its sustained pro-cognitive effects. Such information is of great importance in future pharmacological, toxicological and other pre-clinical studies of COT in rodent species, for the development of COT-based therapeutic agents for the treatment of AD and other types of dementia.

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Table 4.1 Pharmacokinetic parameters of COT obtained from IV bolus experiments (n = 3).

Parameter	Mean ± SD
AUC (hr*ng/mL)	25900.49 ± 2698.80
k_{10} (1/hr)	2.34 ± 0.75
k_{12} (1/hr)	6.84 ± 1.46
k_{21} (1/hr)	0.53 ± 0.08
k_{10} half-life (hr)	0.31 ± 0.09
α (1/hr)	9.58 ± 2.15
β (1/hr)	0.13 ± 0.01
$t_{1/2,\alpha}$ (hr)	0.07 ± 0.02
$t_{1/2,\beta}$ (hr)	5.49 ± 0.54
A (ng/mL)	58182.35 ± 20076.88
B (ng/mL)	2514.35 ± 143.40
C_{max} (ng/mL)	60696.71 ± 20220.19
V_{ss} (mL/kg)	712.01 ± 98.47
V_1 (mL/kg)	53.09 ± 16.76
V_2 (mL/kg)	658.92 ± 81.87
CL (mL/hr/kg)	116.67 ± 12.20
CLD_2 (mL/hr/kg)	352.95 ± 93.09

AUC, area under the concentration – time curve; k_{10}, elimination rate constant; k_{12}, distribution rate constant; k_{21}, redistribution rate constant; α, hybrid rate constant for distribution; β, hybrid rate constant for elimination; $t_{1/2,\alpha}$, distribution half-life; $t_{1/2,\beta}$, elimination half-life; C_{max}, peak plasma concentration; V_{ss}, volume of distribution at steady state; V_1, volume of the central compartment; V_2, volume of the peripheral compartment; *CL*, clearance; CLD_2, inter-compartment clearance.

Table 4.2 Pharmacokinetic parameters of COT obtained from oral experiments (n = 4).

Parameter	Mean ± SD
AUC (hr*ng/mL)	24124.74 ± 2430.79
V (mL/kg)	1030.61 ± 52.08
k_{01} (1/hr)	2.33 ± 0.09
k_{10} (1/hr)	0.12 ± 0.01
k_{01} half-life (hr)	0.30 ± 0.01
k_{10} half-life (hr)	5.75 ± 0.70
CL (mL/hr/kg)	125.25 ± 13.37
T_{max} (hr)	1.34 ± 0.03
C_{max} (ng/mL)	2476.86 ± 116.92

AUC, area under the concentration – time curve; *V*, volume of distribution; k_{01}, absorption rate constant; k_{10}, elimination rate constant; *CL*, clearance; T_{max}, time to reach peak plasma concentration; C_{max}, peak plasma concentration.

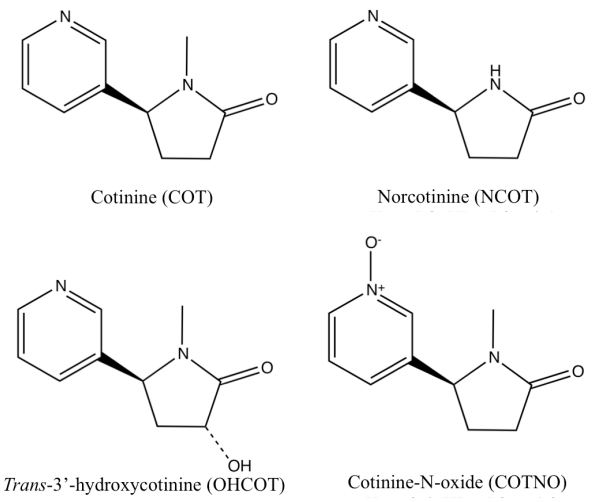


Figure 4.1 Chemical structures of cotinine (COT), norcotinine (NCOT), *trans*-3'-hydroxylcotinine (OHCOT) and cotinine-N-oxide (COTNO).

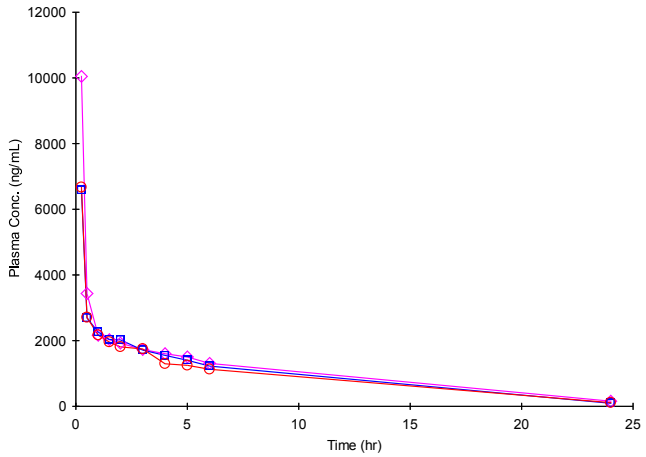


Figure 4.2 Plasma concentration (ng/mL) versus time (h) data obtained from rats (n = 3) intravenously dosed with 3 mg/kg COT.

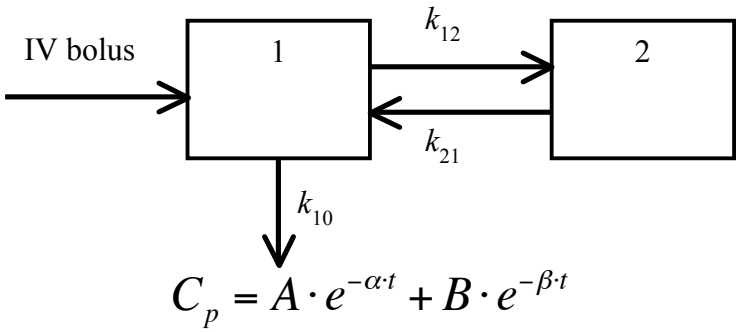


Figure 4.3 Schematic diagram and equation of two-compartment IV-bolus PK model with first order elimination. Compartment 1 is the central compartment and compartment 2 is the peripheral compartment. k_{10}, elimination rate constant; k_{12}, distribution rate constant; k_{21}, redistribution rate constant; C_p, plasma concentration; t, time; α, hybrid rate constant for distribution; β, hybrid rat constant for elimination.

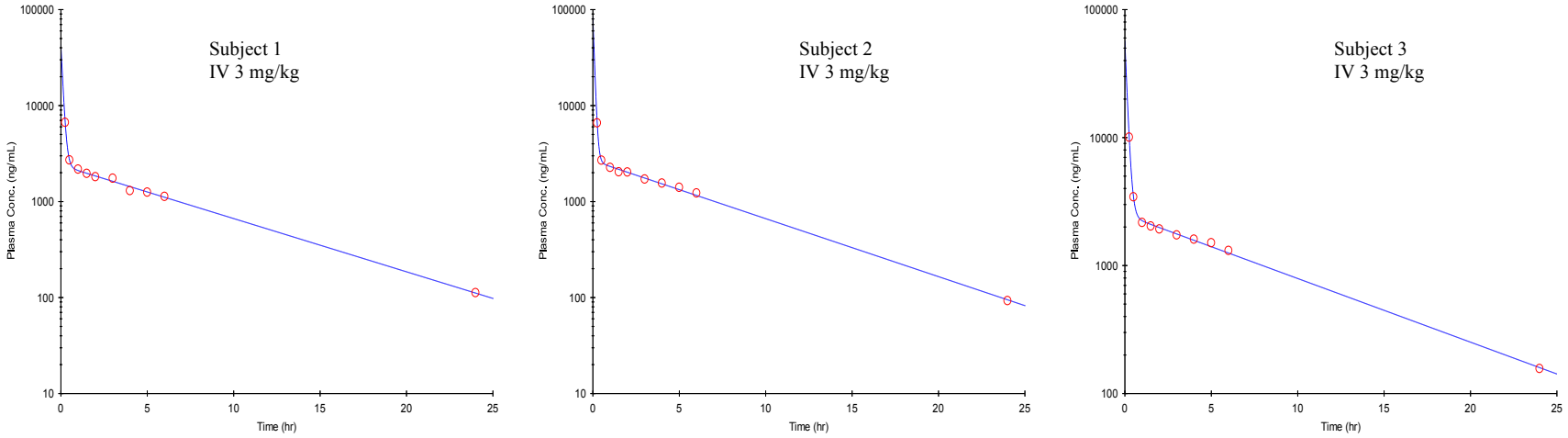


Figure 4.4 PK profiles in semi-logarithmic scale for rats intravenously dosed with 3 mg/kg COT.

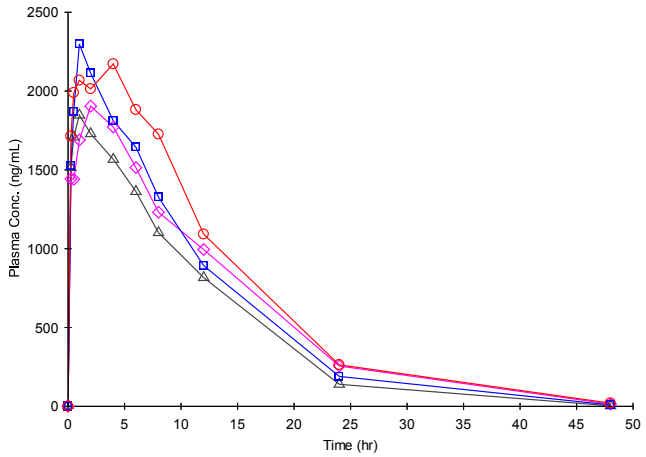
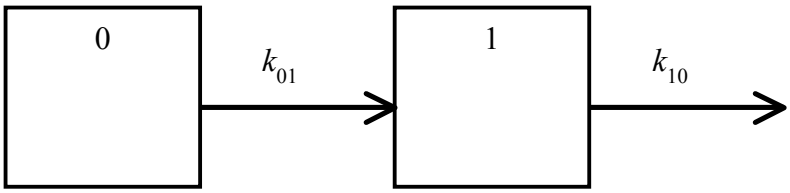


Figure 4.5 Plasma concentration (ng/mL) versus time (h) data obtained from rats (n = 4) orally dosed with 3 mg/kg COT.



$$C_p = \frac{F \cdot D \cdot k_{01}}{V \cdot (k_{01} - k_{10})} \cdot (e^{-k_{10} \cdot t} - e^{-k_{01} \cdot t})$$

Figure 4.6 Schematic diagram and equation of one-compartment oral PK model with first order elimination. Compartment 0 is the GI track and compartment 1 is the central compartment. k_{10}, elimination rate constant; k_{01}, absorption rate constant;; C_p, plasma concentration; F, bioavailability; D, dose; V, volume distribution; t, time.

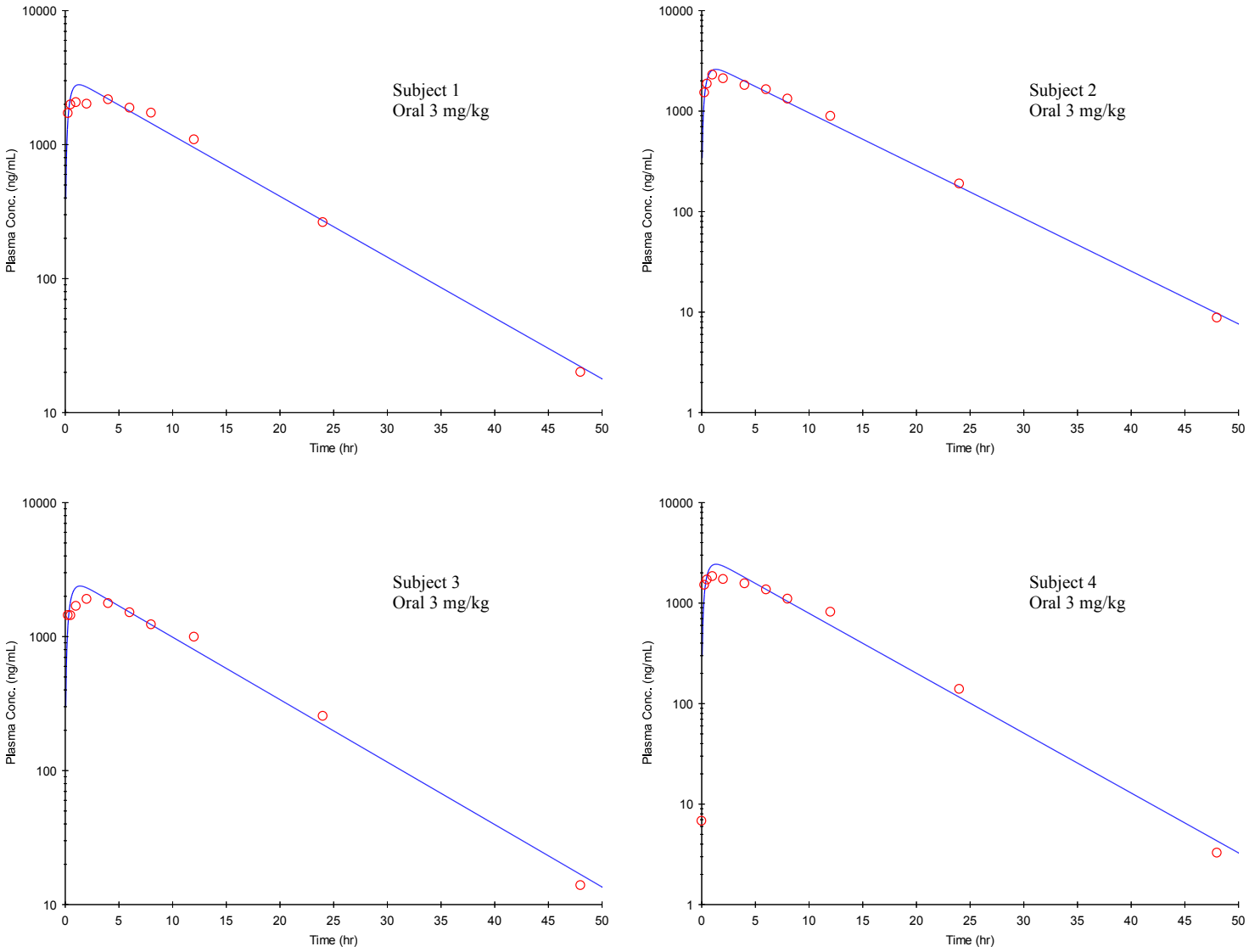


Figure 4.7 PK profiles in semi-logarithmic scale for rats orally dosed with 3 mg/kg COT.

CHAPTER 5

A RAPID ANALYTICAL METHOD FOR THE QUANTIFICATION OF PACLITAXEL IN RAT PLASMA AND BRAIN TISSUE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY (HPLC-MS/MS)

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Abstract

RATIONALE: Paclitaxel, an antitumor agent for the treatment of several types of cancers, has recently been reported to cause impaired cognitive function and neuropathic pain in humans. To assess the effects of paclitaxel on the central nervous system, a sensitive and accurate method is required to quantify paclitaxel concentrations in plasma and brain tissue obtained from rodents receiving paclitaxel.

METHODS: The biological samples were prepared by liquid-liquid extraction and separated by a 3.5 min reverse-phase liquid chromatography (RPLC) method using a BDS Hypersil C8 column under isocratic conditions. Paclitaxel was quantified using a multiple reaction monitoring (MRM) with a triple quadrupole tandem mass spectrometer working in the ESI+ mode. A stable isotope-labeled analogue of paclitaxel was used as the internal standard (IS).

RESULTS: The method was validated to be precise and accurate within the dynamic range of 0.5-100 ng/mL based on 100 μL plasma and 1.5-300 ng/g based on 33 mg of brain tissue in homogenate. This method was applied to samples from 2 mg/kg intravenously dosed rats. The plasma concentrations were observed to be 26.62 ± 8.93 ng/mL and brain concentrations 11.08 ± 4.18 ng/g when measured 4 hours post dose.

CONCLUSIONS: This rapid LC-MS/MS method was validated to be sensitive, specific, precise and accurate for the quantification of paclitaxel in rat plasma and brain tissue homogenate. Application of the method to study samples provided sufficient proof of blood-brain barrier penetration of paclitaxel, allowing further investigation of its influence on the central nervous system.

Key words

Paclitaxel, brain, plasma, quantification, LC-MS/MS.

1. Introduction

Paclitaxel (Taxol), a compound extracted from the trunk bark of the Pacific yew tree *Taxus brevifolia*, was found to have significant cytotoxicity by preventing the depolymerization of microtubules and therefore inhibiting mitosis.[1] Based on its cytotoxic activities, paclitaxel has been confirmed to have a wide spectrum of anticancer activity and can be used as a chemotherapeutic agent for the treatment of different types of cancers, including lung, ovarian, breast, colon, head and neck cancers.[2, 3] In order to further study the activity and toxicity of paclitaxel, it is necessary to investigate its distribution in the circulatory system and organ tissues.

Due to the existence of the blood-brain barrier (BBB), the distribution of paclitaxel in brain tissue could be significantly different from that in plasma or other tissues.[4] Thus the BBB permeability and brain tissue distribution of paclitaxel are of great importance for the study of its efficacy towards brain cancers. Moreover, attention has been drawn to the major side effects of paclitaxel on the nervous system, including impaired cognitive function and neuropathic pain.[5-8] To facilitate further study on the efficacy and toxicity of paclitaxel on the central nervous system (CNS), it is necessary to develop an analytical method that can quantify paclitaxel in the plasma and brain tissue to obtain distribution information.

A number of analytical methods have been developed for the quantitation of paclitaxel in biological samples. Due to the specificity and sensitivity of liquid chromatography-tandem mass spectrometry (LC-MS/MS), most current methods are based on this technique.[9-28] These methods were able to quantify paclitaxel in common biological samples, with the highest sensitivity of 0.1 ng/mL in plasma[9], 5 pg/mL in cell media[23], and 25 ng/mL in urine[18]. Some of these methods required a large sample volume[9, 14, 28] or complicated sample preparation[11, 17,

[28] to achieve the sensitivity they reported. Meanwhile, only very few methods were developed and validated for brain sample analysis. The first method for brain paclitaxel quantitation was developed by P. Guo et al., which had a low sensitivity (54 ng/mL in brain homogenate) and also a complex sample preparation method with solid-phase extraction (SPE).[11] Two newer methods with higher sensitivity were published by W. Guo (10 ng/g in brain tissue) and X. Tong (2.0 ng/g in brain tissue), respectively.[12, 20] All these methods for brain paclitaxel quantitation reported either an incomplete validation or a validation following the guidance from the State Food and Drug Administration (SFDA) of China instead of the guidance from the US Food and Drug Administration (FDA).[29, 30] Therefore, it is still necessary to develop and fully validate an analytical method that demonstrates quantitation of paclitaxel in plasma and brain tissue with high sensitivity, precision and accuracy. Other improvements such as lower sample volume, simpler sample preparation and higher throughput of LC-MS/MS would also make for better method performance.

Based on the physicochemical properties of paclitaxel, we developed an LC-MS/MS method for the quantitation of paclitaxel in both rat plasma and brain tissue samples, which had the advantages of higher sensitivity, simpler sample preparation and higher throughput compared to previous methods. The specificity of the LC-MS/MS method and the utilization of a stable isotope-labeled internal standard (IS) provided the method with great precision and accuracy, which were both well within the requirements of the US FDA guidance. This method has been successfully applied to an animal study of the brain distribution and the BBB permeability of paclitaxel.

2. Experimental

2.1 Chemicals and reagents

Paclitaxel standard was purchased from Sigma-Aldrich (St. Louis, MO). The stable isotope-labeled internal standard, paclitaxel-d5 (benzoylamino), was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Chemical structures of paclitaxel and paclitaxel-d5 are shown in Figure 5.1. Methyl tert-butyl ether (MTBE), ammonium formate, formic acid, acetonitrile (ACN), methanol (MeOH) and water were all LC-MS grade agents from Sigma (St. Louis, MO).

2.2 Instrumentation

The LC-MS/MS analysis was performed with an Agilent 1100 binary pump HPLC system (Santa Clara, CA) interfaced to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer with an electrospray (ESI+) source (Milford, MA). The Masslynx 4.0 software by Waters (Beverly, MA) was used for the instrument control and quantitation analysis.

2.3 LC-MS/MS conditions

The separation was performed on a BDS Hypersil C8 (50 × 2.1 mm, 5 μm) from Thermo Scientific (West Palm Beach, FL) coupled with a SecurityGuard C8 guard column (4 × 3.0 mm ID) from Phenomenex (Torrance, CA). The mobile phase was 10mM ammonium formate aqueous buffer with 0.1% formic acid (A) and MeOH (B). An isocratic elution with a washing gradient was used (time/minute, % mobile phase B): (0, 60), (1.5, 60), (1.8, 95), (2.7, 95), (3.0, 60), (3.5, 60). The flow rate was 0.8 mL/min and the column temperature was 40 °C. The LC system was interfaced by a six-port divert valve to the mass spectrometer, introducing eluents from 0.5 to 1.5 min to the ion source. The injection volume was 20 μL and the injection needle was washed with methanol after each injection.

