Development of LC-MS/MS Methods for Quantitative Analysis of Plant-Derived Anticancer Agent and Synthetic Estrogen in Complex Matrices

Simuli Lindah Wabuye
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DEVELOPMENT OF LC-MS/MS METHODS FOR QUANTITATIVE ANALYSIS OF PLANT-DERIVED ANTICANCER AGENT AND SYNTHETIC ESTROGEN IN COMPLEX MATRICES

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DEDICATION

This dissertation is dedicated to my beloved family
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to God and all the people who have greatly contributed to my successes in life.

First and foremost, I give all the glory and honor to God the father, the Son and the Holy Spirit for sustaining me, keeping me in good health and enabling me to come this far in my life. I’m also thankful for His strength, wisdom, and peace each and every day. Above all, I thank God for surrounding me with great mentors, supportive family members and wonderful friends.

I am very grateful to my advisor, Dr. Yan Xu for his immense guidance, support, and invaluable ideas throughout my graduate studies at CSU. Thank you for being very understanding, for believing in me and inspiring me to be a great and independent researcher. I will always count on you as my mentor.

I would like to thank my dissertation committee members Dr. Baochuan Guo, Dr. Xue-Long Sun, Dr. John F. Turner and Dr. Joshua G. Bagaka’s for your helpful input, and suggestions throughout my annual research reports and candidacy exam. Your direction was necessary and it has greatly contributed to my research work that has lead to this dissertation.

I would also like to thank Dr. Xiang Zhou, for his thorough training on the “state-of-the-art” analytical techniques, and for giving me a great opportunity to work with him as his TA and assistant manager of the mass spectrometry facility and instrumentation center at CSU.
I would like to extend my appreciation to my mentor and friend, Dr. Isaiah Warner, for the advice, encouragement, and immeasurable help that he has always given to me and to my family ever since we came to this country. Special thanks to Mr. and Mrs. Mudasia for showing me the path to follow at a very young age, I must say it all pays off.

I would not be where I am today without the unconditional love and support of my family and relatives, and, in this regard, I would like to THANK:

My grandmother, Robai Makokha, for her words of wisdom and advice.

My parents, Joseph Mukewa and Delilah Nanyama Wabuyele for the discipline they instilled in me, their words of encouragement “be ambitious”, and most of all, for believing in me.

My uncle and aunt, Ainea Kasembeli and Leanne Jerotich Makokha, for being my mentors ever since my childhood and for being very supportive in every aspect.

My brothers and sisters: Christopher Matanda Wabuyele, Margaret Lukoye Wekesa, Jane Nabhdulumbi Malaba, Dr. Frank Busolo Wabuyele, Lilian Sikhoya Wandabwa, Kennedy Zakhayo (Ticho) Wabuyele, Dr. Caroline Lusike Wabomba, Andrew Biketi Wabuyele, Dr. Ben Musundi Wabuyele, and Nancy Namchanja (Nama) Wabuyele. I thank you all for being there for me, for setting an example for me to follow, and for your constant assurance and words of inspiration.

My cousins: Dr. Moses Zhakayo Kasembeli (Mosee), Jane Katila Makokha, Ben Chilungu (Koki) Makokha, Philip and Michael Wekunda, my brother in-laws: Dr. John
Mukire Wabomba, Abraham Wandabwa and Lionel Williams, my sister in-laws: Dr. Margaret Matanda, Hoita Mongi, and Dr. Precious Kasembeli among others, thank you for your help and never ending support.

To all my invaluable friends, I’m very grateful for everything we have shared, and thank you all for being there for me and for making my stay at CSU fun and memorable. Jennifer Williams: the good times we’ve shared ever since the beginning of this journey will always be remembered. Amina Abbadi: the nights we went salsa dancing where the best and they sure did relieve stress. Carol Opee: you have always been there for me through thick and thin and I couldn’t thank you enough. I love you all!

Last, but not least, I would like to thank my church, The Word Church as well as the Beacon young adults ministry at New song, for the spiritual support they provided me. It was a blessing to meet powerful prayer warriors at Word North like sister Pamela Switzer (and others), who helped me understand the power of prayer and intersession. God bless you!

Many thanks to Dr. Yan Xu’s research group, both the former and present members for your friendship, ideas, and support throughout my graduate work. It was a pleasure knowing you and working together. I will miss you a lot! Lastly I would like to thank, the College of Sciences for the Cellular and Molecular Medicine Specialization (CMMS) Fellowship, the College of Graduate Studies for the Doctoral Dissertation Research Expense Award Fellowship Program (DDREAFP) and the Department of Chemistry at Cleveland State University for the financial support.
DEVELOPMENT OF LC-MS/MS METHODS FOR QUANTITATIVE ANALYSIS OF PLANT- DERIVED ANTICANCER AGENT AND SYNTHETIC ESTROGEN IN COMPLEX MATRICES

SIMULI LINDAH WABUYELE

ABSTRACT

Quantitative methods of analysis play an important role in early stages of drug discovery and clinical development, as well as, biomonitoring studies of human exposure to endocrine disrupting chemicals (EDCs). It is significant to develop quantitative methods that are highly sensitive, selective and accurate for potential anticancer drugs and EDCs (or their metabolites) in complex matrices considering the fact that; 1) some anticancer agents have a low therapeutic index and have failed in the clinical trials, and 2) the effects of some EDC have been found to occur at very low concentrations. Therefore, such quantitative methods could help predict the absorption, distribution, metabolism and elimination (ADME) of the anticancer agents, and provide a better understanding of the efficacy of the drug and levels of toxicity. In addition, insight on the biotransformation and toxicokinetics of EDCs could be obtained for better assessment of the exposure levels and dosage effect.