The mass spectrometer was operated in the positive ion ESI mode. Nitrogen was used as the desolvation gas at a flow rate of 800 L/h and a temperature of 400 °C. The cone gas flow was 50 L/h. The source temperature was 110 °C. The capillary voltage and the cone voltage were set at 3.5 kV and 38 V, respectively. Argon was used as the collision gas and the collision cell pressure was maintained at 3.5×10^{-3} mbar. The multiple reaction monitoring (MRM) functions were used to detect and quantify the analyte and IS. With the collision energy at 27 eV and the dwell time at 0.1 sec, ion transitions of *m/z* 876→308 and 881→313 were monitored for paclitaxel and paclitaxel-d5 respectively, which represented the fragmentation at the ester bond and a loss of the taxane structure; while ion transitions of *m/z* 876→591 and 881→591, which represented the fragmentation at the same bond but with charge retentions on the taxane fragments, were used as confirmation transitions. Fragmentation profiles are shown in Figure 5.2.

2.4 Solutions and standards

The stock solutions of paclitaxel and paclitaxel were prepared by dissolving 1.0 mg of solid in 1.0 mL of ACN to yield a concentration of 1.0 mg/mL. Calibration working solutions of paclitaxel were prepared at concentrations of 5, 10, 50, 100, 200, 500, 800 and 1000 ng/mL by serial dilutions from the stock solution. Quality control (QC) working solutions were 5, 15, 300.0 and 750 ng/mL. IS working solution was 250 ng/mL paclitaxel-d5 in ACN. Stock solutions were kept at -20 °C when not in use.

2.5 Spiked samples and real samples

K_2 EDTA-treated blank Sprague–Dawley rat plasma was purchased from Bioreclamation (Westbury, NY). Blank brains obtained from drug-free Sprague–Dawley rats were rinsed by normal saline to remove blood and then homogenized with two volumes of water to prepare the

blank brain homogenate. A Kinematica (Luzern, Switzerland) Polytron® rotating blade homogenizer was used to disperse the brain tissue to form a homogeneous mixture with water. 10 μL of calibration or QC working solution was spiked into 100 μL of plasma or brain homogenate to generate the corresponding calibration standards or QC samples. The final concentrations of the calibration standards were 0.5, 1.0, 5.0, 10.0, 20.0, 50.0, 80.0 and 100.0 ng/mL in rat plasma or brain homogenate. The QC samples were 3.0, 30.0 and 70.0 ng/mL.

Real samples were obtained from 2 mg/kg intravenously (tail vein) dosed rats 4 hours after the treatment. The injection was conducted with a 1 mL paclitaxel solution within 1 min. Plasma was collected via cardiac puncture and treated with EDTA. Brain samples were homogenized in the same manner as blank brain.

All biological samples were stored at -20 °C before use. Fresh standards and QC samples were prepared for each day of validation.

2.6 Sample preparation

Sample preparation was carried out by a one-step liquid-liquid extraction (LLE) method. To each 100 μL of biological sample, 20 μL of IS working solution (250 ng/mL) and 1 mL of MTBE were added. The mixture was vortexed for 10 min and then centrifuged at 21,130 g for 10 min to extract paclitaxel and the IS. The supernatant was transferred to a new tube and then evaporated to complete dryness in a centrifuge evaporator at 40 °C. The residue was reconstituted by 100 μL of 60% MeOH/water (v/v) with 0.1% formic acid. After 5 min of sonication, 5 min of vortex and 5 min of centrifugation at 21,130 g, the supernatant was obtained for LC-MS/MS analysis.

2.7 Method validation

Linearity was tested by spiked standards for both plasma and brain homogenate. Calibration curves were made from peak area ratios between analyte and IS, using 1/x weighted linear regression. The intra-day (n = 5) and inter-day (n = 15) precision and accuracy were assessed by QC samples at 0.5 (LLOQ), 1.5, 30.0 and 75.0 ng/mL.

Autosampler stability (25 °C, 8 hours), bench-top stability (25 °C, 8 hours) and freeze-thaw stability (-20 °C, 3 freeze-thaw cycles, 72 hours) in plasma and brain homogenate were tested for paclitaxel at the low (1.5 ng/mL) and high (75 ng/mL) concentrations (n = 3), by comparing samples before and after the stability tests.

Matrix effects, relative recovery and absolute recovery for both plasma and brain homogenate were calculated from peak areas of spiked samples, post-preparation spiked samples and neat standard solutions of paclitaxel at 1.5, 30.0 and 75.0 ng/mL (n = 3).

3. Results and Discussion

3.1 LC-MS/MS method development

To achieve the separation and quantification of paclitaxel within the biological samples, conditions needed to be determined and optimized for both the high performance liquid chromatography (HPLC) and the tandem mass spectrometry (MS/MS).

The conditions of an HPLC method are usually determined by the physiochemical properties of both the analytes and the biological matrices. Paclitaxel has a partition coefficient value (LogP) of 3.3, suggesting that it is a hydrophobic compound with higher affinity to reverse-phase columns. It is also a weak base (amide) with a pKa value of 10.4, which means more than 99.9% of paclitaxel molecules will be uncharged in a pH 7.0 environment. Based on these properties of the analyte, a BDS Hypersil C8 column was chosen due to its specificity for

hydrophobic and basic compounds. A water/MeOH mobile phase system was used with this column to separate paclitaxel from the matrices by reverse-phase liquid chromatography (RPLC). Peak width and peak symmetry were optimized by the addition of an ammonium formate/formic acid buffer system. Since there is only one analyte and the corresponding stable isotope-labeled IS to be separated, an isocratic condition was used for the separation. A washing step with high organic composition was added after the elution of the analyte to remove all the hydrophobic impurities from the matrices, followed by a re-equilibration step. During the method development, we also tried different gradient conditions, by which better separation could be achieved with sharper peak shapes, higher throughput and higher sensitivity. However, significant carryover was observed when a gradient was used. With the autosampler bypassed, the carryover was confirmed to be in the column, which has been referred to as "column memory effect".[31] The reason for this issue was believed to be incomplete elution of the analyte under gradient conditions, resulting from the strong binding or secondary interactions between the hydrophobic analyte and the stationary phase. This issue could be easily overcome by isocratic conditions with slight compromise of the chromatographic performance.

For the sensitive detection of paclitaxel in the HPLC elute, the triple quadrupole mass spectrometer was set to operate under unit resolution mode. Since paclitaxel is a weak base, positive ion mode was used. With the constant infusion of a paclitaxel neat solution (10 μg/mL in ACN) at a flow rate of 10 μg/min, ion source parameters were optimized to achieve the strongest intensity of the molecular peak of paclitaxel, which was a sodium adduct ion $[M+Na]^+$ at *m/z* 876. According to the MS1 scan spectrum of paclitaxel shown in Figure 5.2A, the most abundant peak was the sodium adduct ion $[M+Na]^+$ at *m/z* 876 instead of the protonated molecule $[M+H]^+$ at *m/z* 854, due to its strong affinity to sodium ions originating from the

glassware, stainless steel parts, solvent impurities and biological matrices (for biological samples).[32] The sodiated molecule of paclitaxel was selected as the precursor ion to be forwarded to further optimization. A product ion scan of the sodiated molecule at *m/z* 876 was conducted, which gave the most intense product ion at *m/z* 308 at the optimized collision energy of 28 eV, shown as Figure 5.2B. Therefore, the MRM transition of *m/z* 876→308 for paclitaxel and the corresponding transition of *m/z* 881→313 for paclitaxel-d5 were set up for quantitation, which provided more than 10 times higher sensitivity than the transition using $[M+H]^+$ ions.

3.2 Sample preparation method development

Considering that paclitaxel is a highly hydrophobic compound, we expected to use liquid-liquid extraction (LLE) to extract it from the biological samples. LLE is one of the most commonly used approaches for biological sample preparation, due to its greater specificity versus protein precipitation (PPT) and higher throughput relative to solid phase extraction (SPE). In all the published methods, LLE with MTBE, which has high volatility and extraction efficiency, was the most used protocol for the preparation of plasma samples.[9, 10, 12-15, 18-22, 24-27] Meanwhile, SPE is more used to prepare tissue samples for a more thorough clean-up.[11, 18, 23] To achieve satisfactory recovery and also high throughput, the LLE method with MTBE was used for the sample preparation of both plasma and brain samples in our study, so that both types of samples could be processed simultaneously. Centrifugation was used to separate the organic layer from the tissue pellet and aqueous layer, instead of a freezing process used in some of the current methods.[9, 19] After evaporation, reconstitution and another round of centrifugation, the extracted samples could be analyzed by LC-MS/MS.

3.3 Linearity and sensitivity

Calibration curves made for paclitaxel in plasma and brain homogenate are shown in Table 5.1. A good linearity ($R^2 > 0.99$) was observed over the range of 0.5–100 ng/mL in plasma and brain homogenate (1.5 – 300 ng/g in brain tissue). A 1/x-weighted linear regression was used for all the calibration curves. Slopes, intercepts and R^2 values are shown in Table 5.1. The sensitivity of the method was defined by the lower limit of quantitation (LLOQ), which was 0.5 ng/mL in plasma or brain homogenate (1.5 ng/g in brain tissue).

3.4 Precision and accuracy

Precision and accuracy of this method were assessed at the LLOQ and three QC concentrations of paclitaxel in both matrices. Precision, defined as the closeness of measurements of the same concentration, was represented by the coefficient of variation (CV) or relative standard deviation (RSD) among measured concentrations. Accuracy, defined as the closeness between measured and true values, was assessed by the relative bias (%bias) between measured concentrations and nominal concentrations. Both intra-day (n = 5) and inter-day (n = 15) precision and accuracy were tested based on a three-day validation for both plasma and brain samples. RSD and %bias values are shown in Table 5.2, which are all well within the FDA acceptance criteria of less than 15% for QCs and less than 20% for LLOQs, suggesting great precision and accuracy of the method.

3.5 Recovery and matrix effects

Recovery and matrix effects of paclitaxel were tested at the three QC concentrations (n = 3) in both plasma and brain samples, shown in Table 5.3.

For each concentration in the respective matrix, three blank biological samples and three spiked samples were prepared. The spiked samples were reconstituted with the initial mobile

phase, while the blank samples were spiked with paclitaxel to prepare "post-extraction spiked" samples. At the same time, three neat standards for each concentration were prepared by diluting the paclitaxel working solution in the same mobile phase. Relative recoveries, which represented the extraction efficiency, were calculated by the ratio between the peak areas of paclitaxel in the "post-extraction spiked" samples and the corresponding spiked samples. Matrix effects, which represented the influence of the matrix on the analyte response, were calculated by the ratio between the peak areas of paclitaxel in the "post-extraction spiked" samples and the corresponding neat standards.

According to the data shown in Table 5.3, the extraction efficiency for paclitaxel was almost 100% in the plasma, due to the high hydrophobicity of paclitaxel. The extraction efficiency was slightly lower in the brain homogenate than plasma, which was caused by interference from lipids and/or protein impurities in the brain tissue. As has been previously reported by Xue et al., the recovery obtained from spiked tissue homogenates may not truly reflect the recovery of compounds extracted from the tissues of dosed animals.[33] This is because the spiked compounds may not penetrate into the cells of tissue homogenate in a manner which accurately mimics the real samples. This potential problem can be minimized when the homogenization process is able to disperse the tissue cells into a homogenous mixture, such that there is no significant difference between the spiked and dosed tissue homogenates. In our study, the brain tissue was homogenized using pure distilled deionized water and a rotating blade homogenizer. Due to the softness of brain tissue and the lysing effects of the water, the brain homogenate prepared in this manner was quite homogeneous. Therefore, the spiked brain homogenate showed great similarities to the real samples and is therefore, representative of the real recovery from tissues.

A consistent level of matrix effect was observed in all the tested concentrations in both matrices, resulting from ion suppression caused by the co-eluting impurities that competed with paclitaxel for ionization. Since the ion suppression effect was consistent and, more importantly, a stable isotope-labeled IS was used to compensate for it, the precision and accuracy were not affected by the presence of the matrix.

3.6 Specificity

Representative chromatograms obtained from double blank samples (blank without IS), zero blank samples (blank with IS) and spiked samples at the LLOQ (0.5 ng/mL for plasma and brain homogenate) are shown in Figure 5.3. A clear peak was shown in the analyte channel and the IS channel respectively for both matrices. The double blank samples showed clean background in both the analyte channel and the IS channel, suggesting there was no endogenous levels or contamination of paclitaxel or the IS. The clear background in the analyte channel of the spiked samples suggested that there was no cross-talk interference from the IS. All the chromatograms above confirmed that the LC-MS/MS method was specific for the separation and quantitation of paclitaxel and the stable isotope-labeled IS.

3.7 Stability

For the stability tests of paclitaxel, three batches of QC samples were spiked at the same time, each of which included three replicates at two concentrations (1.5 and 75.0 ng/mL) in plasma and brain homogenate, respectively. The first batch was processed and analyzed promptly to obtain the concentration data used as time zero control. These analyzed samples were left in the autosampler for 12 hours and reanalyzed for the autosampler stability test. At the same time, the second batch was left unprepared on the bench-top (25 °C) for 8 hours and then prepared and analyzed for the bench-top stability test. The other batch of samples were stored at

-20 °C for 24 hours and then completely thawed at 25 °C on the bench-top without assistance. After another two identical freeze-thaw cycles, these samples were prepared and analyzed for the freeze-thaw stability test. For all the stability tests, response factors (IS concentration times peak area ratio between paclitaxel and IS) were obtained for all the analyzed samples. Stabilities were calculated by the response factor ratio between stability test groups and the time zero control group, shown in Table 5.4. With the deviation from the time zero control within 10%, paclitaxel was confirmed to be stable at the two tested concentrations in both plasma and brain homogenate, in terms of autosampler, bench-top and freeze-thaw stability. Therefore, validation of study samples stored within the conditions of the stability tests have been demonstrated to be stable; and the data obtained are as precise and accurate as those analyzed freshly.

3.8 Application

Plasma and brain samples from rats (n = 3) intravenously dosed with 2 mg/kg of paclitaxel were obtained 4 hours after treatment. The same sample preparation and quantitation method were applied to these samples, giving out the results shown in Table 5.5. The representative chromatograms of these samples are shown in Figure 5.4.

Paclitaxel could be detected in both plasma and brain homogenate samples, with the concentrations within the linear range established in the method. Assuming 1 g of brain tissue is equivalent to 1 mL plasma, paclitaxel showed a brain-to-plasma concentration ratio of 0.42. This value might be slightly higher than the actual value, due to the remaining blood in the brain tissue even after being washed by saline. However, this ratio still showed that paclitaxel could penetrate the blood-brain barrier (BBB) and have significant distribution in the brain tissue. Further investigation of the effects of paclitaxel on the central nervous system can be done with this method.

4. Conclusions

A selective and sensitive LC–MS/MS analytical method for the quantitation of the drug paclitaxel in rat plasma and brain tissue was developed and validated. This method could fulfill the precise and accuracy quantitation of paclitaxel within the linear range of 0.5 – 100 ng/mL in rat plasma and brain homogenate (1.5 – 300 ng/g in brain tissue), with an LLOQ of 0.5 ng/mL and 1.5 ng/g, respectively. A low sample volume, 100 μL of rat plasma or brain homogenate, was needed for this method. The LC-MS/MS method was as short as 3.5 min, providing high throughput together with simple sample preparation using liquid-liquid extraction. This method has been successfully applied to preclinical studies of paclitaxel in rats to assess its blood-brain barrier permeability.

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Table 5.1 Calibration curves paclitaxel in plasma and brain homogenate (n = 3).

Matrix	Slope	Intercept	R2
Plasma	0.0281 ± 0.0008	0.0008 ± 0.0013	0.9993 ± 0.0003
Brain	0.0271 ± 0.0011	-0.0002 ± 0.0007	0.9996 ± 0.0003

Table 5.2 The intra-day (n = 5) and inter-day (n = 15) precision (RSD) and accuracy (bias) paclitaxel measurement at four concentrations in rat plasma and brain homogenate by the LC-MS/MS method.

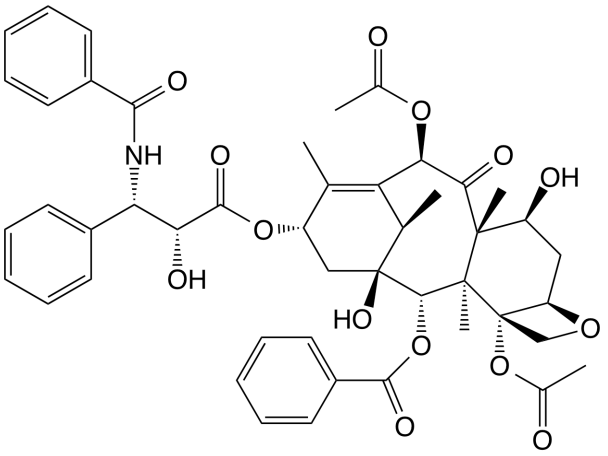
Matrix	Nominal Conc. (ng/mL)	Intra-day			Inter-day		
		Measured Conc. (ng/mL)	RSD (%)	Bias (%)	Measured Conc. (ng/mL)	RSD (%)	Bias (%)
Plasma	0.5	0.49 ± 0.02	4.85	-1.60	0.47 ± 0.02	6.00	-5.07
	1.5	1.50 ± 0.08	5.23	0.00	1.49 ± 0.08	4.96	-0.44
	30.0	30.61 ± 0.87	2.85	2.05	30.16 ± 0.38	2.58	0.52
	75.0	76.40 ± 0.73	0.95	1.87	75.49 ± 0.46	1.68	0.65
Brain	0.5	0.51 ± 0.01	1.77	1.20	0.50 ± 0.02	5.57	-0.67
	1.5	1.50 ± 0.02	1.53	0.27	1.51 ± 0.10	4.47	0.58
	30.0	30.26 ± 0.65	2.14	0.85	30.20 ± 0.16	1.77	0.68
	75.0	75.78 ± 1.04	1.37	1.05	76.41 ± 1.42	1.40	1.88

Table 5.4 Autosampler stability (n = 3), bench-top stability (n = 3) and freeze–thaw stability (n = 3) of paclitaxel at two concentrations in plasma and brain homogenate. Stabilities are shown in forms of percentage of relative concentration to the time zero control (mean ± SD).

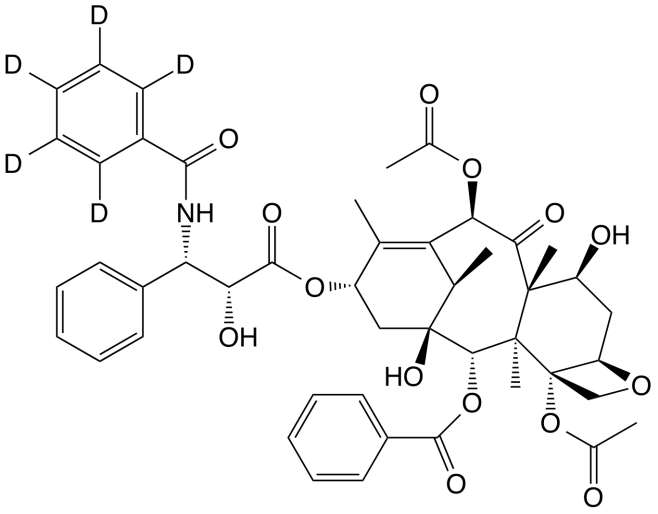
Matrix	Conc. (ng/mL)	Autosampler stability (%)	Bench-top stability (%)	Freeze-thaw stability (%)
Plasma	1.5	100.55 ± 2.19	92.53 ± 3.34	99.09 ± 3.34
	75.0	100.08 ± 1.06	92.72 ± 0.72	98.19 ± 0.32
Brain	1.5	93.75 ± 2.08	98.61 ± 3.18	95.14 ± 2.41
	75.0	99.63 ± 1.70	99.73 ± 2.07	100.52 ± 1.58

Table 5.5 Paclitaxel concentrations (mean ± SD) of plasma and brain samples obtained from rats (n = 4) 4 hours after a single subcutaneous dose of 2 mg/kg paclitaxel.

Matrix	Measured Conc.
Plasma	26.62 ± 8.93 ng/mL
Brain	11.08 ± 4.18 ng/g



Paclitaxel



Paclitaxel-d5

Figure 5.1 Chemical structures of paclitaxel and paclitaxel-d5.