Currently, the most powerful technique for quantitative analysis is liquid chromatography (LC) method of separation coupled to tandem mass spectrometry (ESI-
MS/MS). In this dissertation, a brief overview of the methodology, instrumentation, samples preparation techniques and essential parameters for bioanalytical method validation will be discussed, as well as, the work flow of liquid chromatography tandem mass spectrometry (LC-MS/MS) method development. LC-MS/MS was exploited for quantitative analysis of (-)-Securinine (SE), a plant-derived anticancer agent and Bisphenol A (BPA), a synthetic estrogen.

In order to facilitate the pharmaceutical development of SE natural products, i.e., ensure quality, efficacy and safety; a sample preparation method was developed to isolate SE from its natural source; Securinega suffruticosa plant and an LC-MS/MS method was developed and validated to quantify the SE in raw plant material. The method developed was applied to the quantitative analysis of the distribution and levels of SE in different parts of S. suffruticosa plant (i.e., leaves, roots, stems, bark and branches).

Furthermore, in order to support the pharmacological studies of SE as a potential anticancer drug, an LC-MS/MS method was developed and validated for quantitative determination of SE in mouse plasma and was applied to the measurement of SE concentrations in a mouse study.

Lastly, in order assess human exposure to chemicals that mimic endogenous hormonal action and interfere with the endocrine function (i.e., synthetic estrogens) highly sensitive and accurate techniques are crucial. Due to the prevalence of BPA in laboratory environment and reagents, a metabolite of BPA i.e., Bisphenol A β-D-glucuronide (BPA-G) was used as a urinary biomarker to assess human exposure to BPA. A stable isotope dilution (SID)-LC-MS/MS method was developed, validated and successfully applied to the measurements of BPA-G in 40 patients’ urine samples.
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CHAPTER I

INTRODUCTION TO QUANTITATIVE LC-MS/MS METHOD DEVELOPMENT FOR PLANT-DERIVED ANTICANCER AGENT AND SYNTHETIC ESTROGEN

1.1 General introduction

The early stages of drug discovery encompass the identification of new drugs that target molecular and biological pathways that seem to play a role in various diseases including cancer. This discovery is then followed by isolation, characterization and then clinical development i.e., preclinical studies, clinical trials and pharmaceutical production (Figure 1) [1]. The success of these early stages of drug discovery and development, as well as, biomonitoring studies (i.e., determination of internal dose) of human exposure to endocrine disrupting chemicals EDCs depends on various analytical technologies, such as, qualitative and quantitative methods of analysis, among other disciples of science.
An efficient and high-throughput approach is required in order to characterize drug “lead” compounds and specific EDCs, as well as, measure their concentrations in biological matrices during the pre-clinical developments and biomonitoring studies. This enables the prediction of absorption, distribution, metabolism, and elimination (ADME) of the drug over time throughout the body. Moreover, these allows one to monitor how fast the drug starts to act, the effect and duration of the drug which ultimately affects the administration i.e., the amount and intervals of dosing [2]. Hence, provide a better understanding of the efficacy and toxicity levels; since new drugs have failed in the clinical trials not only due to unpredicted toxicity but also metabolism problems [3, 4]. In addition, since the effects of some EDCs have been found to occur at very low concentrations; the quantitative determination of internal levels and understanding the biotransformation and toxicokinetics of the EDCs is imperative to provide a better assessment of the exposure levels and dosage effect [5-7].

Significant progress over the recent years in liquid chromatography separation, mass spectrometry detection, and sample pre-treatment techniques has facilitated bioanalytical method development and validation. Liquid chromatography tandem mass spectrometry (LC-MS/MS) has emerged as the most powerful technique capable to analyze unknown and structurally diverse molecules in complex matrices, with high performance, sensitivity and selectivity [1, 8]. In this work, LC-MS/MS methods were developed and validated to quantify (-)-Securinine (SE), a plant derived anticancer agent and Biphenol A (BPA), a synthetic estrogen also known as an endocrine disrupting compound.
Figure 1: Drug discovery and development process [9]
### 1.1.1 Natural products in cancer therapy

Cancer is a group of diseases with characteristically abnormal growth of cells that rapidly divide and form tumors, some of which spread and invade (metastasize) other tissues and become life-threatening. It’s the second most common cause of death in the US, following heart disease[10] and has been estimated by the American Cancer Society that it’s responsible for at least 1 of every 4 deaths. In 2013, the total estimated cancer deaths in America is about 580,350 i.e., close to 1,600 people per day. As these numbers keep increasing with new cancers cases every year (i.e.,1,660,290 diagnosed)[10] there has been an increased demand on cancer research to prevent inhibit, delay or reverse the life threatening disease.

Traditional cancer treatments including surgery, chemotherapy, and radiotherapy are commonly used but they are limited as single modalities [11]. Chemotherapy for example, is toxic to both cancer cells as well as the normal non-cancerous cells, whereas, radiotherapy kills the tumor cells as well as affects the nearby normal cells. Due to the limitation of tradition cancer treatment, other therapies including targeted therapies have emerged. In targeted therapy, specific chemotherapeutic agents; naturally occurring or synthetic chemicals, modulate specific signaling pathways involved in the carcinogenesis and block the out of control growth and spread of cancer. This therapy has proved effective and has been used in together with traditional therapies as combination therapies for a higher efficacy and decreased cytotoxicity [12]. Therefore, to establish effective
cancer therapy, there is a need for discovery and development of new molecular targets for cancer prevention and therapy.