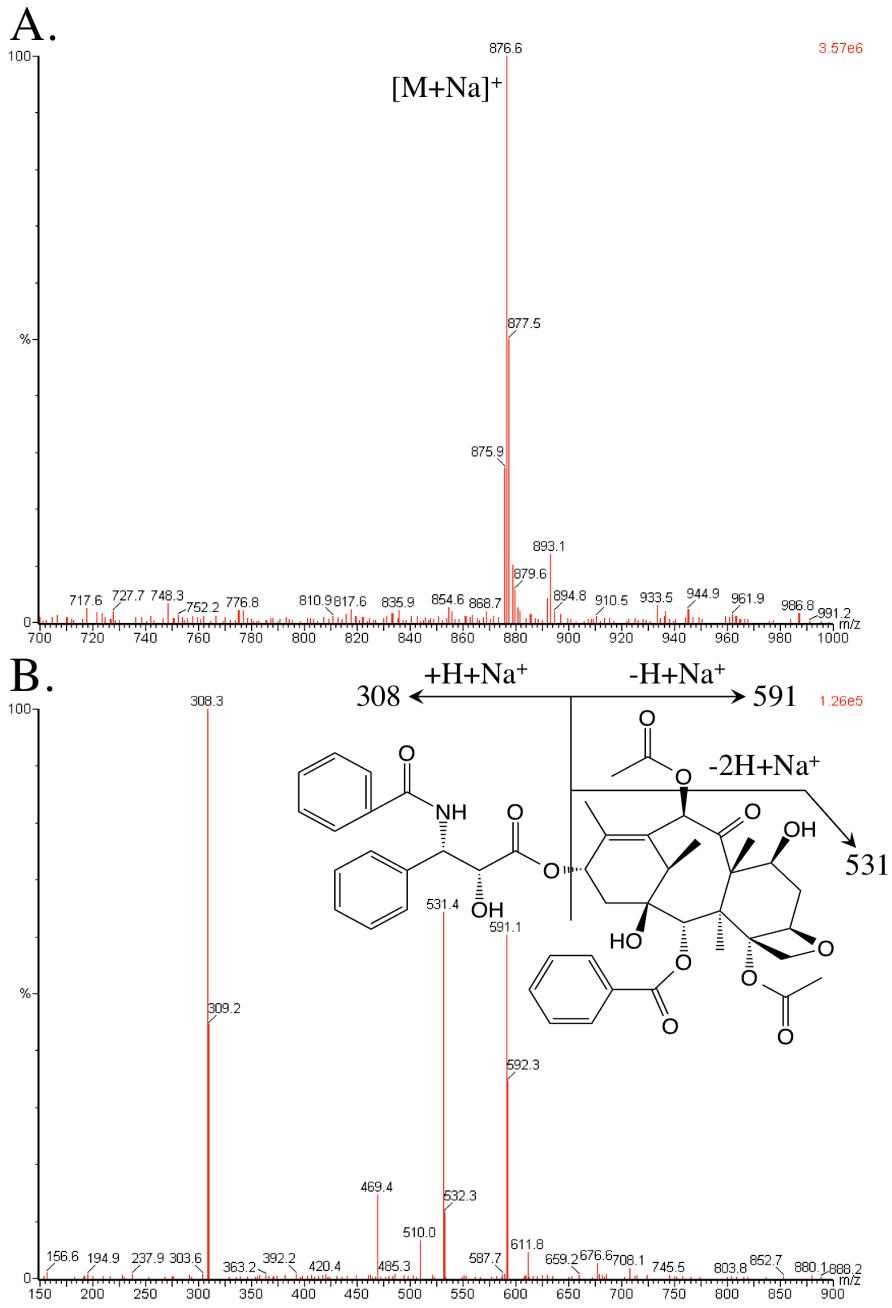


Figure 5.2 A) Mass spectrum of the MS1 scan of paclitaxel in the standard solution; B) mass spectrum of the product ion scan of the sodiated ion $[M+Na]^+$ of paclitaxel (m/z 876) and the fragmentation profile of paclitaxel under the experiment conditions. Charge retentions are presented by arrows.

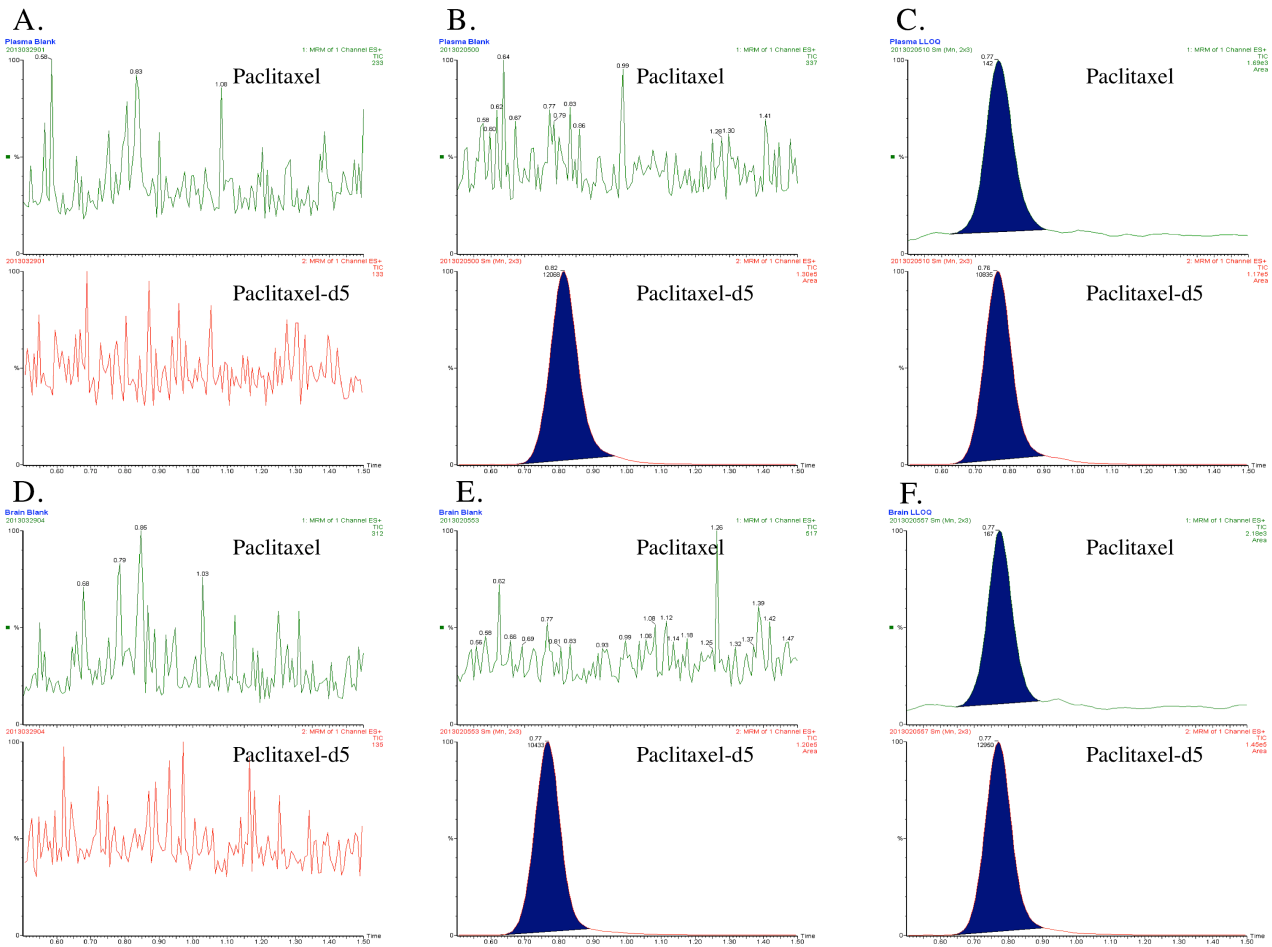


Figure 5.3 Representative chromatograms of A) double blank plasma sample; B) zero blank plasma sample with IS; C) spiked plasma sample at the LLOQ 0.5ng/mL; D) double blank brain homogenate sample; E) zero blank brain homogenate sample with IS; F) spiked brain homogenate sample at the LLOQ 0.5ng/mL. Both channels for paclitaxel and the IS (paclitaxel-d5) are displayed for each sample.

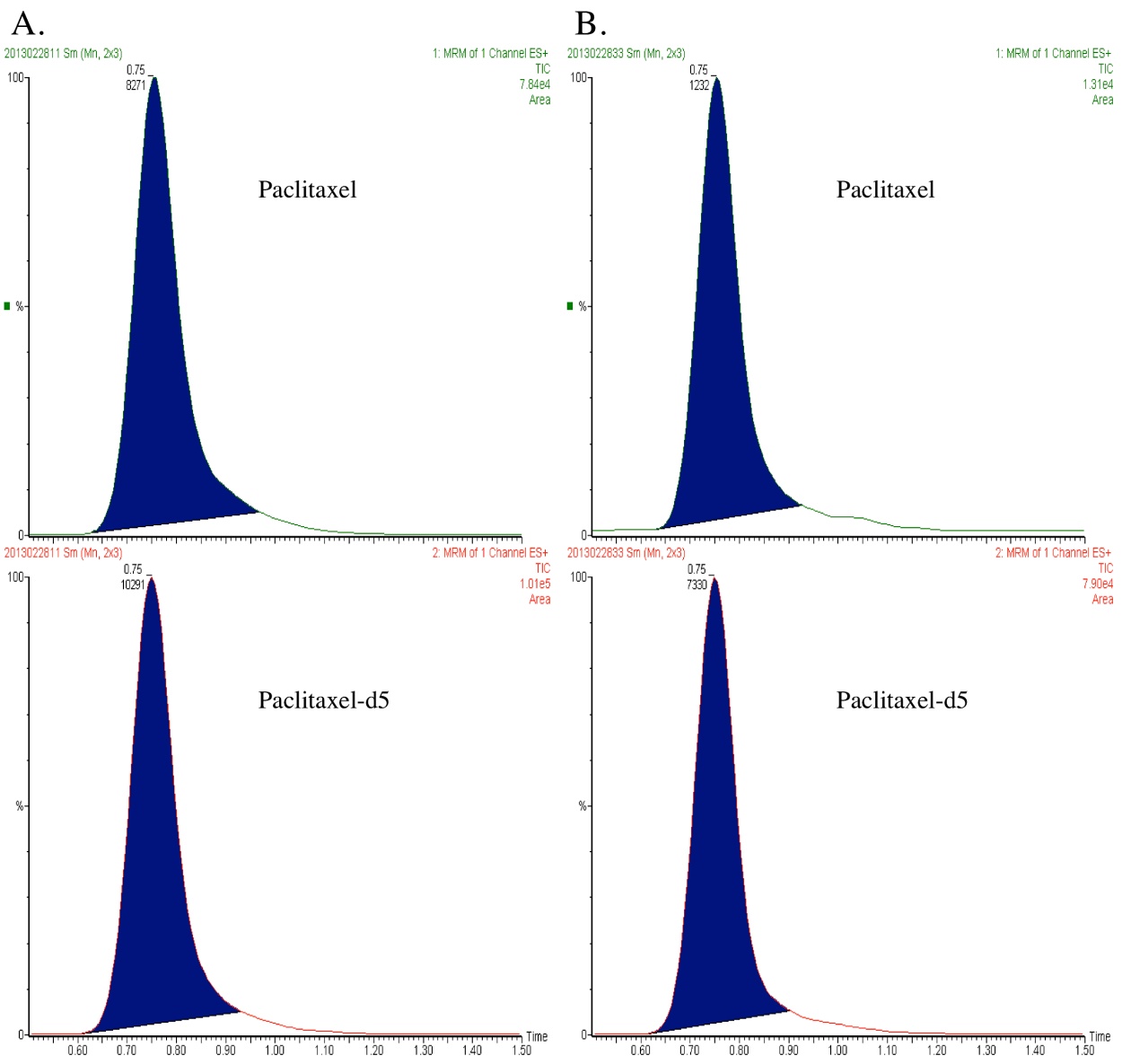


Figure 5.4 Representative chromatograms of real plasma (A) and brain homogenate (B) samples from the animal study. Both channels for paclitaxel and the IS (paclitaxel-d5) are displayed for each sample.

CHAPTER 6

CONCLUSIONS

CNS, composed of brain and spinal cord, is one of the most important physiological systems in human being and higher animals. Cognition is the major function of the CNS, which is responsible for processing, memory and body control. Another important function of the CNS is pain sensation, which detects damaging stimuli to the body and plays a critical role in self-protection. As a lot of different drugs, drug metabolite and endogenous compounds can have direct interactions with the CNS. Therefore, research on analytical methods of such analytes is critical to interrogate cognition and pain in the CNS. In this dissertation, a series of studies is presented for the review, development, validation and application of novel analytical methods for the quantitation of CNS-related analytes.

According to the literature review in Chapter 2, numerous analytical methods have been developed for the quantitation of drugs, drug metabolites and endogenous compounds in brain tissue using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Different sample collection and sample preparation techniques have been utilized to obtain clean, concentrated and LC-MS/MS compatible samples from brain tissue. Sample collection and preparation have been made more efficient by the development of fully automated, high-throughput and highly specific techniques, including automated microdialysis, online solid-phase extraction (SPE) and robotic liquid handling systems, which represent the future directions of bioanalysis.

Cotinine (COT), a major metabolite of nicotine, has been reported to have memory enhancement effects. Efforts have been made to research the pro-cognitive effects of COT for the development of therapeutic agents for the treatment of Alzheimer's disease (AD). Chapter 2 involved the develop and application of a bioanalytical method for the simultaneous quantitation of COT and three major metabolites of COT in plasma and brain tissue samples using LC-MS/MS. In Chapter 3, this method was also applied to a pharmacokinetic (PK) study of COT orally and intravenously dosed to rats, revealing important information on the PK and metabolism of COT. The analytical method and PK parameters can be used for future animal studies involving the mechanism of actions, pharmacological activities and toxicology of COT in rodent species. The current method and information can also be used for research on structural analogues of COT to achieve better activity. A compound with pro-cognitive effects, high bioavailability and low toxicity can potentially be developed into a therapeutic agent for the treatment of AD, mild cognitive impairment and other types of dementia.

It has been reported that paclitaxel and several other chemotherapeutic agents can cause severe neuropathic pain in cancer patients, which usually cannot be alleviated by common analgesic medications. In order to assist revealing the mechanism of the neuropathic pain caused by paclitaxel, a rapid LC-MS/MS method was developed for the quantitation of paclitaxel in rat plasma and brain tissue, as described in Chapter 5. This fast, sensitive, precise and accurate method can be applied to animal studies to determine the dose-effect relationship of paclitaxel. Different animal models can be used to find the signaling pathways involved in the neuropathic pain caused by paclitaxel, leading to the mechanism of actions behind this medical issue. By understanding the neuropathic mechanism, possible therapeutic agents and strategies can be

further developed to alleviate the suffering of cancer patients, improve their life quality and expand their life expectancy.

APPENDIX A

BIO-GENERATION OF STABLE ISOTOPE LABELED INTERNAL STANDARDS FOR ABSOLUTE AND RELATIVE QUANTITATION OF DRUG METABOLITES IN PLASMA SAMPLES BY LC–MS/MS

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Abstract

In order to achieve a better understanding of the toxicity of drug candidates, quantitative characterization of circulatory drug metabolites has been of increasing interest in current pharmaceutical research. Stable isotope labeled (STIL) internal standards (IS) are ideally used to simplify drug metabolite quantitation via liquid chromatography and tandem mass spectrometry (LC–MS/MS) analysis, primarily due to their capability to compensate matrix effects, thereby leading to faster method establishment by using generic assay conditions. However, chemical synthesis of STIL metabolites can often be resource intensive, requiring lengthy exploratory synthesis route development and/or extensive optimization to achieve the required stability for some metabolites. To overcome these challenges, we developed a general method that could generate STIL metabolites in a matter of hours from STIL parent drugs through the utilization of an appropriate in vitro metabolic incubation. This methodology can potentially save valuable synthesis resources, as well as provide timely availability of STIL IS. The following work demonstrates the proof-of-concept that multiple STIL metabolites can be generated simultaneously to provide satisfactory performance for both absolute quantitation of drug metabolites and for potential use in assessment of relative exposure coverage across species in safety tests of drug metabolites (MIST).

1. Introduction

Drug metabolites are considered by some to be one of the leading causes of unexpected drug safety issues in drug research and development [1,2]. Animal experiments are used in nonclinical research to predict drug safety in humans, which, however, may not be able to cover all the major circulatory metabolites in humans at clinical concentrations because of interspecies differences in metabolism and disposition [2,3]. To address this issue, the US Food and Drug Administration (FDA) published a regulatory document "Guidance for Industry, Safety Testing of Drug Metabolites" (MIST) in February 2008 [4]. In addition, the International Conference on Harmonization (ICH) published the M3 (R2) document "Guidance on Nonclinical Safety Studies for The Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals" in June 2009 [5]. Therefore, a rapid and accurate determination of animal-to- human relative exposure is important for evaluation of circulatory metabolites at early stages of drug development [6–10].

Different strategies have been developed to assess animal-to- human relative exposure of drug metabolites. The conventional approach, absolute quantitation, requires both reference standards and internal standards (IS) to determine the actual metabolite concentrations in animals and human. This strategy is the most robust and accurate practice, but it may be delayed by the synthesis of standards (reference and/or internal), especially at early stages of drug development. Accordingly, methods of relative quantitation without reference standards were developed to mitigate this issue. A metabolite-to-parent mass spectrometry (MS) response factor (RF), which can be obtained by MS-to-UV calibration [11,12] MS-to-radioactivity calibration [13,14] or MS-to-NMR calibration [11,15] has been used to normalize analyte-specific response variations, thereby providing a tool to estimate relative exposures across species. Nevertheless, methods

using RF usually do not have high sensitivity and can be labor intensive. Recently, another strategy was developed to determine relative exposure of metabolites by comparing analyte-to-IS peak area ratios between animals and human without the use of calibration samples. Matrix-induced response variations were alleviated by mixing dosed human plasma with equal volumes of blank animal plasma and vice versa [16–18].

Internal standards are recommended for absolute and relative quantitation of drug metabolites. Typically, stable isotope-labeled internal standards (STIL IS) are the ideal choices for bioanalysis by liquid chromatography-tandem mass spectrometry (LC–MS/MS). Variations in analyte recovery during sample preparation, chromatography, MS response and, more importantly, matrix effects can be compensated for by STIL IS, based on the chemical similarities between the STIL analogs and analyte drugs. This can lead to easier and faster method establishment by using generic method conditions. However, synthesis and isotope labeling of metabolites, especially hydroxylated and oxidized metabolites, can be challenging due to the lack of availability of process chemistry routes and the need for exploratory syntheses leading to lengthy development time and sequestration of synthesis resources. Furthermore, metabolite stability issues are often prevalent, which, in combination with intensive synthesis efforts, often leads to delays and increases in the cost of method establishment.

Alternative approaches have been explored to overcome the current challenge of STIL IS synthesis for metabolites. A structural analog IS can be used, but due to the potential dissimilarity between the analyte(s) and the IS, somewhat more method development effort is usually necessary. While it is possible to use STIL parent drugs as the IS to quantify metabolites (because STIL parent is usually available in the early stages of drug development), one needs to

be aware that the co-eluting parent drug may invoke ion suppression on the IS, causing possible quantitation bias [19].

The in vitro approach for investigation of biotransformation from parent drugs to their metabolites has been a common practice in drug metabolism research. This process can also be applied to STIL parent compounds to generate STIL metabolites, which has been previously used in elucidation of metabolite structures and biotransformation mechanisms [20–22]. The advantage of using such approach for quantitative analysis has also been recently discussed in literature [14]. In this study, we adopted this practice to generate STIL metabolites for the use as IS for quantitation of metabolites in biological samples. By incubating the STIL parent compounds with human liver microsomes (HLM) under optimal conditions, metabolites maintaining the original stable isotope labels can be generated by enzymatic reactions. In this case study, we tested the performance of "bio-generated" STIL IS (bio-IS) in comparison to that of chemically synthesized STIL IS (syn-IS) in method qualification and absolute quantitation of major metabolites of two drug candidates, parent compound A (PA) and parent compound B (PB) (Figure A.1). Moreover, the performance of bio-IS in the determination of animal-to-human relative exposure without reference standards was tested for MAs (metabolites of PA). Incubation yields, matrix effects, purity and specificity were also assessed.

2. Experimental

2.1 Chemicals and reagents

PA (parent drug A), MA1 (metabolite 1 of PA), MA2 (metabolite 2 of PA), PB (parent drug B), MB1 (metabolite 1 of PB), cis-MB2 (cis-isomer of metabolite 2 of PB), trans-MB2 (trans-isomer of metabolite 2 of PB), MB3 (metabolite 3 of PB) and their STIL analogs were synthesized by Janssen Research and Development. Gen- test pooled human liver microsomes

were purchased from BD Biosciences (San Jose, CA). β-Nicotinamide adenine dinucleotide phosphate (monosodium salt, MW 833.4) (NADPH), ethylenediaminetetraacetic acid (disodium salt, 0.5M) (EDTA), magnesium chloride (1.0 M) (MgCl2), phosphoric acid (1.0 M), formic acid and ammonium acetate were obtained from Sigma–Aldrich (St. Louis, MO). Blank human, rat, mouse, beagle, cynomolgus monkey and rabbit plasma were purchased from Bioreclamation (Hicksville, NY). HPLC grade acetonitrile (ACN), methanol, isopropyl alcohol (IPA) and trifluoroacetic acid (TFA) were obtained from EMD Chemicals Inc. (Gibbstown, NJ). HPLC grade dimethyl sulfoxide (DMSO) was purchased from Burdick and Jackson (Morristown, NJ). Acetone was from VWR (Philadelphia, PA).

2.2 Instrumentation

The chromatographic separation of analytes and internal standards was carried out on an HPLC system consisting of Shimadzu LC20AD pumps and a SIL-HTC autosampler (Columbia, MD). The HPLC system was interfaced with an API 5000 triple quadrupole mass spectrometer from Applied Biosystems (Foster City, CA) operated in positive Turbo IonsprayTM mode. Instrument control and data processing were performed using AnalystTM ver. 1.5.1 software (Applied Biosystems, Foster City, CA).

2.3. Solutions and standards

Individual stock solutions were prepared at 1 mg/mL for MA1, syn-STIL MA1, MA2 and syn-STIL MA2 in 50:50 DMSO:ACN (v:v), 1 mg/mL for MB1, cis-MB2, trans-MB2 and MB3 in 25:25:50 ACN:DMSO:methanol (v:v:v), and 400 µg/mL for syn-STIL MB1, syn-STIL cis-MB2, syn-STIL trans-MB2 and syn-STIL MB3 in 25:25:50 ACN:DMSO:methanol (v:v:v). All stock solutions were stored at 4 °C when not in use. Commingled, synthesized internal standard (syn-IS) working solutions were prepared at 10 ng/mL for syn-STIL MA1 and syn-STIL MA2,

and at 50 ng/mL for syn-STIL MB1, syn-STIL cis-MB2, syn-STIL trans-MB2 and syn-STIL MB3 with ACN as the solvent.

For the absolute quantitation of MA1 and MA2, a commingled working solution of 2 μg/mL in 20:80 ACN:water (v:v) was used to prepare calibration standards (STD). The commingled STDs were prepared fresh daily by serial dilution of the working solution using blank rat plasma to concentrations of 0.5, 1, 2, 5, 20, 50, 80 and 100 ng/mL for each analyte. Quality control (QC) samples were prepared at concentrations of 0.5, 1.5, 45 and 75 ng/mL.

For the absolute quantitation of MB1, cis-MB2, trans-MB2 and MB3, a commingled working solution of 20 μg/mL in ACN was used to prepare STDs at 5, 10, 20, 50, 200, 400, 800 and 1000 ng/mL, and QCs at 5, 15, 450 and 750 ng/mL for each analyte in mouse plasma.

For the relative exposure analysis of MA1 and MA2, STDs were prepared at concentrations of 25, 50, 200 and 600 ng/mL for each analyte in human, rat, mouse, beagle, monkey and rabbit plasma.

All the spiking solutions and QC samples were kept at −20 °C when not in use.

2.4. Microsomal incubation

A generic microsomal incubation method was used to prepare bio-generated internal standards (bio-IS). Before the experiments, all solutions were kept at 4 °C and the HLM was thawed slowly on ice. To each reaction tube, 40 μL of 50 mM EDTA, 50 μL of 100 mM $MgCl_2$, 700 μL of 0.1 M phosphate buffer (pH 7.4, sparged with O_2 for 5 min), 100 μL of HLM (pH 7.4), 10 μL of 5 mM STIL parent drug solution (STIL PA in 25:75 DMSO:ACN or STIL PB in 50:50 ACN:water, final concentration of 50 μM) and 100 μL of 10 mM NADPH were added and mixed well. The reaction system was incubated in a 37 °C water bath for 1 h, followed by the addition of 6 mL of 1:1 ACN:acetone to stop the reaction. After vortex-mixing for 5 min, and

centrifugation at 1200 × *g* for 10 min, the supernatant was transferred to a clean test tube and evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was reconstituted in 250 μL of 90:10 water:ACN (v:v) with 0.02% formic acid and then transferred to a 0.2 μm centrifugal filter tube, followed by centrifugation at 7200 × *g* for 5 min. Finally, the filtered solution was diluted with ACN (50 mL for STIL PA and 25 mL for STIL PB) to generate the bio-IS working solution used in the sample preparation.

2.5. Sample preparation

Protein precipitation was used as the sample preparation technique for all biological samples. After adding 100 μL of water into each well of a 96-well plate, a protein precipitation filtration 96-well plate from Analytical Sales and Services, Inc. (Pompton Plains, NJ) was taped on top of the 96-well plate to form a filtration assembly. To each well of the protein precipitation plate, 15 μL of biological sample was added, followed by 100 μL of IS working solution (bio-IS or syn-IS). After vortexing on a plate shaker at 1000 rpm for 2 min, the filtration assembly was centrifuged at 1480 × *g* for 3 min. The 96-well plate was then separated from the filter plate and capped with a silica cover. After another 1 min of vortexing at 1000 rpm, the samples were ready for LC–MS/MS analysis.

2.6. LC–MS/MS conditions

A Phenomenex Gemini C18 110 Å Column (150 mm × 4.6 mm, 3 μm) was used for chromatographic separation of each MA and their respective IS. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 75:25 ACN:methanol (v:v). With a flow rate at 0.6 mL/min, the separation was carried out by a gradient (min, %B): (0, 30), (1.0, 30), (3.5, 45), (3.6, 95), (4.0, 95), (4.1, 30), (4.5, 30). The injection volume was 10 μL for absolute quantitation and 2 μL for relative exposure analysis.

The separation of each MB and their respective IS was carried out by a Phenomenex Kinetex PFP 100 Å Column (30 mm × 2.1 mm, 2.6 μm). Mobile phase A was 0.01% formic acid in 5 mM ammonium acetate (pH 4.75) and mobile phase B was methanol. The flow rate was 0.5 mL/min. The gradient was (min, %B): (0, 20), (0.15, 20), (3.3, 90), (3.3, curve 5), (5.0, 99), (5.2, 99), (5.3, 20), (6.0, 20). The injection volume was 3 μL. The challenging separation of cis-MB2 from trans-MB2 (positional isomers) was the justification for the unusual mobile phase and the complex gradient.

The LC–MS/MS detection and quantitation of analytes were conducted by multiple reaction monitoring (MRM). The operating parameters of the ion source were optimized for each group of metabolites in general, while declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were optimized for each pair of analyte and IS. The MRM transitions were: MA1, 439 → 403; syn-STIL MA1, 443 → 407; bio-STIL MA1, 444 → 408; MA2, 439 → 405; syn/bio-STIL MA2, 445 → 408; MB1, 423 → 140; syn/bio-STIL MB1, 427 → 144; cis-MB2, 409 → 377; syn/bio-STIL cis-MB2, 413 → 377; trans-MB2, 409 → 377; syn/bio-STIL trans-MB2, 413 → 266; MB3, 407 → 254; syn/bio-STIL MB3, 411 → 254. All the pairs of syn-IS and bio-IS showed identical MRM due to their same structures, except for those of MA1.

2.7. Method qualification

To assess the intra-day precision and accuracy, one calibration curve and six replicates of four QC concentrations were analyzed for both groups of analytes (MAs and MBs) using syn-IS and bio-IS on each day of method qualification. Qualifications were repeated for three days to evaluate inter-day precision and accuracy (n = 18).

2.8. Matrix effects evaluation

Post-column infusion was used to assess the matrix effects introduced by bio-IS. For each group of analytes, two blank plasma samples were prepared with syn-IS and bio-IS, respectively. Injections of the blank samples onto the column were conducted while a syringe pump continuously infused analyte standard solutions (10 ng/mL in ACN) at 20 μL/min into the column eluent by a tee connected to the mass spectrometer inlet. MRM chromatograms of the analytes were recorded and compared between syn-IS and bio-IS.

2.9. Quantitation of yield of bio-IS

The bio-IS working solutions prepared by microsomal incubation were quantified to calculate the yields from in vitro incubation and for establishing the appropriate amount for spiking as IS. Synthesized STIL metabolites were used to prepare calibration standards, and non-labeled metabolites were used as IS. For quantitation of bio-STIL MAs, the calibration standards were prepared at concentrations of 0.5, 1, 2, 4 and 8 ng/mL by serial dilution of syn-STIL MAs in ACN. For quantitation of bio-STIL MBs, the calibration standards were 1, 2, 5, 10, 50, 100 and 250 ng/mL of syn-STIL MBs in ACN. To each 25 μL of calibration standards or bio-IS solutions, 100 μL of the corresponding non-labeled metabolite ACN solution (10 ng/mL for MAs and MBs) and 100 μL water were added and mixed well before injection. The same LC–MS/MS methods as used for the biological samples were used for the quantitation of bio-IS.

2.10. Absolute quantitation

Plasma samples were obtained from rats (n = 4) before and 1, 2, 4, 7, 24 h after receiving a single oral dose of 10 mg/kg of PA. Plasma samples were obtained from mice (n = 3) at the same time points after receiving three single oral doses of 50 mg/kg, 100 mg/kg and 200 mg/kg

of PB. All samples were analyzed separately with syn-IS and bio-IS, resulting in absolute metabolite concentrations used to compare the agreement in data generated by syn-IS and bio-IS.

2.11. Relative exposure evaluation

MA1 and MA2 were spiked at four concentration levels in plasma from six species. Each one of the spiked samples was analyzed by using bio-IS. Without a calibration curve, peak area ratios between analytes and IS were used to represent analyte concentrations (exposure), which were used to calculate animal-to-human relative exposure. Comparison between measured relative exposure using bio-IS and the calculated nominal values was used to evaluate the performance of bio-IS.

3. Results and discussion

3.1. Microsomal incubation

STIL analogs corresponding to metabolites of interest from PA and PB were successfully generated using the same microsomal incubation method. The incubation samples were precipitated, evaporated, reconstituted, filtered, and diluted to generate the IS working solutions used for protein precipitation in the bioanalysis process. Higher concentrations of IS were needed for MBs due to the higher concentration range requirement of their calibration curves. Therefore, less ACN (25 mL) was used for dilution for each microsomal incubation tube in the case of MBs than that of MAs (50 mL). The concentrations of STIL metabolites in the IS working solutions were quantified by LC–MS/MS, which were used to calculate the incubation yields (Table A.1). Despite the low yield of each STIL metabolite from microsomal incubation, enough STIL metabolites were generated from each tube to support analysis of 500 and 250 plasma samples for MAs and MBs, respectively.

The bio-generation of STIL metabolites needs to satisfy two fundamental prerequisites. First, identification of the appropriate in vitro metabolic system for generation of STIL metabolites of interest is needed. Usually, this information is readily available in early development following characterization of in vitro metabolism of a new drug entity. In current particular examples, microsomal incubation was selected since the Phase I hydroxylated metabolites (MA1, MA2), carboxylated metabolite (MB1), oxidized metabolites (cis/trans-MB2) and lactamized metabolites (MB3) had been demonstrated previously to be produced from incubation of the respective cold parent compound in microsomal incubations during in vitro metabolism studies. Second, the stable isotope atoms on the STIL parent compound must not be fully removed during Phase I metabolism. Ideally, a minimum mass shift of 3 Da should be maintained, which should be considered when synthesizing STIL parent drugs.

In order to produce STIL metabolites in sufficient quantities for use as IS in bioanalysis, the microsomal incubation should be optimized for maximum production of the desired STIL metabolites. First, it is important to take into consideration the CYP450 inhibition data during optimization of yield with respect to substrate concentration to prevent unnecessary waste of precious STIL parent drug. For both STIL PA and STIL PB, we tested concentrations of 5 μM, 10 μM and 50 μM and it turned out that there was a noticeable decrease in percent yield as the concentration was increased (data not shown). Despite this trend, 50 μM was finally used for both compounds since the absolute production of metabolites was still higher. Second, the percentage of organic solvent in the parent drug solution should be kept as low as possible because organic solvents like ACN, DMSO and ethanol can all inhibit CYP450 activity. Typically, it is recommended to have <0.1% DMSO and <1% ACN or ethanol in the final mixture, unless the parent drug has solubility issues. In fact, we needed 0.25% DMSO in the

final mixture of STIL PA due to its lipophilicity. Third, microsomes from relevant species, or pooled from multiple-species, can be used to produce the highest yield of each metabolite especially for compounds with marked species differences in metabolism. To pool microsomes from multiple species for optimum yield, it is necessary to consider potential interference from isobaric metabolites produced by different species. Fourth, incubation time is another parameter that can impact the yield of metabolites. Here, we used only one hour since there was no significant increase in yield using a longer incubation time due to secondary metabolism. Fifth, it should be noted that the relative concentration of different metabolites is usually fixed from incubations in one species. This may give limited leverage for the analyst to adjust the concentration of one IS, which is an obvious disadvantage. In cases where the profile of the metabolites produced in vitro is very different from that seen in plasma samples, the concentrations in the IS solution may be adjusted, for example, by pooling incubation products from multiple species to achieve optimal IS level for each metabolite. Finally, a deuterium-labeled drug may behave differently from the unlabeled drug in terms of metabolic profile and yield [23]. Use of ^{13}C, or ^{15}N labeled parent drug may generate more predictable results. However, synthesis of ^{13}C, or ^{15}N labeled parent drug may be more costly and time-consuming.

If such prerequisites are met, and the yield-related factors are optimized, STIL metabolites that are sufficient for an average-sized study can be generated with a minimum amount of STIL parent compound. In current study, 10 μL of 5 mM solution (pmol level) of STIL parent drug was used for each incubation tube. This method can be applied to the bio-generation of STIL analogs of most Phase I metabolites, with very little adjustment of conditions during the in vitro incubation. Whole process usually takes no more than a day or two.

Moreover, CYP450 can catalyze multiple reactions to produce multiple STIL metabolites at the same time, making this method even more efficient.

In terms of reproducibility, we have repeated our protocol several times for both PA and PB. The results suggested that, as long as the same incubation conditions had been followed, this bio-generation method was highly reproducible, generating very similar amounts of STIL metabolites from batch to batch. The products and their yield were predominantly determined by the parent compound properties and the incubation conditions, both of which could be well controlled in a single project.

3.2. Specificity and purity

The bio-IS working solutions contained impurities from HLM, incubation by-products and, more importantly, high remaining concentrations of STIL parent compound, making specificity of the LC–MS/MS method of great importance. Ideally, there should be no interference introduced by the addition of bio-IS to the plasma samples. Especially important to evaluate is the presence of any unlabeled metabolite (D_0) which would be generated if the stable isotope atoms were removed during metabolism, or if the STIL parent drug had significant contamination with unlabeled content.

Figure A.2 shows representative chromatograms of blank plasma prepared with syn-IS and bio-IS. In chromatograms of MA1 and MA2, the background of samples containing bio-IS (Figure A.2B) were completely comparable to those from syn-IS (Figure A.2A), showing no extra peaks or D_0 in the analyte channels. Meanwhile, the chromatograms of MB1 and MB3 each showed noticeable signal for D_0 at the retention time of MB1 and MB3, which happened with both the bio-IS (Figure A.2D) and the syn-IS (Figure A.2C). Since isotopic interference was quite unlikely when using multiple stable isotope labels, one possible reason for D_0 was that

MB1 and MB3 were generated in the microsomal incubation from non-labeled PB, a potential impurity in the synthesized STIL PB. Nevertheless, the D_0 level was consistent and comparatively low relative to the lower limit of quantitation (LLOQ), and therefore, did not impact the performance of bio-IS in quantitation.

In terms of chromatographic separation, all the metabolites were well separated from each other. The parent compounds had greater hydrophobicity and eluted significantly later (3.55 min for PA and 4.85 for PB), leaving no ion suppression effects on the analytes or IS. There was an extra peak showing up in the MB3 channel of blank plasma sample with bio-IS (Figure A.2D), which was assumed to be a by-product or impurities from the incubation. Since all the metabolites and interferences were completely separated, the purity of the bio-IS did not affect the quantitative performance.

3.3. Precision and accuracy

The precision and accuracy of both syn-IS and bio-IS for all the analytes at four different concentrations of QC samples were assessed. The results are shown in Table A.2, which demonstrates that both syn-IS and bio-IS could meet the acceptance criteria for method validation set by the FDA. All %CV's were within 15% and all the values for relative bias were within ±15%, for both syn-IS and bio-IS. Therefore, we concluded the performance of bio-IS was equivalent to that of syn-IS in terms of precision and accuracy. In addition, it was demonstrated that bio-generated IS provided satisfying performance for quantitation at both low (0.5 – 100 ng/mL for MAs) and high (5 – 1000 ng/mL for MBs) concentration ranges.

3.4. Matrix effects

There was no clean-up of the HLM incubation products, the reaction mixtures were simply precipitated, evaporated, reconstituted, filtered and diluted with ACN. Therefore,

biological impurities from HLM might be introduced during sample preparation, which could cause matrix effects if they co-eluted with any of the analytes. Therefore, a post-column infusion experiment was conducted to assess the extra matrix effects caused by bio-IS compared to syn-IS, the result of which is shown in Figure A.3. Based on the chromatograms of the analytes, there was no significant change to the ion suppression or enhancement trends observed for bio-IS compared to that of syn-IS.

3.5. Absolute quantitation

The bioanalytical methods were applied to real samples from preclinical studies to test the performance of bio-IS in conventional absolute quantitation. All the samples were analyzed twice using syn-IS and bio-IS, respectively, to create concentration-time curves which are shown in Figure A.4.

From the figure, it was apparent that the PK profiles created by syn-IS and bio-IS were in excellent agreement since the curves almost completely overlap. To be more specific, relative bias (difference divided by average) between syn-IS and bio-IS was calculated for all values above the LLOQ. For the 31 values of MAs, none exceeded ±20% bias and there was only one (3% of total) between ±15% and ±20% bias. For all 156 values of MB samples at three dose levels, there were only 3 (2% of total) above ±20% bias, and 4 (3% of total) between ±15% and ±20% bias. All other values measured by syn-IS and bio-IS had bias less than ±15%, which meant that the bio-IS demonstrated satisfactory performance compared to the syn-IS for absolute quantitation.

3.6. Relative exposure evaluation

Recent regulatory guidance recommends that circulating metabolites identified in human should be present at equal or greater concentration levels in at least one of the preclinical species

used in safety assessment [4] and [5]. Gao et al. proposed an approach to evaluate the relative exposure of metabolites in preclinical species versus human in the absence of authentic standards of the metabolites, calibration curves, and other attributes of standard bioanalytical methodology [16]. This was conducted by mixing equal volumes of dosed human plasma with blank animal plasma and vice versa, followed by LC – MS/MS analysis. Gao et al. and Ma et al. have demonstrated that relative exposure coverage of the metabolites in animals versus humans can be obtained by comparing the peak area ratios of metabolite/IS in animals versus humans [16] and [18]. The matrix difference across species was theoretically eliminated by mixing the plasma. However, in Gao's work, a generic analog IS was used for multiple model compounds, which may not track the ionization efficiency of the individual analyte. In that case, even by mixing the plasma, it could be not guaranteed that biases caused by matrix effects were completely eliminated. This could be the reason why only semi-quantitative results were obtained in their work. In our current project, a similar approach was evaluated while replacing an analog IS with bio-generated STIL IS, which tracks the ionization efficient sufficiently. By using STIL IS for each individual metabolite, the need for mixing the plasma was eliminated and more accurate results could be obtained.

For this experiment, MA1 and MA2 were spiked into the plasma of different animal species (mouse, rat, dog, monkey, rabbit) and human at different concentrations and processed using bio-IS without calibration curves. Analyte-to-IS peak area ratios were used to represent metabolite abundance. The animal-to-human relative exposure was measured by comparing the analyte-to-IS peak area ratio of animal to that of human for every combination of concentrations. The performance of bio-IS was assessed by comparing the above measured animal-to-human relative exposure values to nominal values, which were calculated by dividing the spiked

concentration in animal by the spiked concentration in human as shown in Table A.3A. Relative biases from nominal values of MA1 and MA2 at all the concentration combinations are shown in Table A.3B and C.

It was apparent that all the relative exposure values measured with bio-IS had bias within ±15% from the calculated nominal values, demonstrating acceptable performance of bio-IS in accurately measuring animal-to-human relative exposure. Among all the animal/human combinations, the concentration ratios ranged from 0.042 (25 ng/mL to 600 ng/mL) to 24 (600 ng/mL to 25 ng/mL), which covers the extreme cases, and all could be well quantified by bio-IS. It was demonstrated that accurate relative exposure values could be obtained with bio-IS even without calibration curves. This is of great value in the rapid determination of species exposure coverage for MIST strategy decisions at the early stages of drug development when reference standards of metabolites may not be readily available.