Natural products have been exploited as a source for new drug targets since ancient times [13-15]. It’s reported that over 60% of the anticancer drugs currently approved are obtained from natural sources including plants, marine organisms and micro-organisms [16]. Its been estimated that about 80% of the people in developing countries take traditional medicine for their primarily health care and about 85% of traditional medicine involves the use of plant extracts [9]. A majority of plants used for medicinal purposes, have been the primary source of chemotherapeutic agents and served as leads for the early drug discovery and development. In the US, a few plant-derived anti-cancer agents have been approved for use i.e., vinblastine (VLB), vincristine (VCR), etoposide (VM 26), teniposide (VP 16-213), vinorelbine (VRLB), vindesine (VDS), Paclitaxel (taxol®), docetaxel (Taxotere®), Topotecan and Irinotecan (CPT-11; Camptosar) [14, 17]. Besides being used to obtain the active compounds, natural plant products has been used the source of the starting drug for semi-synthesis and as models for new synthetic drugs [9].

The development of novel chemotherapeutic agents from natural sources is faced with various challenges and has been constrained by the lack of sufficient plant resources, inability to access the pertinent sources as well as identification, isolation and production of the active compound in large quantities necessary[14]. Despite the challenges faced, natural sources/products continue to significantly impact the development of new molecular targets for modern combination therapies to minimize or solve the problems inherent to the traditional cancer treatments.
1.1.1.1 Plant-derived anticancer agents

In this work, naturally occurring Securinega alkaloids will be discussed. These are a group of polycyclic compounds isolated from the plants of Securinega and Phyllanthus specie of Euphorbiaceae family. The four known alkaloids (Figure 2) from this class include; securinine, virosecurinine (viroSE), allosecurinine (alloSE) and viroallosecurinine (viroalloSE). Securinine has been found to be the major alkaloid in the leaves of Securinega suffruticosa and alloSE as the minor alkaloid and the roots of the plant contain securinine and alloSE as minor and major alkaloids respectively. On the hand, viroSE is the major and viroalloSE is the minor alkaloid from the leaves of Securinega virosa [18-23].

Securinine was the first member of these alkaloids to be isolated from its natural source i.e., Securinega suffruticosa a plant that is well distributed in north eastern parts of Asia and has been used in Chinese folk medicines [22]. (-)-Securinine (SE) exhibits a wider range of biological activities and recently it has been found to have potential clinical use as anticancer agent for acute myeloid leukemia (AML) and colon cancer [24-26].

In order to facilitate the pharmaceutical development and ensure quality efficacy and safety of SE natural products, a sample preparation method and an LC-MS/MS methods were developed to isolate SE from Securinega suffruticosa plant, and quantify the levels of accumulation in different parts of the plant (Chapter I). Also, to support the
Figure 2: The Securinega alkaloids

Securinine

Virosecurinine

Allosecurinine

Viroallosecurinine

Figure 2: The Securinega alkaloids
pharmacological studies of SE, an LC-MS/MS method was developed, validated and applied to the measurement of SE concentrations in a mouse study (Chapter II).

1.1.2 Endocrine disrupting chemicals (EDCs)

The human endocrine system (Figure 3) is known to control various biological processes in the human body during the early stages of development (i.e., cell differentiation and organ formation), as well as, upon maturity. The endocrine glands are responsible for producing hormones involved in various signaling pathways for normal functioning of various tissues and organs [27].

Endocrine disruptors are exogenous chemicals that act like hormones via binding to hormone-receptor protein complex (Figure 4) and disrupt hormonal action. These compounds interfere with the endocrine function consequently causing adverse physiological effects which could result in various cancers i.e. breast cancer, prostate cancer, and other reproductive disorders. EDCs are known to affect the hormonal action of estrogen, androgen, thyroid among other biological processes [27].

1.1.2.1 Synthetic estrogen

Synthetic estrogens (i.e., xenoestrogens) are chemicals that enter the body and bind to estrogen receptor and mimic its actions. Examples include chemicals i.e.,
Figure 3: Human endocrine system [27].
Figure 4: Hormonal action via receptor binding [27].
polychlorinated biphenyls (PCBs), biphenyl A (BPA) and phthalates [28]. In work, BPA will be discussed.

BPA is used to make polycarbonate plastic and epoxy resins which are found in many consumer products i.e., food containers, water bottles, dental sealants, baby bottles and also, used as a linings / inner coating of food cans or beverages to prevent rusting [29, 30]. BPA is first synthesized via condensation (Figure 5) of two equivalents of phenol with acetone (thus the use of the suffix A in the name) then, BPA can react with phosgene or diphenyl carbonate to form polycarbonate polymers (Figure 6). However, when incomplete polymerization occurs or the ester linkages are subject to high temperature or exposed to acidic/basic conditions, BPA leaches out into the food or beverages [31]. This frequently exposes humans to BPA which could result in adverse physiological effects [32-34].

BPA was discovered as a synthetic estrogen in 1930s [35]. The structure of BPA closely resembles that of estradiol.; the endogenous estrogen (Figure 7), in that, both compounds share the phenol groups, which has been reported to be the important site for receptor recognition [36]. Hence, BPA can bind to the estrogen receptor (ER) α and β, with 10 times higher affinity for ERβ and mimics its hormonal action [37, 38] acting as an endocrine disrupting compound.