4. Conclusions

In vitro microsomal incubation can generate multiple STIL metabolites from the corresponding STIL parent drug with sufficient quantity and purity for direct use as IS in bioanalytical applications, in particular during early stages of drug development. STIL IS generated in this approach demonstrated acceptable performance in absolute quantification of drug metabolites, showing good correlation with results generated by synthesized STIL IS, as well as in cross-species relative exposure determination, providing excellent agreement with theoretical values. Therefore, this simple, low-cost and efficient method of making STIL IS can be a valuable tool for assisting faster method establishment to generate reliable bioanalytical data.

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Table A.1 Concentrations of bio-IS in the reconstituted IS working solution and incubation yields (%).

Parent	bio-IS	Conc. (ng/mL)	Yield (%)
STIL PA	STIL MA1	0.84 ± 0.02	0.19
	STIL MA2	1.61 ± 0.07	0.37
STIL PB	STIL MB1	79.40 ± 1.09	9.30
	STIL *trans*-MB2	31.46 ± 1.06	3.81
	STIL *cis*-MB2	6.82 ± 0.24	0.83
	STIL MB3	1.74 ± 0.08	0.21

Table A.2 Intra-day (n = 6) and inter-day (n = 18) precision and accuracy of all the analytes by syn-IS and bio-IS.

Parent	Metabolite	Nominal Conc. (ng/mL)	Intra-day (n = 6)				Inter-day (n = 18)			
			CV (%)		Bias (%)		CV (%)		Bias (%)	
			syn-IS	bio-IS	syn-IS	bio-IS	syn-IS	bio-IS	syn-IS	bio-IS
PA	MA1	0.5	3.97	5.89	2.81	1.21	5.22	4.66	1.48	1.56
		1.5	2.91	2.49	2.67	0.57	2.63	2.38	1.93	0.14
		45	1.06	2.62	7.50	4.97	1.92	2.64	5.71	2.98
		75	1.06	2.33	7.62	6.39	2.79	3.30	5.01	3.01
	MA2	0.5	4.30	5.81	6.06	0.45	4.79	5.84	3.56	-0.48
		1.5	2.96	2.84	5.27	-1.12	3.33	2.83	2.82	-1.21
		45	2.01	2.30	7.53	0.26	2.28	2.04	5.48	1.32
		75	1.81	1.79	7.68	2.39	3.18	2.47	4.28	1.48
PB	MB1	5	4.16	6.58	4.56	12.61	5.04	10.97	5.18	3.57
		15	2.72	3.35	8.39	7.13	3.76	4.98	4.85	2.31
		450	5.64	3.68	2.67	3.64	4.36	4.94	1.97	3.52
		750	0.96	2.36	4.04	4.49	2.61	2.94	2.90	4.07
	trans-MB2	5	4.75	5.45	7.88	3.63	8.41	13.69	9.95	7.13
		15	4.88	3.07	7.97	1.29	6.27	5.51	5.73	0.87
		450	4.08	3.77	5.13	0.16	6.09	6.02	2.90	2.23
		750	3.07	4.60	6.10	6.12	4.48	4.32	5.45	4.53
	cis-MB2	5	3.53	4.59	12.56	12.70	6.03	9.28	7.17	4.48
		15	2.55	2.14	7.31	8.72	4.38	6.14	4.69	2.30
		450	4.68	4.82	6.37	7.29	6.22	6.47	6.26	6.45
		750	1.97	2.09	9.26	7.03	5.79	4.23	7.01	7.47
	MB2	5	2.29	5.69	6.10	12.34	5.99	13.89	4.37	4.01
		15	1.95	3.67	8.62	3.49	4.31	5.71	5.29	1.09
		450	2.51	2.28	7.77	2.44	4.85	5.17	6.65	3.44
		750	3.67	3.96	7.08	4.68	5.54	4.52	7.02	5.45

Table A.3 A) Nominal values of animal-to-human relative exposure for each animal/human combination. **B)** Bias (%) between measured and nominal values of animal-to-human relative exposure of MA1; **C)** Bias (%) between measured and nominal values of animal-to-human relative exposure of MA2.

A.

	Nominal Conc. (ng/mL)	Human			
		25	50	200	600
Animal	25	1.00	0.50	0.13	0.04
	50	2.00	1.00	0.25	0.08
	200	8.00	4.00	1.00	0.33
	600	24.00	12.00	3.00	1.00

B.

Animal Species	Nominal Conc. (ng/mL)	Human			
		25	50	200	600
Mouse	25	-0.64	2.35	1.82	5.91
	50	-3.88	-0.99	-1.50	2.46
	200	-4.59	-1.72	-2.22	1.71
	600	-5.39	-2.54	-3.04	0.85
Rat	25	3.46	6.57	6.03	10.29
	50	1.03	4.06	3.53	7.69
	200	-1.30	1.67	1.15	5.21
	600	-1.65	1.31	0.79	4.84
Dog	25	-1.73	1.22	0.71	4.75
	50	-2.50	0.43	-0.08	3.93
	200	-1.54	1.42	0.90	4.96
	600	-4.85	-1.99	-2.49	1.42
Monkey	25	-5.52	-2.68	-3.17	0.72
	50	-4.46	-1.59	-2.09	1.85

	200	-4.91	-2.05	-2.55	1.37
	600	-4.05	-1.17	-1.67	2.28
Rabbit	25	-1.80	1.16	0.64	4.68
	50	-2.92	0.00	-0.51	3.49
	200	-3.78	-0.89	-1.40	2.56
	600	-3.78	-0.89	-1.40	2.56

C.

Animal Species	Nominal Conc. (ng/mL)	Human			
		25	50	200	600
Mouse	25	-6.52	0.55	0.74	0.72
	50	-5.41	1.75	1.95	1.92
	200	-13.56	-7.02	-6.84	-6.86
	600	-2.34	5.05	5.25	5.23
Rat	25	-3.26	4.06	4.26	4.23
	50	-7.50	-0.50	-0.31	-0.33
	200	-5.11	2.07	2.26	2.24
	600	-6.61	0.46	0.65	0.63
Dog	25	-1.40	6.07	6.26	6.24
	50	-6.80	0.25	0.44	0.42
	200	-8.61	-1.69	-1.51	-1.53
	600	-9.13	-2.26	-2.07	-2.09
Monkey	25	-7.32	-0.30	-0.11	-0.13
	50	0.89	8.52	8.73	8.70
	200	-6.98	0.06	0.25	0.23
	600	-9.72	-2.88	-2.70	-2.72
Rabbit	25	-0.79	6.72	6.92	6.90
	50	-4.94	2.26	2.45	2.43
	200	-7.15	-0.13	0.06	0.04
	600	-10.30	-3.51	-3.33	-3.35

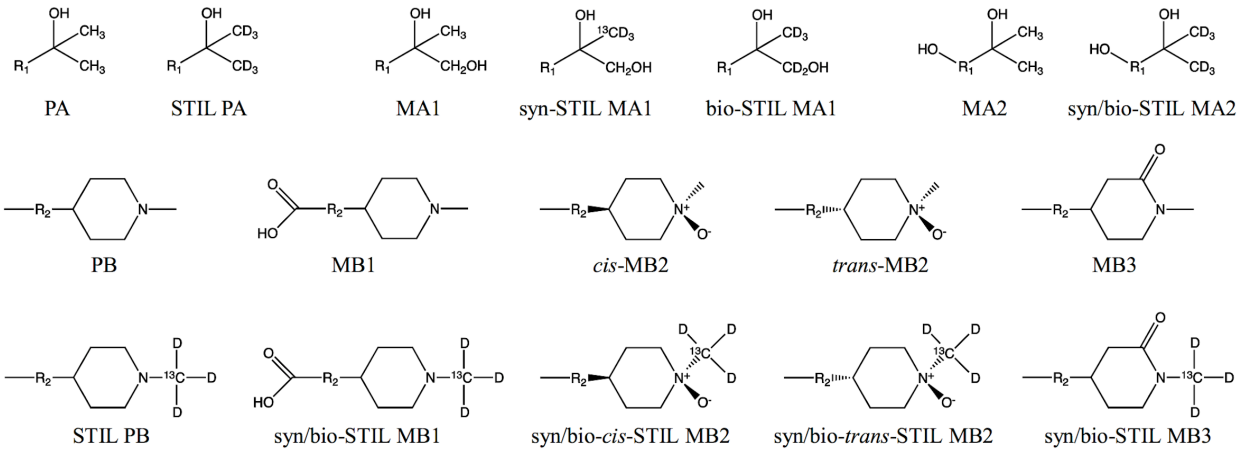

Figure A.1 Partial structures of analytes and IS. The chemical structures were identical for all the syn-IS and bio-IS except for those of MA1.

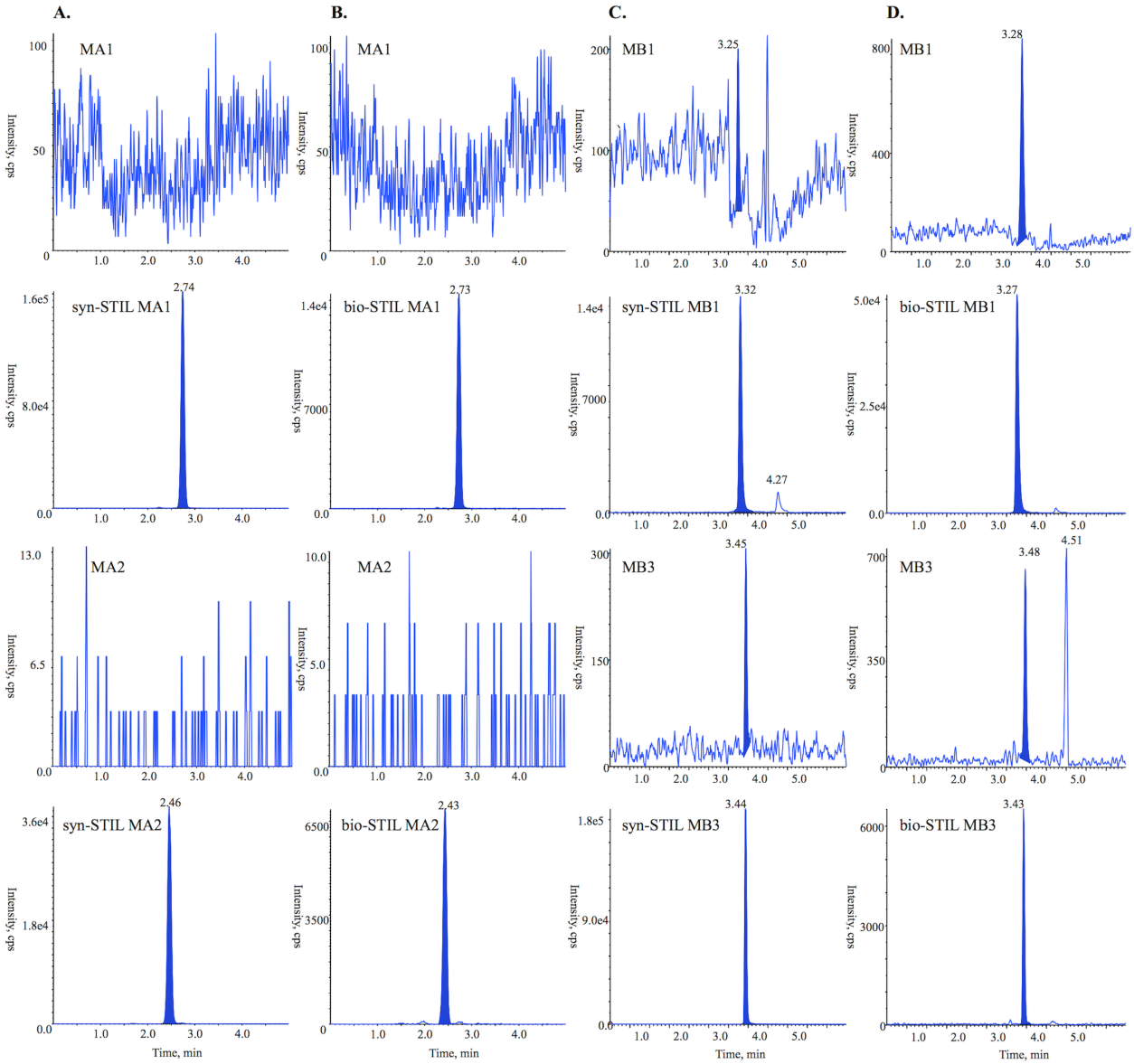

Figure A.2 Chromatograms of blank plasma with IS: A) MA1 and MA2 with syn-IS; B) MA1 and MA2 with bio-IS; C) MB1 and MB3 by syn-IS; D) MB1 and MB3 by bio-IS.

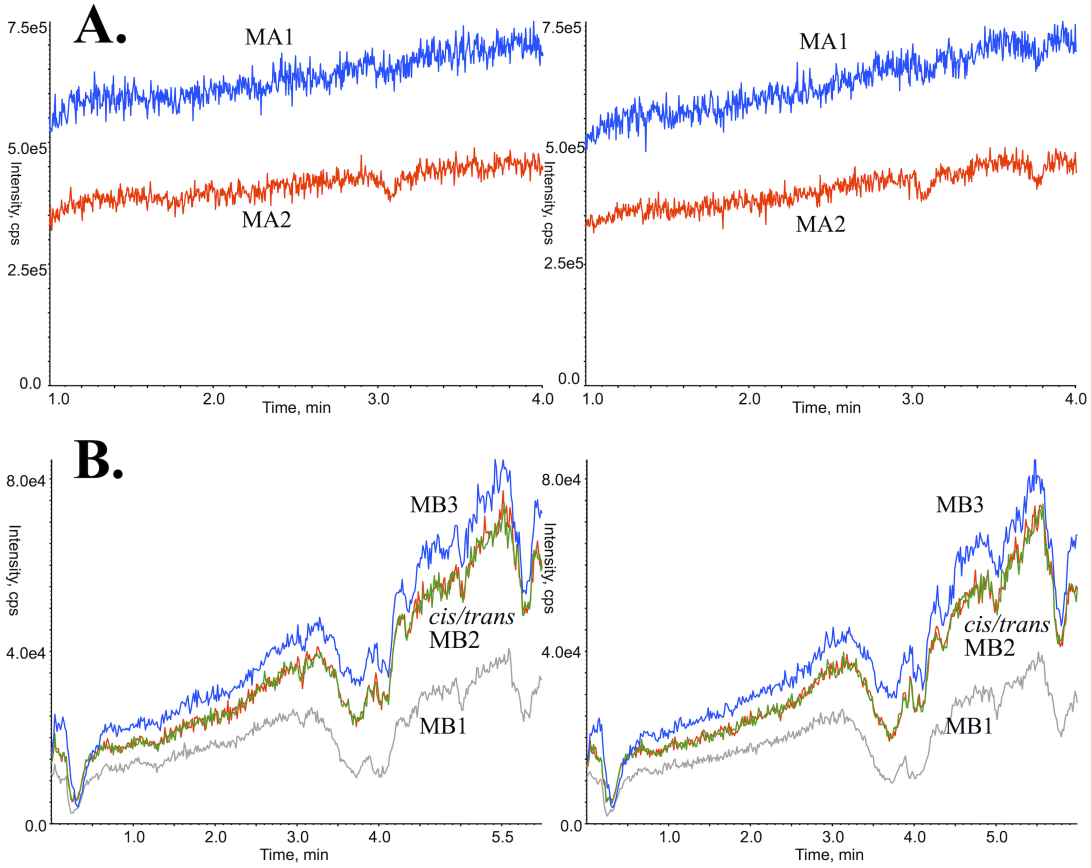

Figure A.3 Post column infusion chromatograms of blank plasma with syn-IS (left) and bio-IS (right) of A) MA1 and MA2; B) MB1, *cis*-MB2, *trans*-MB2 and MB3.

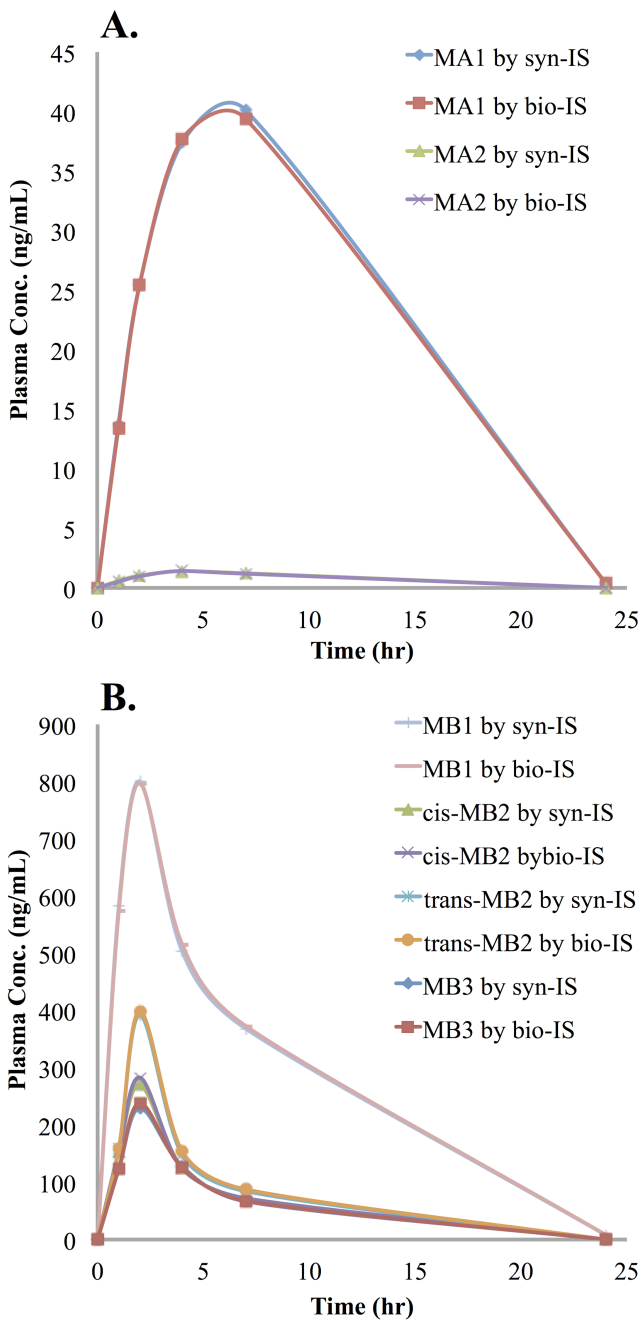

Figure A.4 A) MA1 and MA2 PK profiles of rat study samples by syn-IS and bio-IS; B) MB1, *cis*-MB2, *trans*-MB2 and MB3 mouse PK profiles by syn-IS and bio-IS. All the measured values below the LLOQ were displayed as zero in the curves.

APPENDIX B

BIO-GENERATION OF STABLE ISOTOPE-LABELED INTERNAL STANDARDS FOR ABSOLUTE AND RELATIVE QUANTITATION OF PHASE II DRUG METABOLITES IN PLASMA SAMPLES USING LC-MS/MS

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Abstract

Quantification of drug metabolites in biological samples has been of great interest in current pharmaceutical research, since the metabolite concentrations and pharmacokinetics can contribute to a better understanding of the toxicity of drug candidates. Two major categories of Phase II metabolites, glucuronide conjugates and glutathione conjugates, may cause significant drug toxicity and therefore require close monitoring at early stages of drug development. In order to achieve high precision, accuracy and robustness, stable isotope-labeled (SIL) internal standards (IS) are widely used in quantitative bioanalytical methods using liquid chromatography and tandem mass spectrometry (LC-MS/MS), due to their capability of compensating for matrix effects. However, chemical synthesis of SIL analogues of Phase II metabolites can often be very difficult and require extensive exploratory research, leading to higher cost and significant delays in drug research and development. To overcome these challenges, we have developed a generic method which can synthesize SIL analogues of Phase II metabolites from more available SIL parent drugs or SIL conjugation co-factors, using *in vitro* biotransformation. This methodology was successfully applied to the biogeneration of SIL glucuronide conjugates and glutathione conjugates. The method demonstrated satisfactory performance in both absolute quantitation and assessment of relative exposure coverage across species in safety tests of drug metabolites (MIST). This generic technique can be utilized as an alternative to chemical synthesis and potentially save time and cost for drug research and development.

Key words

Stable isotope labeled internal standard, microsomal incubation, glucuronide conjugate, glutathione conjugate, absolute quantitation, relative exposure, LC-MS/MS, MIST.

Introduction

Drug toxicity has always been one of the most important considerations during the research and development of new drug candidates. In addition to the toxicity caused by the parent drugs directly dosed to patients, drug metabolites are also considered to be a major source of toxicity.(Nicholson, Connelly et al. 2002) Due to the polymorphism of metabolism, unknown metabolic pathways and unknown metabolite pharmacokinetics, toxicity induced by drug metabolites can sometimes cause unexpected drug safety issues.(Baillie, Cayen et al. 2002, Hastings, El-Hage et al. 2003) To ensure the safety of clinical tests on humans, nonclinical and preclinical drug safety tests on animals are widely used to predict toxicity in humans. However, in some instances, such animal experiments may not be able to cover all the drug metabolites at clinical concentrations observed in humans, due to interspecies and/or interracial differences in drug metabolism and disposition.(Leclercq, Cuyckens et al. 2009) In order to address this issue, regulatory documents have been released by different organizations. In 2008, the US Food and Drug Administration (FDA) published the "Guidance for Industry, Safety Testing of Drug Metabolites" (MIST) which has drawn increasing attention from the pharmaceutical industry.(FDA 2008) In addition, the International Conference on Harmonization (ICH) also published the M3 (R2) document "Guidance on Nonclinical Safety Studies for The Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals" in 2009.(ICH 2009) According to both regulatory documents, major drug metabolites generated in humans must be covered by animal experiments, which requires quantitative bioanalytical assays for rapid and accurate determinations of animal-to-human relative exposure ratios at early stages of drug development. (Atrakchi 2009, Vishwanathan, Babalola et al. 2009, Gao and Obach 2011)

To assess the animal-to-human relative exposure, different strategies have been developed and validated in drug development. The most straightforward strategy involves the absolute quantitation of drug metabolite concentrations in animals and humans, which requires reference standards and internal standards (IS) for all the studied metabolites. Though this strategy provides the most robust and reliable data, the synthesis of such standards can be very challenging and may cause a significant delay at the early stage of drug research and development. Another widely accepted strategy was developed to determine relative exposure using analyte-to-IS peak area ratios between animals and human without the use of calibration samples.(Gao, Deng et al. 2010, Ma, Li et al. 2010, Gao and Obach 2012) Rapid determination of animal-to-human relative exposure of major metabolites was fulfilled without building calibration curves, which speeds up the progress of early-stage screening. Moreover, interspecies differences in the biological matrices were compensated by using mixed plasma samples.

In both absolute and relative quantitation of drug metabolites based on liquid chromatography-tandem mass spectrometry (LC–MS/MS), internal standards are recommended to achieve satisfactory precision, accuracy and method robustness. Ideally, stable isotope-labeled internal standards (SIL IS) should be used in quantitative LC-MS/MS assays. Since SIL analogues have the same physical and chemical properties as the analytes but different molecular weights, they can effectively compensate for variations in extraction, chromatography, MS response and, more importantly, matrix effects. However, the synthesis of SIL analogues of drug metabolites can be very difficult, and usually involves extensive exploratory synthesis, leading to delays and increases in the cost of method development. To address this issue, a new method was developed to generate SIL analogues for Phase I drug metabolites from SIL parent drugs using microsomal incubation.(Li, Gong et al. 2013) These biogenerated SIL metabolites demonstrated

satisfactory performances in both absolute and relative quantitation of drug metabolites in plasma samples from different species.

Phase I metabolites, which usually result from oxidation and hydroxylation of the parent drugs, are commonly observed for most drug candidates and extensively studied in drug research and development. However, some drugs show significant Phase II metabolism, generating Phase II metabolites via conjugation reactions. Among all the Phase II metabolites, glucuronide conjugates and glutathione conjugates always garner great interest from researchers. Glucuronidation reactions are catalyzed by UDP-glucuronosyltransferases (UGTs), conjugating carboxylic acids with uridine 5'-diphosphoglucuronic acid (UDP-glucuronic acid, UDPGA).(Ritter 2000) Acyl glucuronide drug metabolites have been considered a "red flag" during drug research and development, since such metabolites can reach appreciable concentrations in the circulatory system and contribute to significant toxicity.(Shipkova, Armstrong et al. 2003) Glutathionylation is another important Phase II metabolism pathway for drug molecules that contain carboxylic acid groups. Under the catalysis of glutathione S-transferases (GSTs), parent drugs are conjugated with the co-factor glutathione (GSH), generating a stable, polar and less toxic metabolite.(van Bladeren 2000) However, glutathione conjugation may also cause serious toxicity by depleting GSH or generating toxic metabolites.(Monks, Anders et al. 1990) Therefore, close attention should always be paid to glucuronide and glutathione conjugate metabolites in terms of the estimation of drug toxicity.

Similar to the synthesis of SIL Phase I metabolites, SIL Phase II metabolites are extremely difficult to synthesize and/or purify to achieve satisfactory quantity and purity for use as IS in LC-MS/MS-based bioanalysis. To address this issue, we adopted the concept of the biosynthesis of SIL Phase I metabolites and developed a new protocol for the bio-generation of

SIL Phase II metabolites. By incubating the SIL parent drugs and the corresponding conjugation co-factors together with human liver microsomes (HLM) under optimized conditions, glucuronide and glutathione conjugate metabolites could be generated to meet the requirements of quantity and purity as ISs. Gemfibrozil (GFZ) and telmisartan (TMS) were used for the demonstration of the bio-generation of glucuronide conjugates, while acetaminophen (APAP) was used for the bio-generation of glutathione conjugates. In addition to the combination of SIL parent drugs and non-labeled co-factors, we also tested the conjugation between non-labeled parent drugs and SIL co-factors (SIL UDPGA and SIL GSH). In this case, SIL parent drugs are not required, and SIL co-factors can be generically used for different parent drugs to generate SIL Phase II metabolites, making this strategy more widely applicable. Structures of synthesized SIL IS (syn-IS, only for GFZ-GA and TMS-GA), biogenerated SIL IS with labels on the parent drug moiety (bio-IS I) and biogenerated SIL IS with labels on the co-factor moiety (bio-IS II) of gemfibrozil glucuronide (GFZ-GA), telmisartan glucuronide (TMS-GA) and acetaminophen glutathione (APAP-GS) are shown in Figure B.1. The performance of biogenerated SIL ISs were tested in method validation, real sample quantitation and relative exposure determination, demonstrating satisfactory specificity, precision and accuracy in both absolute and relative quantitative analysis of Phase II drug metabolites using LC-MS/MS. This generic, low-cost and convenient strategy can be used as a potential alternative to chemical synthesis of SIL ISs to save cost, labor and time during the early stages of drug research and development.

Experimental

1. Chemicals and reagents

Gemfibrozil (GFZ), gemfibrozil-d6 (GFZ-d6), gemfibrozil 1-O-β-glucuronide (GFZ-GA), gemfibrozil 1-O-β-glucuronide-d6 (GFZ-GA-d6), telmisartan (TMS), telmisartan-d3

(TMS-d3), telmisartan acyl-β-D-glucuronide (TMS-GA), acetaminophen-d3 (APAP-d3), acetaminophen glutathione disodium salt (APAP-GS) were purchased from Toronto Research Chemicals (Toronto, Canada). Telmisartan-d3 acyl-β-D-glucuronide (TMS-GA-d3) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). UDP-glucuronic acid-$^{13}C_6$ was synthesized using engineered *Escherichia coli* by the group of Dr. Maor Bar-Peled from the Complex Carbohydrate Research Center of the University of Georgia (Athens, GA).(Broach, Gu et al. 2012, Yang, Bar-Peled et al. 2012) UGT Reaction Mix Solution A (25mM UDPGA), UGT Reaction Mix Solution B (250 mM Tris-HCl, 40 mM $MgCl_2$, and 0.125 mg/mL alamethicin) and Gentest pooled human liver microsome (HLM, 20 mg/mL) were purchased from BD Biosciences (San Jose, CA). Acetaminophen (APAP), glutathione (GSH), glutathione-(glycine-$^{13}C_2$, ^{15}N), β-Nicotinamide adenine dinucleotide phosphate (monosodium salt, MW 833.4) (NADPH), glutathione S-transferase (GST), magnesium chloride ($MgCl_2$), phosphate buffer (100 mM), formic acid, ammonium acetate, hydrochloric acid (HCl), methyl *tert*-butyl ether (MTBE), HPLC grade dimethyl sulfoxide (DMSO), LC-MS grade acetonitrile (ACN) and LC-MS grade water were obtained from Sigma-Aldrich (St. Louis, MO). Blank human plasma, rat plasma, mouse plasma, beagle plasma, cynomolgus monkey plasma and rabbit plasma were purchased from Bioreclamation (Hicksville, NY).

2. Instrumentation

The separation of analytes and internal standards were carried out using an Agilent 1100 HPLC system (Santa Clara, CA). The HPLC system was interfaced to a Waters Quattro Micro triple quadrupole mass spectrometer with an ESI(+) source (Milford, MA). Instrument control, data acquisition and data processing were performed using Waters MassLynx 4.0 software (Milford, MA).

3. Solutions and standards

Individual stock solutions of GFZ-GA, syn-SIL GFZ-GA, TMS-GA, syn-SIL TMS-GA and APAP-GA were prepared at 1 mg/mL in water with 0.1% formic acid. Drug substrates, GFZ, SIL GFZ, TMS and SIL TMS were dissolved in ACN at 5 mM. SIL UDPGA was dissolved in water yielding a final concentration of 0.25 mM. APAP and SIL APAP were dissolved in ACN at 100 mM. All the drug solutions were stored at -20 °C when not in use.

For the absolute quantitation of GFZ-GA, serial dilutions by water were used to obtain working solutions at concentrations of 50, 100, 200, 500, 2000, 5000, 8000 and 10000 ng/mL. Calibration standards were prepared by spiking 10 µL of respective working solution into 100 µL of blank rat plasma, yielding concentrations of 5, 10, 20, 50, 200, 500, 800 and 1000 ng/mL. Quality control (QC) samples were prepared at the concentrations of 5, 15, 150 and 750 ng/mL in rat plasma. The linear range was 5 – 1000 ng/mL. For the relative exposure analysis of GFZ-GA, standards were prepared in a similar manner to yield concentrations of 10, 20, 200 and 800 ng/mL in human, rat, mouse, beagle, monkey and rabbit plasma.

For the absolute quantitation of TMS-GA, serial dilutions with water were used to obtain working solutions at concentrations of 5, 10, 20, 50, 200, 500, 800 and 1000 ng/mL. Calibration standards were prepared by spiking 10 µL of the respective working solution into 100 µL of blank rat plasma, yielding concentrations of 0.5, 1, 2, 5, 20, 50, 80 and 100 ng/mL. Quality control (QC) samples were prepared at the concentrations of 0.5, 1.5, 15 and 75 ng/mL in rat plasma. The linear range was 0.5 – 100 ng/mL. For the relative exposure analysis of TMS-GA, standards were prepared in a similar manner to yield concentrations of 1, 2, 20 and 80 ng/mL in human, rat, mouse, beagle, monkey and rabbit plasma.

For the absolute quantitation of APAP-GS, serial dilutions by water were used to obtain working solutions at concentrations of 100, 200, 500, 1000, 2000, 5000 and 10000 ng/mL. Calibration standards were prepared by spiking 10 μL of respective working solution into 100 μL blank rat plasma, yielding concentrations of 10, 20, 50, 100, 200, 500 and 1000 ng/mL. Quality control (QC) samples were prepared at concentrations of 10, 30, 150 and 750 ng/mL in rat plasma. The linear range was 10 – 1000 ng/mL. For the relative exposure analysis of APAP-GS, standards were prepared in a similar manner to yield concentrations of 20, 40, 200 and 800 ng/mL in human, rat, mouse, beagle, monkey and rabbit plasma.

All calibration standard samples and relative exposure standard samples were prepared fresh on the day of analysis. All QC samples were stored at -20 °C when not in use.

4. Microsomal incubation

The bio-generation of bio-IS I of GFZ-GA and TMS-GA were carried out using a protocol provided with the UGT reaction mix from BD Biosciences (San Jose, CA). To each reaction system, 693 μL of water, 80 μL of UGT Reaction Mix Solution A, 200 μL of UGT Reaction Mix Solution B, 2 μL of drug solution (5 mM SIL GFZ or SIL TMS) were mixed and pre-heated in a 37 °C water bath for 5 min. After 25 μL of HLM suspension was added, the reaction mixture was gently vortexed and placed in a 37 °C water bath for 6 hours. The final 1 mL of the reaction system contained 50 mM of Tris-HCl, 8 mM of $MgCl_2$, 25 μg/mL of alamethicin, 2 mM UDPGA, 0.5 mg/mL of HLM and 0.01 mM of SIL drug substrates. The reaction was terminated by the addition of 1 mL of ACN containing 0.1% formic acid, followed by vortexing for 5 min and centrifugation at 21130 × *g* for 5 min. Supernatant from two reaction systems were combined and, if needed, diluted with 0.1% formic acid to proper concentrations to

fit in the linear range of the analytical methods. The resulting bio-IS I solutions were stored at -80 °C before use.

The bio-generation of bio-IS II for GFZ-GA and TMS-GA was conducted using a similar protocol but with lower concentrations. Each reaction mixture contained 185 μL of water, 80 μL of SIL UDPGA solution, 100 μL of UGT Reaction Mix Solution B and 10 μL of 1% drug substrate solutions (0.05 mM GFZ or TMS). After pre-heating in a 37 °C water bath for 5 min, 25 μL of HLM was added, yielding a final mixture with 50 mM of Tris-HCl, 8 mM of $MgCl_2$, 25 μg/mL of alamethicin, 0.05 mM SIL UDPGA, 0.5 mg/mL of HLM and 1.25 μM of non-labeled drug substrates. After gentle vortexing and placing in a water bath at 37 °C for 6 hours, the reaction was terminated by the addition of 1 mL of ACN containing 0.1% formic acid, followed by vortexing for 5 min and centrifugation at 21130 × *g* for 5 min. Supernatant from two reaction systems was combined and, if needed, diluted with 0.1% formic acid to proper concentrations to fit in the linear range of the analytical methods. The resulting bio-IS II solutions were stored at -80 °C before use.

The bio-generation of bio-IS I of APAP-GS was carried out using a generic incubation protocol. To each reaction system, 10 μL of 100 mM SIL APAP solution, 10 μL of 100 mM GSH solution, 100 μL of 10 mg/mL GST solution, 100 μL of 10 mM NADPH, 50 μL of 100 mM $MgCl_2$ solution and 700 μL of phosphate buffer (pH 7.4) were mixed and pre-heated at 37 °C for 5 min. After 50 μL of HLM suspension was added, the reaction was started and maintained at 37 °C using a water bath. The final reaction mixture contained 1 mM of SIL APAP, 1 mM of GSH, 1 mg/mL of GST, 1 mM of NADPH, 5 mM of $MgCl_2$ and 1 mg/mL of HLM. After 3 hours, the reaction was terminated by the addition of 3 mL of ACN, followed by vortexing for 5 min and centrifugation at 21130 × *g* for 5 min. Supernatant from two reaction systems was combined and

diluted with 1% formic acid to 20 mL. The resulting bio-IS I solutions were stored at -80 °C before use.

The bio-generation of bio-IS II of APAP-GS was carried out using a similar incubation protocol as described above. Non-labeled APAP and SIL GSH were used instead to generate the SIL IS with different labeling sites.

5. Sample preparation

Protein precipitation was used as the sample preparation method for the plasma samples of TMS-GA. To each 100 µL plasma sample, 10 µL of the IS solution and 300 µL of ACN were added, vortexed and centrifuged at 21130 × *g* for 5 min. The supernatant was obtained and evaporated to dryness at 50 °C using a centrifugal vacuum evaporator. The residue was reconstituted in 100 µL of 10:90 ACN:water (v:v) with 0.1% formic acid. After 5 min of vortexing and 5 min of centrifugation at 21130 × *g*, the supernatant was transferred to HPLC vials for LC-MS/MS analysis.

Liquid-liquid extraction was used for the preparation of the plasma samples of GFZ-GA. To each 100 µL of plasma, 10 µL of the IS solution, 50 µL of 1 M HCl and 1 mL MTBE was added. After 5 min of vortexing and 5 min of centrifugation at 21130 × *g*, the supernatant was transferred to a new tube, followed by evaporation at 50 °C using a centrifugal vacuum evaporator. The residue was reconstituted in 100 µL of 10:90 ACN:water (v:v) with 0.1% formic acid. After 5 min of vortexing and 5 min of centrifugation at 21130 × *g*, the supernatant was transferred to HPLC vials for LC-MS/MS analysis.

Sample preparation for plasma samples of APAP-GS was carried out in a similar manner as TMS-GA samples, with the exception of adding 50 µL of IS solution instead of 10 µL. The final samples were reconstituted in 5:95 ACN:water (v:v) with 0.1% formic acid.

6. LC-MS/MS conditions

The separation of GFZ-GA, TMS-GA and respective ISs was carried out using the same HPLC method. A Thermo Betasil C18 Column (50 × 2.1 mm, 5 μm) coupled with a Phenomenex SecurityGuard C18 guard column (4 × 2.0 mm) was used for the separation of analytes and respective ISs. The mobile phase A was 10 mM ammonium formate buffer with 0.1% formic acid and the mobile phase B was ACN. At a flow rate at 0.6 mL/min, a gradient condition was used as follows (min, %B): (0, 10), (0.5, 30), (4.0, 95), (5.0, 95), (5.01, 10) and (6, 10). The injection volume was 10 μL. The solvent delay function was used to minimize the contamination of the ion source, introducing LC eluents from 1.3 to 4.3 min for GFZ-GA and from 1.0 to 4.0 min for TMS-GA into the mass spectrometer.

The separation of APAP-GS and ISs was carried out using an Agilent Zorbax Eclipse Plus C18 Column (50 × 2.1 mm, 1.8 μm) coupled with a Phenomenex SecurityGuard C18 guard column (4 × 2.0 mm). The mobile phase A was 10 mM ammonium formate buffer with 0.1% formic acid and the mobile phase B was ACN. At a flow rate at 0.3 mL/min, a gradient condition was used as follows (min, %B): (0, 5), (1, 5), (5.0, 95), (5.5, 95), (5.6, 5) and (10, 5). The injection volume was 10 μL. The solvent delay function was used to minimize the contamination of the ion source, introducing LC eluents from 2.0 to 5.5 min into the mass spectrometer.

The mass spectrometer was operated in positive ion ESI mode. For the detection of GFZ-GA, TMS-GA and respective ISs, the nebulizer gas was set at a flow rate of 600 L/h and a temperature of 400 °C. The cone gas flow was set to 50 L/h. For the detection of APAP-GS and ISs, the nebulizer gas was set at a flow rate of 450 L/h and a temperature of 450 °C. The cone gas flow was set to 20 L/h. For both methods, the source temperature and capillary voltage were set at 120 °C and 4.0 kV, respectively. Multiple reaction monitoring (MRM) functions were

employed for the quantification of analytes and ISs, using the ion transition, cone voltage and collision energy parameters shown in Table B.1.

7. Method validation

To assess the intra-day (n = 5) and inter-day (n = 15) precision and accuracy, calibration curves and five replicates of four QC concentrations were analyzed for GFZ-GA and TMS-GA using syn-IS, bio-IS I and bio-IS II, respectively. The intra-day (n = 5) and inter-day (n = 15) precision and accuracy of the APAP-GS analysis were tested using only bio-IS I and bio-IS II, since the syn-IS of APAP-GA was not available.

8. Absolute quantitation

Plasma samples were obtained from rats (n = 3) receiving a single oral dose of 10 mg/kg of TMS at time points of 0, 0.5, 1, 3, 6 and 24 hours. These plasma samples were analyzed using syn-IS, bio-IS I and bio-IS II of TMS-GA.

9. Relative exposure

Similar quantitative strategies were used for the relative exposure studies of GFZ-GA, TMS-GA and APAP-GS. Spiked plasma samples were prepared at four concentration levels using plasma from six different species, each one of which was analyzed by bio-IS II and bio-IS II separately. Instead of measuring the absolute concentrations with calibration curves, peak area ratios between analytes and IS were used to represent analyte concentrations (exposure), which were used to calculate animal-to-human relative exposure. Animal-to-human relative exposure ratios measured by different ISs were compared with the nominal values.

Results and Discussion

1. Microsomal incubation

Since Phase II metabolism usually involves conjugation between the parent drug and a co-factor, the stable isotope label can be located at either the drug substrate or the conjugation co-factor, both of which can yield SIL conjugation metabolites. In this study, two different types of STIL analogues were generated for GFZ-GA, TMS-GA and APAP-GS, respectively. Type I bio-generated internal standards (bio-IS I) represented for SIL Phase II metabolites with stable isotope labels on the parent drug moiety, while Type II bio-generated internal standards (bio-IS II) represented for SIL Phase II metabolites with stable isotope labels on the conjugation co-factor moiety.