Initially, BPA was considered a weak environmental estrogen because of its relatively low affinity for the nuclear ERs compared with estradiol in some assays [39, 40]. However, recently it’s been found that BPA can activate rapid cellular responses at very low concentrations; below the levels where BPA was initially expected to bind to
Figure 5: Synthesis of BPA

http://en.m.wikipedia.org/wiki/Bisphenol_A
Figure 6: Synthesis of polycarbonate plastic

http://en.m.wikipedia.org/wiki/Bisphenol
Bisphenol A (BPA)
MW = 228.3

Estradiol (endogenous estrogen)

Figure 7: Chemical structure of BPA and endogenous estrogen
the receptor [41]. Also, animal studies suggest that BPA could cause effects in animal models at levels in the range of human exposure [31, 42-44].

Therefore, biomonitoring studies (i.e., assessment of internal dose) of human exposure to BPA require highly specific and accurate analytical methods of quantitation. In order to minimize BPA contamination, in this work, the metabolite of BPA i.e., bisphenol A β-D-glucuronide (BPA-G) was used as a urinary biomarker to assess human exposure to BPA. A stable isotope dilution (SID)-LC-MS/MS method was developed, validated and successfully applied to the measurements of BPA-G in 40 patients’ urine samples (Chapter IV).

1.2 Quantitative methods of analysis

Traditionally, quantitative analysis was performed using immunoassay methods i.e., radio-immunoassay (RIA), enzyme-multiplied immunoassays (EMIT) and fluorescence polarization immunoassay [FPIA]. Although, the estimate of the free drug concentration with these methods is comparable to LC; they tend to overestimate the values, due to non-specific binding of the antibody to the drugs. Also, the interferences from the biological matrices result in decreased accuracy of the assays, because they are not highly specific.

In addition, evaporative light scattering detection (ELSD), and chemiluminescent nitrogen detection (CLND) coupled to liquid chromatography (LC) has also been used for quantitative analysis. ELSD is a technique sensitive to mass of the analyte remaining
after solvent evaporation and is thus limited by the fact that volatile, low melting point compounds cannot be detected. CLND on the other hand depends on the number of nitrogen atoms in the sample, therefore, its nitrogen selective and could be limited by impurities and solvents that contain nitrogen [1]. These techniques with their limitations are not applicable for drug analysis on a large scale basis.

Improvements have been made towards liquid chromatography coupled to ultraviolet (UV), fluorescence, electrochemical detection and gas chromatography (GC) coupled to mass spectrometry techniques. These methods have a better sensitivity, linearity, precision and could analyze small sample volumes simultaneously in biomatrices than the earlier methods and thus have become the most commonly employed techniques.

UV detects only molecules that are able to absorb UV light and the absorbed energy is proportional to the concentration of the compounds of interest at certain concentration ranges. It’s therefore not specific in that, various analogues of the compound of interest could also absorb in the same UV region.

Electrochemical and fluorescence detection are more sensitive and specific than UV, but are limited by the fact that they requires compounds that have electro-active groups or are fluorescent. Therefore, derivatization of the compounds will be required, which could alter the physical chemical properties of the drug analyzed and could also be time consuming.

GC-MS on the other hand, couples the unsurpassed specificity of the MS to capillary GC for analysis of compounds of low polarity. However, some of this compounds despite their low polarity, they are too large to be analyzed without
decomposition. Moreover, many pharmaceutical drugs are polar, non-volatile and thermolabile, for this reason, they cannot be analyzed by this technique [45].

All the aforementioned methods are limited in their application to quantitative analysis; however, with further improvements in the areas of hyphenation (combination) of analytical techniques, liquid chromatography tandem mass spectrometry (LC-MS/MS) emerged as the most powerful method for the analysis of pharmaceutical compounds. Due to its unsurpassed capability of high through-put analysis, sensitivity and selectivity it has been used for identification of novel drug targets, chiral impurities, and degradation products/metabolites etc.[8] So far, it’s the unrivalled method for pharmaceutical drug and metabolite quantification.

1.2.1 Liquid chromatography-mass spectrometry (LC-MS)

1.2.1.1 Liquid chromatography (LC)

LC is used to separate the analyte of interest from biological matrix components and complex mixtures. Also, it’s a way of introducing the analyte, into the MS for analysis, in solution. The LC system consist of the mobile phase (in the solvent reservoirs), binary pumps that keep the mobile phase flowing at a constant flow in the system, an autosampler for sample injection, a guard column to protect the column from
contamination, a column for separation and finally, a detector which is connected to a data processing software (Figure 8).

There various fundamentally different modes of separation which include; reverse-phase chromatography (RPC), normal-phase chromatography (NPC), non-aqueous reverse-phase chromatography (NARP), hydrophilic interaction chromatography (HILIC), ion-exchange chromatography (IEC), ion-pair chromatography (IPC), size-exclusion chromatography (SEC) (TABLE I) [46]. Each of these techniques has its own advantages and disadvantages, which ultimately determine their suitability as separation techniques hyphenated to MS.

Reverse-phase chromatography (RPC) or Reverse phase liquid-chromatography (RPLC) is the most commonly used method and is utilized in this work. It’s based on the partitioning of the analyte between a polar mobile phase and a hydrophobic stationary phase on the column. The mobile phases used includes a mixture of aqueous (e.g. water or buffer) and organic solvents (e.g. methanol or acetonitrile). The stationary phase is a non-polar alkyl hydrocarbons \( i.e., \) C-8 or C-18 chains bonded on silica support (or other inert supports) in the analytical column [47]. In RPLC, the non-polar compounds are retained longer on the column than polar compounds, but by varying the percent content of the organic additive in the mobile phase, analytes can be eluted. Therefore, the choice of the solvents used, the modifiers (acids, bases or ion pairing agents) added, and the pH selected, greatly influence the selectivity and the retention of the analytes in RPLC.