Generic microsomal incubation methods were used to generate SIL analogues for GFZ-GA, TMS-GA and APAP-GS. The concentrations of bio-ISs were estimated by comparing the peak areas of bio-ISs with those of the reference standards at known concentrations, which were used to calculate the yield of the biotransformation reactions (Table B.2). The amount of bio-IS I for GFZ-GA, bio-IS I and bio-IS II for APAP-GS generated from two tubes of 1 mL reaction mixture was enough to generate IS solutions suitable for the linear range used in the validation of each analytical method. The bio-IS I concentration of TMS-GA was too high and needed to be diluted three times before analysis, due to the lower linear range of TMS-GA. Since the concentration of SIL UDPGA stock solution was about 100 times lower than the commercial UDPGA solution, multiple tubes of reaction systems (4 for GFZ-GA and 2 for TMS-GA) needed to be combined to yield enough bio-IS II to fit the linear range of each analyte. After adjustment by combination or dilution, concentrations of bio-IS I and bio-IS II of all the studied analytes could fit within the linear range of their respective methods. The result suggested that, though

only a very small amount of SIL parent drug or co-factor was used in each tube of reaction, enough SIL metabolites to support the whole project were produced by small amounts of replicates of the microsomal incubation systems (usually 2 to 4).

To effectively generate SIL Phase II metabolites using microsomal incubation, several basic requirements have to be met, based on the properties of parent drugs and available resources. First, either SIL parent drug or SIL conjugation co-factor needs to be available for the bio-generation of SIL metabolites. Because Phase II drug metabolism is primarily based on conjugation, at least one of the conjugation moieties needs to be labeled. When SIL parent drug analogues are available at the early stage of drug development, bio-IS I is recommended for higher abundance and lower cost. However, when SIL parent drug analogues are not available, bio-IS II can be chosen with the use of the universal SIL conjugation co-factors. Second, the SIL atom should be conserved during Phase II metabolism. Since a water molecule will be generated in glucuronidation or glutathionylation reactions, the stable isotope label should not be on the carboxylate group of the parent drug or the reactive hydrogen atom of the conjugation co-factor. Ideally, multiple deuterium, ^{13}C or ^{15}N atoms will provide better performances by higher stability, as well as greater mass differences between the IS and analytes.

The choice of microsome species and/or enzymes is another critical factor that can greatly affect the yield of bio-IS. For glucuronidation reactions, UGT is the primary enzyme catalyzing the conjugation of the parent drug and UDPGA. In this study, we used HLM as the source of UGTs. However, HLM contains a large number of different enzymes that may trigger different metabolic pathways, *e.g.* Phase I metabolism, lowering the yield of the glucuronidation reaction. In this case, a proper microsome species or even multiple-species mixed microsome may need to be optimized for the highest yield. Ideally, recombinant UGT can be used to

minimize the generation of unwanted metabolites and indirectly increase the yield of the SIL Phase II metabolites. For the glutathionylation reactions, GST is the primary enzyme involved in the biotransformation. However, we found that it was not mandatory to use GST in the reaction systems. By adding GST to the reaction mixture, the time needed to reach the highest yield of SIL APAP-GA was shortened from 3 hours to 2 hours. The yield, when a final steady state was reached, was not affected. We also used HLM in the bio-generation of SIL APAP-GA, which was due to the specific chemical properties of APAP. A Phase I metabolite of APAP, *N*-acetyl-*p*-benzoquinone imine (NAPQI), has to be formed at first and will act as an intermediate for the glutathionylation reaction. Therefore, a Phase I microsomal incubation system was also included in the reaction system.

In addition to these very basic requirements, several other factors also need to be considered in microsomal incubation, so as to produce SIL metabolites of enough quantity and quality for the use as IS in bioanalysis. First, minimum amount of organic solvents should be used in the reaction mixture. Unlike drug metabolites that are usually more water soluble, parent drugs often have low water solubility and require organic solvents to dissolve. Because organic solvents like ACN, DMSO and ethanol can inhibit microsomal activities, it is recommended to have less than 0.1% DMSO and 1% ACN or ethanol in the final mixture. Second, incubation times can be variable and may need optimization to achieve the highest metabolite yield. For the glucuronidation reactions using our conditions, 6 hours were tested to be the optimal time length for the incubation. The bio-IS concentrations started to drop after 6 hours, because glucuronide conjugates were not stable in the high pH environment (pH 7.4) of the reaction systems. For the glutathionylation reactions, the highest yield was achieved after 2 hours when GST was used and 3 hours when GST was not used. After the highest points were reached, the yield kept at a

constant level and no concentration drop was observed within 24 hours of continuous incubation. Third, alamethicin was added in the glucuronidation reaction systems. Alamethicin is a fungal derived peptide that can form pores in membranes and reduce latency in UGT activity.(Little, Lehman et al. 1997, Fisher, Campanale et al. 2000) Fourth, stability of glucuronide conjugates and glutathione conjugates can be affected by degradation and/or rearrangements. Low pH (0.1 % formic acid) and low temperature -80 °C conditions were used for the storage of bio-IS solutions to improve the stability of SIL glucuronide conjugates and glutathione conjugates. During the whole project, no significant concentration drop or rearranged structures were observed.

With all of the basic requirements and important factors considered, SIL Phase II metabolites that are enough for an average-sized pre-clinical study can be generated with minimum resources, even though the yields are not as high as chemical synthesis. The generic protocols demonstrated in this paper can be applied to the bio-generation of SIL analogues of glucuronide conjugates and glutathione conjugates, with none or little adjustment or optimization. More importantly, purifications of SIL metabolites are not needed in most occasions, as long as the LC-MS/MS method has good specificity. A constant SIL analogue concentration within the linear range of the analytical method can usually meet the requirement for an IS, even though other impurities are still present in the samples. Therefore, this microsomal incubation method is a simple, low-cost and efficient approach to supply SIL Phase II metabolites for use as ISs in bioanalysis

2. Specificity and purity

Since the bio-IS solutions obtained from microsomal incubations were only processed by simple protein precipitation, there were still large amounts of impurities present in the mixture,

including excess parent drugs (SIL parent drugs for bio-IS II), excess conjugation co-factors (SIL co-factors for bio-IS I), HLM residues, buffer components and other metabolism byproducts. Therefore, specificity and purity needed to be assessed when bio-ISs were added to the biological samples analyzed by respective LC-MS/MS methods. Ideally, there should be no analytes (D_0) or any other interference introduced by the addition of bio-IS.

Figure B.2 showed the representative chromatograms of blank plasma prepared with bio-IS I and bio-IS II of GFZ-GA, TMS-GA and APAP-GS, respectively. According to the chromatograms, separation of analytes and ISs were successful from the background, showing no significant peak distortions or extra impurity peaks. For GFZ-GA, the bio-IS I showed very good intensity, due to the higher concentration of the bio-IS solutions. Blank plasma prepared with bio-IS II for GFZ-GA showed a small peak at the retention time of GFZ-GA in the analyte channel, suggesting a negligible D_0 level. Since the method sensitivity and the D_0 level were both very low, the D_0 observed should not affect the quantitative performances of the bio-IS. In contrast, both bio-IS I and bio-IS II showed significant D_0 levels in the blank samples prepared by such SIL bio-ISs. Considering the bio-IS I and bio-IS II both had mass differences greater than 3 amu from the analytes, inter-channel crosstalk was unlikely to be caused by the D_0 levels. Therefore, it was more likely that the D_0 in bio-IS I was from the unlabeled TMS in the SIL TMS, and the D_0 in bio-IS II was from the unlabeled UDPGA in the SIL UDPGA. The observed D_0 in bio-IS I and bio-IS II were significantly lower than the LLOQ, which should not affect the absolute quantitation since the D_0 levels were constant. For APAP-GS, clean backgrounds were observed in blank samples prepared by bio-IS I and bio-IS II, suggesting undetectable D_0 levels in the bio-ISs. To better demonstrate the performances of bio-IS I and bio-IS II in terms of specificity and sensitivity, spiked plasma samples at each respective LLOQ were analyzed using

bio-IS I and bio-IS II of GFZ-GA, TMS-GA and APAP-GS. The representative chromatograms are shown in Supplemental Figure 1.

3. Precision and accuracy

Intra-day (n = 5) and inter-day (n = 15) precision and accuracy were tested for GFZ-GA, TMS-GA and APAP-GS using different types of ISs. For GFZ-GA and TMS-GA, syn-ISs were available, so that the method validation experiments for precision and accuracy were repeated three times using syn-IS, bio-IS I and bio-IS II, respectively. For APAP-GA, method validation was conducted using bio-IS I and bio-IS II, since the syn-IS was not available. Precision was assessed by the coefficient of variation (CV) among measure values, while accuracy was assessed by relative error from nominal values. According to the results shown in Table B.3, the validations using syn-IS, bio-IS I and bio-IS II all passed the acceptance criteria for method validation by the U.S. FDA.(FDA 2001) All the CV and relative errors were within 20% at the lower limit of quantitation (LLOQ) and 15% at other QC concentrations, suggesting satisfactory performance of both the bio-IS I and bio-IS II in terms of precision and accuracy.

4. Absolute quantitation

The bioanalytical methods were applied to real samples containing TMS-GA to test the performance of bio-IS I and bio-IS II in terms of conventional absolute quantitation. Plasma samples were obtained from rats (n = 3) before and 0, 0.5, 1, 3, 6 and 24 hours after a single oral dose of 10 mg/kg of TMS. All the samples were analyzed three times using syn-IS, bio-IS I and bio-IS II, respectively. Plasma concentrations were plotted against time to create pharmacokinetic (PK) profiles of TMS-GA, shown in Figure B.3.

The PK profiles demonstrated strong agreement between the bio-IS I, bio-IS II and syn-IS methods. To be more exact, the relative bias (difference divided by average) between bio-ISs

and syn-IS was calculated for all of the measured data points. The relative bias from syn-IS ranged from -4.95% to 13.56% for bio-IS I, while the relative bias from syn-IS ranged from -11.10% to 18.58%. Considering the FDA guidance for bioanalytical method development allows for 15% bias for each measured values, these results suggested that bio-IS I and bio-IS II for TMS-GA demonstrated equivalent performance to the syn-IS in absolute quantitation.

5. Relative exposure

By analyzing spiked plasma samples at different concentrations in different species without calibration curves, metabolite concentrations were quantified by response factors calculated by peak area ratios between analytes and IS. The animal-to-human relative exposure was calculated by the ratio between animal and human plasma concentrations for every pair of samples. The performances of bio-ISs were assessed by comparing measured animal-to-human relative exposure values to corresponding nominal values. Table B.4 shows the relative biases between the measured rat-to-human relative exposure values from the nominal values, when different concentrations of GFZ-GA, TMS-GA and APAP-GS were analyzed by bio-IS I and bio-IS II. The same experiments were repeated with four more different animal species (mice, rabbits, beagles and cynomolgus monkey), with the data included in Supplemental Table 1.

All the relative exposure values measured with bio-IS I and bio-IS II had relative biases within 20% from nominal values. Since the most widely accepted precision and accuracy of absolute quantitation are 15%, relative exposure values measured by conventional absolute quantitation methods have a theoretical range of -26.1% to 35.3% deviation from the nominal values. Therefore, the relative exposure results measured by bio-IS I and bio-IS II were considered to be very accurate, demonstrating excellent performance of the bio-ISs in the measurements of animal-to-human relative exposure. With different combinations of animal and

human samples at different concentrations, animal-to-human relative exposure values ranged from 0.0125 to 80 for GFZ-GA and TMS-GA, and from 0.025 to 50 for APAP-GS, suggesting that bio-IS I and bio-IS II generated in this study had a very wide range of coverage in relative quantitation. According to the relative bias values shown in Table B.4, measured animal-to-human relative exposure values tended to have greater biases from the nominal values, when one or both of the tested samples had low analyte concentrations. One of the most probable reasons for this issue was the D_0 in the bio-ISs, which was not compensated for without a calibration curve and therefore had more significant influence on samples with lower concentrations. Therefore, D_0 levels should be well controlled when bio-ISs are used for measurements of animal-to-human relative exposure. Dilution of the bio-IS solutions might be needed to minimize the impact of D_0 on the accuracy of relative quantitation.

Based on the results demonstrated in this section, accurate relative exposure values could be obtained with bio-IS I and bio-IS II without the need for building calibration curves, which was of great value in the rapid determination of species exposure during the early stages of MIST experimental design.

Conclusions

In vitro microsomal incubation can generate SIL Phase II metabolites, including acyl glucuronide conjugates and glutathione conjugates, with satisfactory quantity and purity for use as ISs in LC-MS/MS-based bioanalytical applications. In this study, generic protocols were used to generate two types of different bio-generated SIL ISs, bio-IS I with SIL parent drugs and bio-IS II with SIL conjugation co-factors. Respective bio-IS I and bio-IS II generated for GFZ-GA, TMS-GA and APAP-GS demonstrated excellent performance in method validation, absolute quantitation and cross-species relative exposure determinations of drug metabolites. Matrix

effects and interspecies matrix differences were well compensated for by the use of bio-ISs, providing great precision, accuracy and method robustness in LC-MS/MS-based quantitative bioanalysis. Therefore, this simple, low-cost and efficient method can be used as an alternative to chemically synthesized SIL IS for Phase II drug metabolites in bioanalytical applications.

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Table B.1 Ion transitions and MRM conditions for GFZ-GA, TMS-GA, APAP-GS and respective bio-IS I and bio-IS II.

Compound Type	Compound Name	MRM Transitions	Cone Voltage (V)	Collision Energy (eV)
Analyte	GFZ-GA	425.5 > 121.5	24	25
Syn-IS	GFZ-GA-d6	431.5 > 121.5	24	25
Bio-IS I	GFZ-GA-d6	431.5 > 121.5	24	25
Bio-IS II	GFZ-GA-$^{13}C_6$	431.5 > 121.5	24	25
Analyte	TMS-GA	691.5 > 515.5	24	32
Syn-IS	TMS-GA-d3	694.5 > 518.5	24	32
Bio-IS I	TMS-GA-d3	694.5 > 518.5	24	32
Bio-IS II	TMS-GA-$^{13}C_6$	697.5 > 515.5	24	32
Analyte	APAP-GS	457.4 > 182.3	28	26
Bio-IS I	APAP-GS-d3	460.4 > 185.3	28	26
Bio-IS II	APAP-GS-$^{13}C_2$, ^{15}N	460.4 > 182.3	28	26

Table B.2 Concentrations of bio-IS I and bio-IS II in the final IS working solutions and incubation yields (%).

Parent Drug	Co-factor	IS Type	IS Name	IS Solution Conc. (ng/mL)	Yield (%)
GFZ-d6	UDPGA	Bio-IS I	GFZ-GA-d6	270.7 ± 33.3	6.26%
GFZ	UDPGA-$^{13}C_6$	Bio-IS II	GFZ-GA-13C6	55.0 ± 12.3	6.37%
TMS-d3	UDPGA	Bio-IS I	TMS-GA-d3	277.1 ± 69.3	5.98%
TMS	UDPGA-$^{13}C_6$	Bio-IS II	TMS-GA-13C6	58.2 ± 7.8	8.38%
APAP-d3	GSH	Bio-IS I	APAP-GS-d3	298.4 ± 62.6	1.30%
APAP	GSH-$^{13}C_2$, ^{15}N	Bio-IS II	APAP-GS-13C2, 15N	188.4 ± 37.8	0.82%

Table B.3 Intra-day (n = 5) and inter-day (n = 15) precision (CV%) and accuracy (RE%) for QC samples of GFZ-GA, TMS-GA and APAP-GS analyzed by bio-IS I, bio-IS II and syn-IS (only for GFZ-GA and TMS-GA).

Analytes	Nominal conc. (ng/mL)	Bio-IS I				Bio-IS II				Syn-IS			
		Intra-day		Inter-day		Intra-day		Inter-day		Intra-day		Inter-day	
		CV%	RE%	CV%	RE%	CV%	RE%	CV%	RE%	CV%	RE%	CV%	RE%
GFZ-GA	5.0	8.69	-0.36	8.32	-0.07	12.96	6.60	4.06	12.40	11.74	-2.67	10.86	-4.31
	15.0	3.64	10.65	5.78	7.31	8.01	-0.15	8.83	0.95	8.72	-1.52	6.82	-2.23
	150.0	2.73	1.93	2.64	2.47	3.90	1.27	8.72	0.21	2.15	2.80	3.43	1.68
	750.0	4.20	-1.01	3.84	0.42	1.33	-3.56	9.57	2.72	2.31	4.57	3.17	2.17
TMS-GA	0.5	4.02	-4.40	4.03	-2.80	6.20	0.40	8.25	0.40	5.50	6.00	5.13	9.07
	1.5	3.03	0.27	2.63	0.62	4.23	-6.27	7.46	-2.80	2.25	8.67	2.33	10.31
	15.0	1.50	-0.33	1.15	-0.43	5.19	-4.84	6.52	-1.74	5.33	5.43	4.60	2.92
	75.0	1.76	-3.48	1.13	-3.56	2.72	-0.52	2.61	0.22	2.30	-3.05	3.06	-1.79
APAP-GS	10.0	8.94	-2.80	11.42	0.69	5.89	3.36	8.07	1.79	N/A			
	30.0	7.64	1.21	8.84	-0.18	7.44	7.76	7.96	5.42				
	150.0	3.86	8.05	5.39	5.97	2.61	10.13	3.95	9.08				
	750.0	6.59	3.42	5.43	0.74	3.26	-0.34	4.73	0.83				

Table B.4 Nominal animal-to-human concentration ratios, bias (%) of measured values using bio-IS I and bias (%) of measured values using bio-IS II for rat and human samples of (A) GFZ-GA, (B) TMS-GA and (C) APAP-GS.

A. GFZ-GA

Rat (ng/mL)	Nominal Relative Exposures				Bias from Nonimal by Bio-IS I				Bias from Nonimal by Bio-IS II			
	Human (ng/mL)				Human (ng/mL)				Human (ng/mL)			
	10	20	200	800	10	20	200	800	10	20	200	800
10	1	0.5	0.05	0.0125	2.32	3.08	0.75	1.40	-3.76	-4.03	-5.65	-5.10
20	2	1	0.1	0.025	2.84	3.61	1.26	1.92	5.51	5.22	3.44	4.05
200	20	10	1	0.25	3.54	4.31	1.95	2.61	5.66	5.37	3.59	4.20
800	80	40	4	1	2.16	2.92	0.59	1.24	8.22	7.93	6.10	6.72

B. TMS-GA

Rat (ng/mL)	Nominal Relative Exposures				Bias from Nonimal by Bio-IS I				Bias from Nonimal by Bio-IS II			
	Human (ng/mL)				Human (ng/mL)				Human (ng/mL)			
	1	2	20	80	1	2	20	80	1	2	20	80
1	1	0.5	0.05	0.0125	2.94	7.69	9.50	12.96	-4.30	-1.41	-8.94	-8.08
2	2	1	0.1	0.025	-4.78	-0.38	1.29	4.49	-2.05	0.90	-6.80	-5.92
20	20	10	1	0.25	-5.00	-0.62	1.06	4.25	-0.57	2.42	-5.39	-4.50
80	80	40	4	1	-13.56	-9.57	-8.05	-5.14	0.43	3.45	-4.44	-3.54

C. APAP-GS

Rat (ng/mL)	Nominal Relative Exposures				Bias from Nonimal by Bio-IS I				Bias from Nonimal by Bio-IS II			
	Human (ng/mL)				Human (ng/mL)				Human (ng/mL)			
	20	40	200	800	10	20	200	800	10	20	200	800
20	1	0.5	0.1	0.025	-0.57	14.19	14.34	13.67	6.48	3.31	6.34	8.64
40	2	1	0.2	0.05	-14.37	-1.65	-1.52	-2.10	-9.73	-12.42	-9.85	-7.90
200	10	5	1	0.25	-14.66	-1.98	-1.85	-2.43	-0.78	-3.74	-0.92	1.23
800	40	20	4	1	-16.74	-4.37	-4.25	-4.81	-17.42	-19.88	-17.54	-15.75