RPLC is the widely used method of separation because if its ability to analyze polar, medium and non-polar analytes as well as ionic analytes in some cases. In addition,
Figure 8: Liquid chromatography system
<table>
<thead>
<tr>
<th>Chromatographic Mode</th>
<th>Abrv.</th>
<th>Column Type</th>
<th>Mobile Phase</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse-phase chromatography</td>
<td>RPC</td>
<td>Non-polar (e.g. C&lt;sub&gt;18&lt;/sub&gt;)</td>
<td>Polar mixture of water and organic solvent (e.g. acetonitrile)</td>
<td>Water-soluble samples</td>
</tr>
<tr>
<td>Normal-phase chromatography</td>
<td>NPC</td>
<td>Polar (e.g. unbonded silica)</td>
<td>Less-polar (than stationary phase) mixture of organic solvents (e.g. hexane, ethyl ether, chloroform, methylene chloride)</td>
<td>Water-insoluble samples, isomer separation, and preparative HPLC</td>
</tr>
<tr>
<td>Non-aqueous reverse-phase chromatography</td>
<td>NARP</td>
<td>Non-polar (e.g. C&lt;sub&gt;18&lt;/sub&gt;)</td>
<td>Mixture of organic solvents (e.g. ACN + methylene chloride)</td>
<td>Very hydrophobic samples</td>
</tr>
<tr>
<td>Hydrophilic interaction chromatography</td>
<td>HILIC</td>
<td>Polar (e.g. silica or amide-bonded)</td>
<td>Mixture of water organic solvents (e.g. ACN + H&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>Highly polar samples that are poorly retained by reverse-phase mode</td>
</tr>
<tr>
<td>Ion-exchange chromatography</td>
<td>IEC</td>
<td>Usually an organic resin that has charged groups able to bind ions of opposite charge</td>
<td>Aqueous solution of a salt with buffer.</td>
<td>Separating ionizable samples, large biomolecules (e.g. proteins, carbohydrates)</td>
</tr>
<tr>
<td>Ion-pair chromatography</td>
<td>IPC</td>
<td>Non-polar (e.g. C&lt;sub&gt;18&lt;/sub&gt;)</td>
<td>Polar mixture of water and organic solvent (e.g. ACN) with an ion-pairing reagent (e.g. alkylsulfonates, trifluoroacetic acid) that interacts with sample ions of opposite charge</td>
<td>Acids and bases that are weakly retained by reverse-phase</td>
</tr>
<tr>
<td>Size-exclusion chromatography</td>
<td>SEC</td>
<td>Inert</td>
<td>Aqueous or organic</td>
<td>Large biomolecules, polymers. Separates by molecular weight.</td>
</tr>
</tbody>
</table>
RPLC is more compatible with MS in terms of the solvents used and flow rates at which they enter the MS. Unlike the other separation techniques such as, NPC or IEC that require the use of nonvolatile salts (i.e. phosphates), additives (i.e. ion pairing agents) and organic solvents that tend to significantly reduce the MS detection signal [4].

The pharmaceutical industry works towards the reduction in the sample analysis time and high sample throughput, without compromising the separation efficiency and selectivity of the analytes of interest. To achieve this, various columns have been design with a versatile stationary phase and technology is moving towards shorter lengths and small particle sizes. Ultra high performance LC (UPLC) has also emerged that use columns with sub-2 μm particle size and an LC system that can handle high pressures. UPLC has the advantage of decreased run time and increase sensitivity over conventional LC method hence; it provides the needed high sample throughput without affecting the chromatographic performance.

Nevertheless, the optimization of the separation process is also influenced by the choice of detector. The “start of the art” configuration i.e., hyphenation of LC to mass spectrometry (MS), has proved to be more efficient and of high selectivity compared to most techniques, therefore, it’s the method of detection used in this work.

1.2.1.2 Mass spectrometry (MS)

Mass spectrometry is a powerful tool that is highly selective, and provides a wealth of structural information useful for analysis and quantitation of small molecules.
The main components of a mass spectrometer are shown in Figure 9 and include; a method to introduce the compounds into the MS. Normally, a direct flow injection can be done using a syringe pump, which is the fastest approach or a flow injection analysis (FIA) by LC can be used [48]. The sample in solution is then evaporated in the ion source and gas phase ions are formed. After which, this ions enter the mass analyzer where the electromagnetic field separates the ions based on their mass to charge (m/z) ratio and are then sent to the detector, that amplifies the signal and generates a mass spectrum which is recorded by the computer.

There various techniques that have been developed to couple the LC to MS and serve as an interface i.e., electro spray ionizations (ESI), atmospheric pressure chemical (APCI) and atmospheric pressure photo ionization (APPI). ESI and APCI are the ionization techniques most commonly used for quantitation.

In ESI (Figure 10), the sample solution passes through a charged capillary tube that has a high electric potential (either positive or negative) applied at the end of it. This causes a solvent spray to form, made up of charged droplets. The flow of the nebulizing gas in the same directions, helps increase the spray efficiency, whereas, the nitrogen gas drying gas assists with the desolvation. As the solvent evaporates and the droplet decreases in size, the surface charge density is increased and so is the repulsion force. The droplets break up and then charged ions are formed. The formation of these ions is also depends on the mobile introduced and could results in formation of other adducts in the mobile phase [49]. Therefore the use of volatile buffers (i.e., ammonium formate, ammonium acetate etc) containing counters ions could help suppress this phenomenon.
Figure 9: The basic components of a mass spectrometer
Figure 10: A schematic of an ESI interface

ESI is considered a “soft ionization technique” and it has the ability to analyze polar to ionic compounds, as well as, high molecular weight bio-molecules i.e. proteins.

APCI (Figure 11) on the other hand, differs with ESI in that ionization occurs in the gas phase and not in solution. The sample is sprayed through a capillary, then a nebulizing gas assists to evaporate the liquid in the source and the sample is vaporized with the heater gas. The vapor formed collides with sample solvent molecules ionized by the corona discharge, generating ions. APCI can analyze less polar compounds, and it’s less prone to matrix effects; however, it requires the compounds to be thermally stable.

The mass analyzer is the heart of the MS. It basically distinguishes and separates molecular ions and the ions formed from the dissociated products according to their \( m/z \) ratio, before finally entering the detector: where they are measured and their abundance determined. The Mass analyzer is operated under high vacuum, such that the ions that exit the detector travel with a sufficient yield. There various types of mass analyzers i.e., quadrupole (Q), ion trap (IT), and time of flight (TOF), but not all are applicable for drug analysis and quantitation. In this work, the quadrupole (Q) and ion trap (IT) will be discussed.

Quadrupole mass analyzer are the frequently used for small molecules such as drugs and metabolites in the mass range of 80 \( m/z \) to 700 \( m/z \) [48]. The quadrupole analyzer is made of four parallel circular/hyperbolic rods connected together as two
Figure 11: A schematic of an APCI interface

opposite pairs (Figure 12). These rods are under direct current (DC) potential and radio frequency (RF) potential is applied to them in an alternating manner. The ions from that enter the quadrupole mass filter from the ion source, are focused along the central axis of the rods and oscillate in X, Y and Z directions i.e., some have stable trajectories other have unstable ones. The ions traveling along the Z axis are the only ones affected by the total electric field, resulting in stable trajectories and are able to reach the detector. All the other ions are filtered out; due to the unstable trajectories they hit the rods and are not detected [50, 51].

The quadrupole can be operated in two modes: the scan mode or single ion monitoring (SIM) mode. In the scan mode, the amplitude of the DC and RF voltages are set to monitor a series of masses in a certain mass range. This mode is suitable for qualitative work. In the SIM mode, the MS is tuned to a particular $m/z$ window that overlaps with the ion of interest hence, more sensitive and suitable for quantitation. The SIM mode enables the determination of the structure of the compound based on the precursor (parent) / molecular ion; the ion with the highest $m/z$ value which represents the molecular weight (MW) of the compound of interest. This mode is not selective when components in the sample matrix have the same $m/z$ with the target analyte [51].

Ion trap mass analyzer as depicted in the name, traps ion prior to reaching the detector. The trap consists of; an entrance end cap electrode, ring electrode on two sides and an exit end cap electrode (Figure 13). The voltages applied to the electrodes enable ions to enter the trap and based on these voltages, as well as, the $m/z$ of the ions, they
Figure 12: Quadrupole mass analyzer [50]
Figure 13: Ion trap mass analyzer [50]
oscillate with stable trajectories. When the voltages are altered, the ions are destabilized and exit the trap into the detector. The limitation of the ion trap is that, as ions of the same charge accumulate in the trap, they tend to repel one another. To overcome this problem inert gases \textit{i.e.}, nitrogen and helium have been added into the trap to stabilize the ions and minimize collision with the sides of the trap [50].

### 1.2.1.3 Tandem mass spectrometry (MS/MS)

In addition to single quadrupole or ion trap use, the quadrupole analyzer can be operated in the MS/MS mode where by multiple analyzers (of the same or different kind) are used in tandem \textit{i.e.} the triple quadrupole (QqQ) and the quadrupole ion trap (QTrap).

The QqQ instrument consists of two quadrupoles, separated by collision cell. The first quadrupole selects the precursor ion, the second quadrupole is the collision induced dissociation (CID) cell where the ions are fragmented (at different degrees) in the presence of a collision gas. The resulting fragments are then sent into the third quadrupole, where they are analyzed in scan mode or by single ion monitoring (SIM). The QTrap works slightly different than the QqQ in that, the instrument consists of a quadrupole, which selects the precursor ion and an ion trap which as discussed traps ions. The ions undergo fragmentation by CID in the same trap and then isolated and detected.

In MS/MS there various modes used (Figure 14), but in this work, we only consider the selected-reaction-monitoring (SRM) also known as the multiple-reaction-
Figure 14: Tandem MS modes of operation

monitoring (MRM). In this mode, fragmentation is carried out in Q2 with a collision gas; Q1 and Q3 are used to select specific molecular (precursor) and product ions ($m/z$) respectively, without scanning (Figure 15). MRM is similar to the SIM mode; however, the ions selected in Q1 are only detected if they fragment by the selected mass transition of precursor/product ion pair. This allows for the structural analysis of the compound of interest based upon the fragmentation pattern, thus offers a better sensitivity with an added selectivity for quantitative analysis of complex sample matrices [51].

### 1.2.2 Stable isotope dilution (SID)-LC-MS/MS

Stable isotopes are naturally occurring elements that have the same number of protons, but differ from each other in molecular masses due to having different number neutrons. They have extremely low abundances in nature (TABLE II) i.e., the abundance of 2H relative to 100 parts of 1H is 0.015. Therefore, these stable isotopes are used in combination with LC-MS/MS to provide the highest possible analytical specificity for quantitative analysis [52].