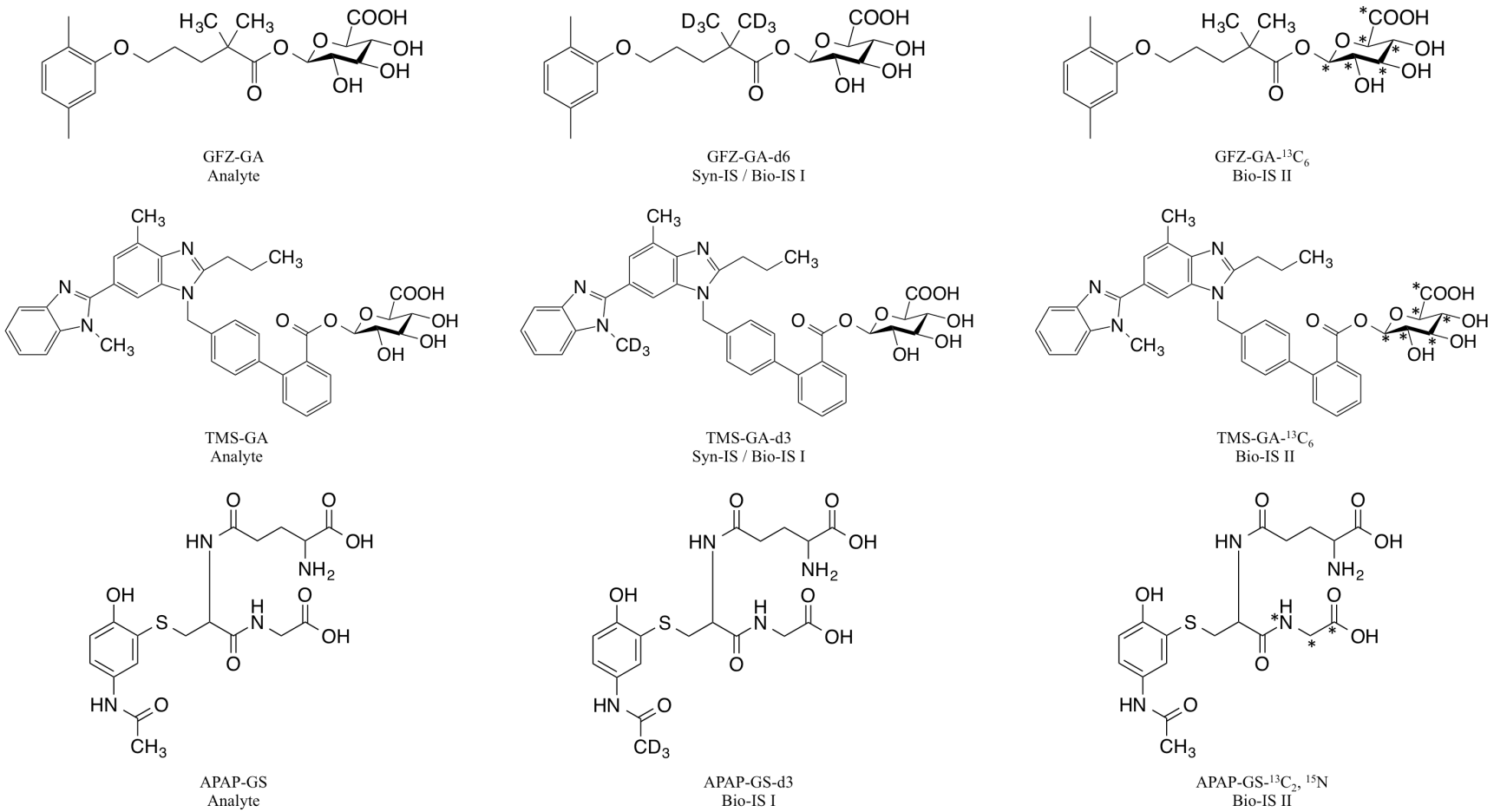


Figure B.1 Chemical structures of GFZ-GA, TMS-GA, APAP-GS and respective bio-IS I and bio-IS II.

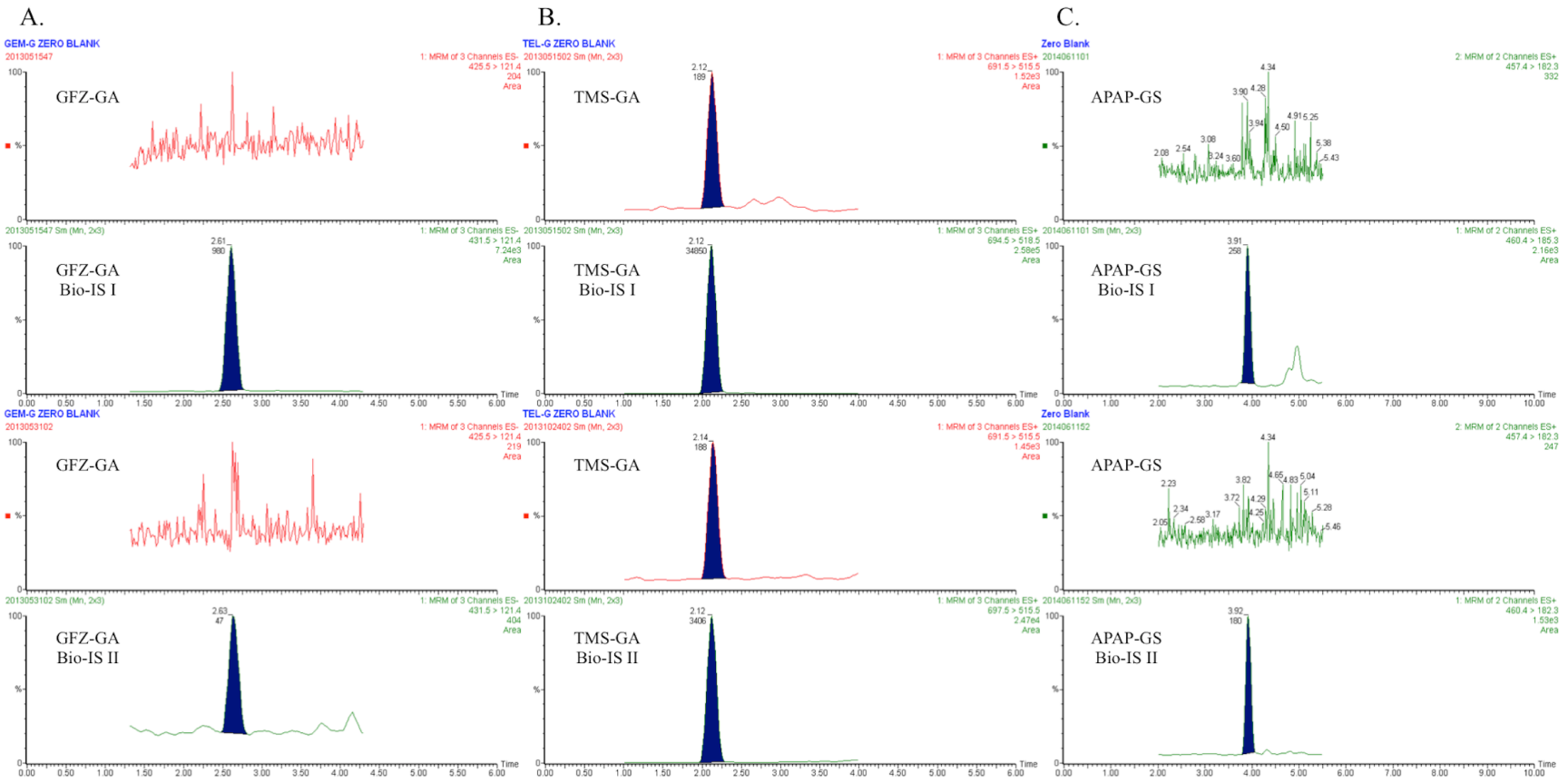


Figure B.2 Representative chromatograms of blank samples prepared by bio-IS I and bio-IS II of (A) GFZ-GA, (B) TMS-GA and (C) APAP-GS.

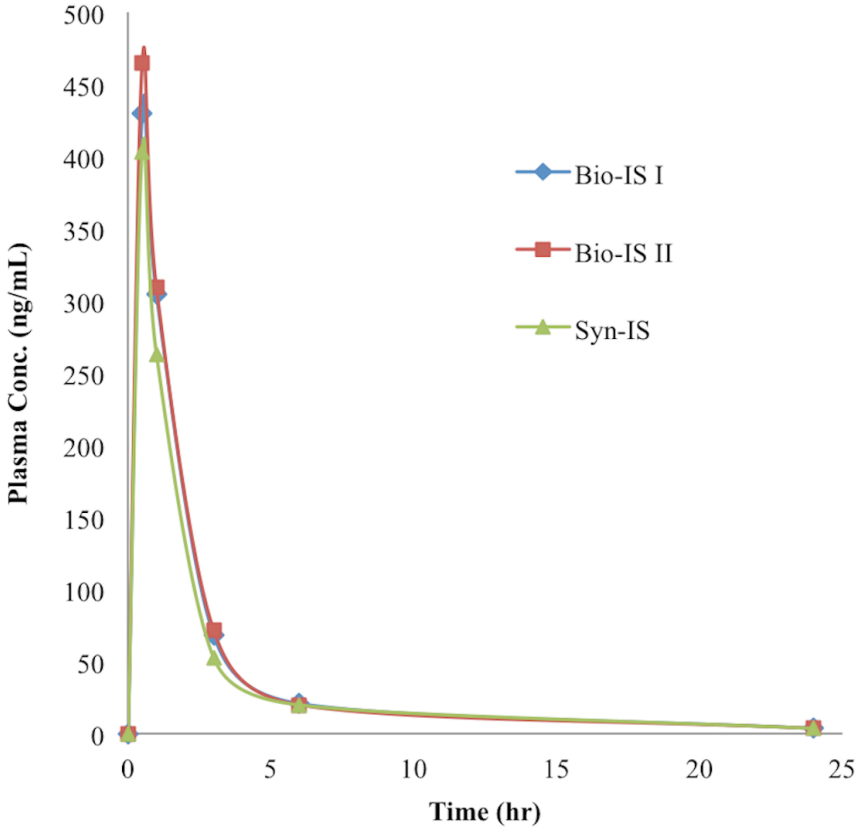


Figure B.3 Pharmacokinetic profiles of TMS-GA obtained using syn-IS, bio-IS I and bio-IS II.

Supplemental data

Supplemental Table 1. Nominal animal-to-human concentration ratios, bias (%) of measured values using bio-IS I and bias (%) of measured values using bio-IS II for mouse, rabbit, dog and monkey samples of (A) GFZ-GA, (B) TMS-GA and (C) APAP-GS.

A. GFZ-GA

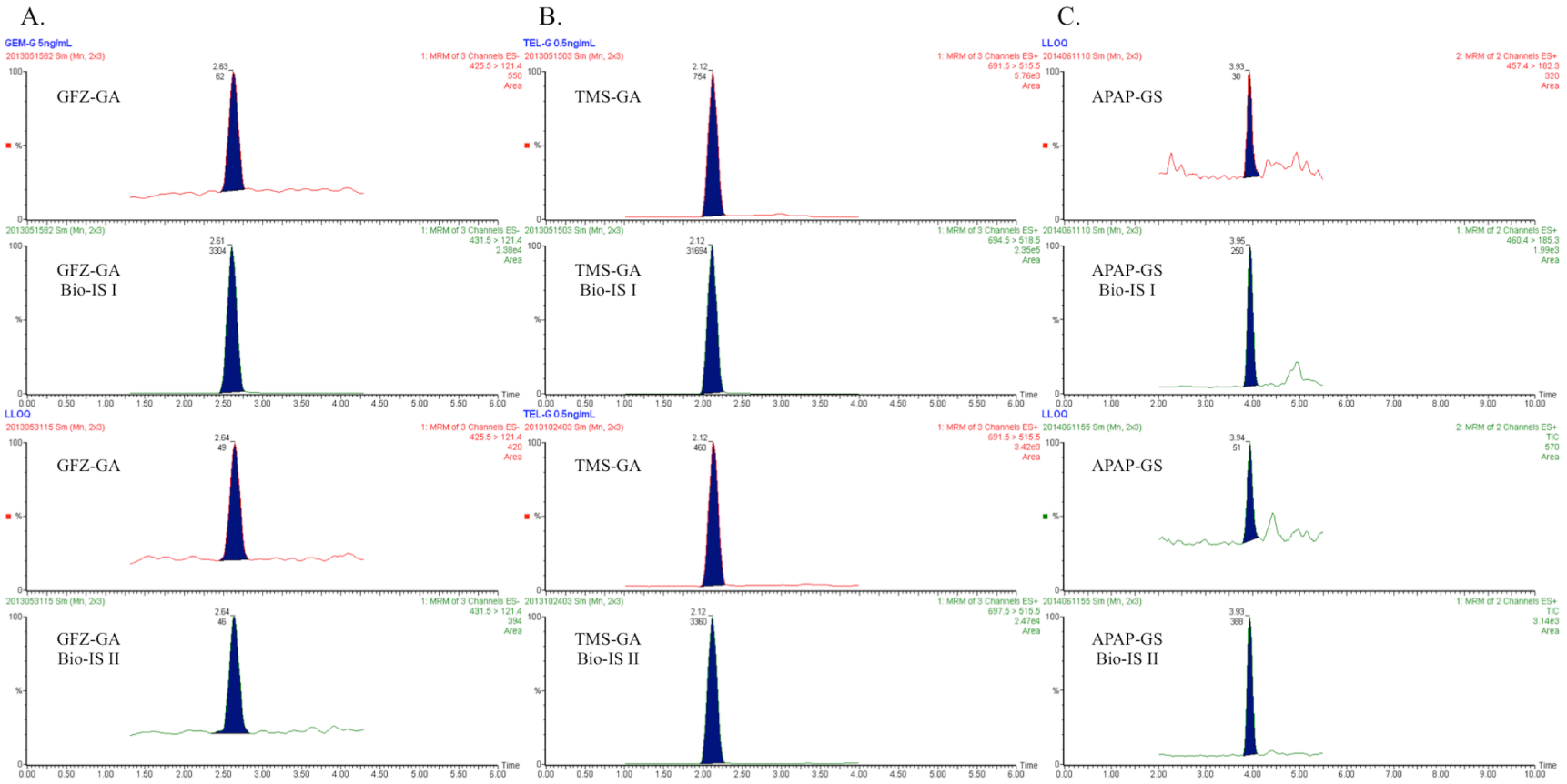
		Nominal Relative Exposures				Bias from Nonimal by Bio-IS I				Bias from Nonimal by Bio-IS II			
		Human (ng/mL)				Human (ng/mL)				Human (ng/mL)			
		10	20	200	800	10	20	200	800	10	20	200	800
Mouse (ng/mL)	10	1	0.5	0.05	0.0125	-0.21	0.53	-1.74	-1.11	-8.25	-8.50	-10.05	-9.52
	20	2	1	0.1	0.025	8.21	9.01	6.55	7.24	1.24	0.97	-0.75	-0.16
	200	20	10	1	0.25	2.57	3.33	1.00	1.65	10.69	10.39	8.52	9.16
	800	80	40	4	1	6.59	7.39	4.96	5.64	6.15	5.86	4.06	4.68
Rabbit (ng/mL)	10	1	0.5	0.05	0.0125	3.58	4.35	1.99	2.65	-4.36	-4.62	-6.24	-5.69
	20	2	1	0.1	0.025	11.68	12.51	9.97	10.68	-3.53	-3.79	-5.42	-4.87
	200	20	10	1	0.25	0.66	1.41	-0.88	-0.24	-0.03	-0.31	-2.00	-1.42
	800	80	40	4	1	7.77	8.57	6.11	6.80	12.59	12.29	10.38	11.03
Dog (ng/mL)	10	1	0.5	0.05	0.0125	6.11	6.89	4.48	5.15	-6.46	-6.71	-8.29	-7.75
	20	2	1	0.1	0.025	2.42	3.18	0.85	1.50	12.81	12.50	10.60	11.25
	200	20	10	1	0.25	0.35	1.09	-1.19	-0.55	8.31	8.01	6.18	6.80
	800	80	40	4	1	5.48	6.26	3.86	4.54	0.71	0.44	-1.27	-0.69
Monkey (ng/mL)	10	1	0.5	0.05	0.0125	7.58	8.38	5.93	6.61	-9.53	-9.77	-11.30	-10.78
	20	2	1	0.1	0.025	9.37	10.18	7.69	8.39	1.13	0.85	-0.86	-0.27
	200	20	10	1	0.25	3.67	4.44	2.08	2.74	2.71	2.43	0.69	1.29
	800	80	40	4	1	5.41	6.19	3.79	4.46	1.10	0.82	-0.89	-0.30

B. TMS-GA

		Nominal Relative Exposures				Bias from Nonimal by Bio-IS I				Bias from Nonimal by Bio-IS II			
		Human (ng/mL)				Human (ng/mL)				Human (ng/mL)			
		1	2	20	80	1	2	20	80	1	2	20	80
	10	1	0.5	0.05	0.0125	0.00	4.62	6.37	9.73	-5.78	-2.94	-10.35	-9.51
Mouse	20	2	1	0.1	0.025	-4.41	0.00	1.68	4.89	-1.03	1.96	-5.83	-4.94
(ng/mL)	200	20	10	1	0.25	-8.49	-4.27	-2.66	0.41	4.78	7.94	-0.30	0.64
	800	80	40	4	1	-12.22	-8.17	-6.63	-3.68	2.51	5.60	-2.46	-1.54
	10	1	0.5	0.05	0.0125	-2.21	2.31	4.03	7.31	-9.47	-6.74	-13.86	-13.05
Rabbit	20	2	1	0.1	0.025	-2.57	1.92	3.64	6.91	-2.39	0.55	-7.13	-6.25
(ng/mL)	200	20	10	1	0.25	-6.91	-2.62	-0.98	2.15	0.39	3.42	-4.48	-3.57
	800	80	40	4	1	-4.87	-0.48	1.19	4.39	5.72	8.91	0.60	1.55
	10	1	0.5	0.05	0.0125	-2.21	2.31	4.03	7.31	-3.01	-0.09	-7.72	-6.84
Dog	20	2	1	0.1	0.025	-3.68	0.77	2.46	5.70	1.89	4.96	-3.05	-2.13
(ng/mL)	200	20	10	1	0.25	-9.63	-5.46	-3.87	-0.84	2.39	5.48	-2.57	-1.65
	800	80	40	4	1	-12.58	-8.55	-7.01	-4.07	2.00	5.07	-2.95	-2.03
	10	1	0.5	0.05	0.0125	4.41	9.23	11.07	14.57	0.38	3.40	-4.49	-3.59
Monkey	20	2	1	0.1	0.025	-5.88	-1.54	0.12	3.28	1.36	4.42	-3.55	-2.64
(ng/mL)	200	20	10	1	0.25	-6.03	-1.69	-0.04	3.12	2.17	5.25	-2.78	-1.87
	800	80	40	4	1	-7.20	-2.91	-1.28	1.84	5.46	8.64	0.35	1.30

C. APAP-GS

		Nominal Relative Exposures				Bias from Nonimal by Bio-IS I				Bias from Nonimal by Bio-IS II			
		Human (ng/mL)				Human (ng/mL)				Human (ng/mL)			
		20	40	200	800	20	40	200	800	20	40	200	800
	20	1	0.5	0.1	0.025	1.72	16.83	16.99	16.29	7.17	3.97	7.02	9.34
Mouse	40	2	1	0.2	0.05	0.29	15.18	15.33	14.65	11.95	8.61	11.79	14.22
(ng/mL)	200	10	5	1	0.25	-6.38	7.52	7.67	7.03	1.91	-1.13	1.77	3.98
	800	40	20	4	1	-16.68	-4.31	-4.18	-4.75	-5.08	-7.91	-5.21	-3.15
	20	0.5	1	5	20	2.87	18.15	18.31	17.61	-1.71	-4.64	-1.84	0.29
Rabbit	40	1	2	10	40	-6.61	7.26	7.40	6.77	6.14	2.98	6.00	8.30
(ng/mL)	200	5	10	50	200	-5.92	8.05	8.20	7.56	-10.89	-13.54	-11.01	-9.08
	800	20	40	200	800	-16.15	-3.70	-3.57	-4.14	-4.21	-7.06	-4.34	-2.26
	20	1	0.5	0.1	0.025	-9.77	3.63	3.77	3.15	-0.68	-3.64	-0.82	1.33
Dog	40	2	1	0.2	0.05	-6.32	7.59	7.73	7.10	-10.75	-13.41	-10.87	-8.94
(ng/mL)	200	10	5	1	0.25	-5.46	8.58	8.72	8.08	-3.96	-6.82	-4.09	-2.01
	800	40	20	4	1	-10.92	2.31	2.45	1.84	-10.96	-13.61	-11.08	-9.15
	20	0.5	1	5	20	-5.17	8.91	9.05	8.41	11.95	8.61	11.79	14.22
Monkey	40	1	2	10	40	-1.72	12.87	13.02	12.35	-3.24	-6.13	-3.37	-1.28
(ng/mL)	200	5	10	50	200	-13.68	-0.86	-0.73	-1.31	-14.03	-16.59	-14.14	-12.28
	800	20	40	200	800	-12.74	0.21	0.35	-0.25	-3.00	-5.89	-3.14	-1.04



Supplemental Figure 1. Representative chromatograms of LLOQ samples prepared by bio-IS I and bio-IS II of (A) GFZ-GA at 5 ng/mL, (B) TMS-GA at 0.5 ng/mL and (C) APAP-GS at 10 ng/mL.