Stable isotope analogs are added to complex biological matrices as internal standards (IS) prior to sample preparation to correct for matrix effect, loss of analyte and variation in sample extraction, hence, improve the method’s precision and accuracy. In this case, the response ratio between the analyte and labeled IS, is used to construct a
Figure 15: Schematic of triple quadrupole (QqQ)

http://www.mrmatlas.org/mrmassays.php


TABLE II: LIST OF COMMON ORGANIC ELEMENTS AND THEIR ISOTOPES [52]

<table>
<thead>
<tr>
<th>Element</th>
<th>m/z</th>
<th>% Natural Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>1.008</td>
<td>99.985</td>
</tr>
<tr>
<td>$^2$H</td>
<td>2.014</td>
<td>0.015</td>
</tr>
<tr>
<td>$^{12}$C</td>
<td>12.000</td>
<td>98.89</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>13.003</td>
<td>1.11</td>
</tr>
<tr>
<td>$^{14}$N</td>
<td>14.003</td>
<td>99.63</td>
</tr>
<tr>
<td>$^{15}$N</td>
<td>15.000</td>
<td>0.37</td>
</tr>
<tr>
<td>$^{16}$O</td>
<td>15.995</td>
<td>99.759</td>
</tr>
<tr>
<td>$^{17}$O</td>
<td>16.999</td>
<td>0.037</td>
</tr>
<tr>
<td>$^{18}$O</td>
<td>17.999</td>
<td>0.204</td>
</tr>
<tr>
<td>$^{28}$Si</td>
<td>27.977</td>
<td>92.21</td>
</tr>
<tr>
<td>$^{29}$Si</td>
<td>28.976</td>
<td>4.70</td>
</tr>
<tr>
<td>$^{30}$Si</td>
<td>29.974</td>
<td>3.09</td>
</tr>
<tr>
<td>$^{32}$S</td>
<td>31.972</td>
<td>95.05</td>
</tr>
<tr>
<td>$^{33}$S</td>
<td>32.968</td>
<td>0.76</td>
</tr>
<tr>
<td>$^{34}$S</td>
<td>33.967</td>
<td>4.22</td>
</tr>
</tbody>
</table>
standard calibration curve and determine the amount of the analyte in the unknown samples.

In addition, since stable isotope labeled analogs of a compound share the same chemical structure and properties as the unlabeled compound, they behave in a similar or identical manner. Therefore, they can be used as surrogates to verify the presence of the analyte of interest, as well as, quantify the analyte in complex matrices, due to the lack of blank (analyte-free) matrices. In this work, stable isotope dilution (SID)- LC-MS/MS was employed in Chapter 4 for specific and accurate measurement of the metabolite of BPA in human urine samples.

1.3 Sample preparation for complex matrices

Sample preparation is critical prior to LC-MS/MS analysis especially when dealing with complex matrices. This helps to remove any impurities and other endogenous matrix components that could possibly interfere with the MS detection. The techniques commonly used include; “dilute and shoot”, protein precipitation (PPT), liquid-liquid extraction (LLE), salting-out assisted liquid-liquid extraction (SALLE) and solid phase extraction (SPE) [53].

Dilute and shoot as the name implies, the samples are simply diluted and directly injected into the LC-MS/MS. This method is applicable in case where the concentration of the analyte in the sample matrix is relatively high and the matrix effect is not a concern.
In protein precipitation (PPT), a precipitation agent/solution \textit{i.e.}, an organic solvent is added to a volume of plasma or serum sample, which typically contain proteins. This disrupts protein association with each other and the proteins precipitate out of solution. The sample is then centrifuged and the supernatant removed and analyzed. PPT technique is popular due to its ease of use and simplicity; however, it does not sufficiently remove all the interferences and as a result matrix effect is common with this technique.

Salting-out assisted liquid-liquid extraction (SALLE) is similar to PPT but instead concentrated inorganic or organic salt is utilized as the salt-out agent to separate the aqueous phase from the organic phase after protein precipitation with an organic solvent. The commonly used salts include; ammonium sulfate, zinc sulfate, sodium chloride and potassium carbonate. However, some of these salts are detrimental to the lifetime of the column and MS. Therefore, salts that are more compatible with MS \textit{i.e.}, ammonium acetate, ammonium formate, and saturated ammonium bicarbonate can be used.

In liquid-liquid extraction (LLE) the compounds are extracted based on solubility in two different immiscible liquids \textit{i.e.} water and organic solvent such as those in TABLE III. Since most biological matrices are in aqueous solution, the organic solvents with a density less than that of water will settle at the top and can be removed easily. Also, depending on the polarity of the analyte and that of the solvent, analytes can be effectively extracted from one solvent into another by vortex-mixing, then centrifuged and the organic layer is removed, evaporated and the residue reconstituted for analysis. The recovery and selectivity of LLE for the analyte is not only dependant on the solubility of the analyte but also the pKa, the solution pH and ionic strength.
<table>
<thead>
<tr>
<th>Solvent</th>
<th>Density (g mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>0.685</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>0.713</td>
</tr>
<tr>
<td>Methyl-tert butyl ether</td>
<td>0.740</td>
</tr>
<tr>
<td>Methylethyl Ketone</td>
<td>0.805</td>
</tr>
<tr>
<td>N-butanol</td>
<td>0.810</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>0.897</td>
</tr>
<tr>
<td>Methylene Chloride</td>
<td>1.33</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.48</td>
</tr>
</tbody>
</table>
By manipulating these factors LLE can provide clean extracts with high recoveries.

Solid phase extraction (SPE) is based on the affinity of solutes in mobile phase for the stationary phase, similar to LC. The analytes are retained on the stationary phase, packed in a column/cartridge. There are various forms of stationary phases or sorbents used, some include: silica, polymeric based (C8, C18), cation and anion exchange, as well as mixed –modes. SPE is performed in various steps shown in Figure 16. First, there is a conditioning step to activate (wet) the sorbent, which is normally done using an organic solvent. Then, the sorbent is equilibrated with the sample solvent or loading buffer prior to the analyte sample being added on to the sorbent. Once the sample is loaded, the analyte interacts with the sorbents upon contact and it’s retained; any other impurities can be washed off. Finally, the analyte is eluted off the sorbent with a stronger elution solvent that can dissociate the analyte-sorbent interactions.

1.3.1 Matrix effect

When LC-MS/MS is used for quantitation of drugs in highly complex matrices (i.e., biological fluids, or plants samples), the presence of interfering components in these matrices co-elute with analyte of interest and hinder ionization [54]. This leads to a less volatile solution, which affects the efficiency of droplet formation and the evaporation process. Consequently, it alters the amount of charge (ionization) reaching the detector, such that the signal is either suppressed or enhanced. Matrix effect has been known as the “achilles heel” (a fatal weakness in spite of overall strength results) of MS quantitation.
Figure 16: SPE procedure
Matrix effect can seriously affect the LC-MS/MS analytical assay: it could result in an increased background noise, random variation in the signal and decreased sensitivity, limiting the accuracy and precision of the method [55]. Matrix effect can occurs as a result of poor sample clean-up, presence of endogenous lipids, use of ion-pairing additives *i.e.*, triethylamine (TEA), or presence of salts in the sample etc. Therefore, the use of column switching valve could divert the salts into waste and decrease the contamination of the MS. However, to minimize matrix effect, an appropriate sample preparation method should be developed. For example LLE has been found to provide cleaner samples than PPT. Also proper optimization of the separation conditions and choice of ionization technique can help minimize or eliminate this problem [54].

Matrix effect is evaluated in two ways (Figure 17). First, by post-column infusion whereby, the analyte is infused into the MS with a syringe pump, at the same time, a blank matrix is injected on to the column and eluted by the mobile phase. Normally, a steady response should be observed on the mass chromatograms; however, in the case of ion suppression or enhancement the signal will decrease or increase respectively, at a certain point on the chromatograms. This is representative of the time at which the inferences elutes off the column. This method is qualitative and would require each analyte to be infused individually each time, so it’s not ideal [54].

The other method used is post-extraction addition where by the analyte is spiked at a certain concentration into the extracted blank matrix and the signal response compared with the corresponding concentration in a neat solution (equation 1.1).
The matrix effect is assessed as matrix factor (MF) where a value of 1 indicates no matrix effect is observed whereas <1 indicates ion suppression and >1 is ion enhancement.

\[
MF_{analyte} = \frac{\text{Peak Area of Analyte in Extracted Matrix}}{\text{Peak Area of Analyte in Solvent}}
\]  

(1.1)

Selection of an appropriate internal standard (IS), is important since it can be used to correct not only variability in sample extraction, LC injections but also MS ionization and hence matrix effect. Ideally, when analyzing complex biological matrices a stable isotope labeled IS would be appropriate because it has similar physicochemical properties to the analyte and would behave in a close manner or identical to the analyte in all stages of the analytical assay (i.e. extraction, injection, ionization etc). However, heavy isotopes are not readily available for some compounds, which would require custom synthesis and it’s relatively expensive. As an alternative, a structural analog of the compound (i.e., one that differs by one functional group) can be used, despite the fact that they could be less representative of the analyte of interest and differ in the retention time and ionization properties compared to analyte of interest.

When the IS is used, matrix effect of the IS is also calculated using equation 1.2 and the normalize MF reported (equation 1.3).

\[
MF_{IS} = \frac{\text{Peak Area of IS in Extracted Matrix}}{\text{Peak Area of IS in Solvent}}
\]  

(1.2)

\[
\text{IS Normalized MF or } MF_{\text{normalized}} = \left( \frac{MF_{\text{analyte}}}{MF_{IS}} \right)
\]  

(1.3)
A) Post Column infusion

B) Post-extraction addition

Figure 17: Method used to assess matrix effect [54]
1.4 Method validation

The U.S Food and Drug administration (FDA) requires bioanalytical methods developed for quantitation of small molecules be validated before they are implemented into preclinical and clinical trials. The FDA guidances are standard procedures that demonstrate that the analytical method developed is reliable and reproducible for analysis of small molecules in biological matrices. The parameters required for validation include selectivity, sensitivity, linearity of the calibration curve, matrix effect, recovery of the assay, accuracy, precision, and stability studies [56-58].

Below is summarized protocol of analytical method validation used in our laboratory [59]

1. **Selectivity** is the ability to measure and differentiate analytes of interest in presence of interfering components.
   a. 6 sources of blank matrix samples are used and the mass chromatograms at \(m/z\) of analyte and \(m/z\) of IS is recorded (5 replicates for each sample).
   b. If interference is present in matrix blank at the same retention time (tR) and the same \(m/z\) of analyte and IS, their mean peak area should be \(\leq 20\%\) of the mean peak area of the analyte at lower limit of quantitation (LLOQ), as well as, that of the IS.

2. **Sensitivity** (correctly should be LLOQ), which is the lowest amount that can be quantified with an acceptable precision and accuracy.
c. LLOQ should be determined by analyzing at least 5 replicates of the sample at the LLOQ concentration on at least one of the validation days. Intra-run precision (%CV) and accuracy (%RE) should be ≤± 20%.

d. Also, the analyte with its IS are determined at the LLOQ in 6 sources of blank matrix and the mass chromatograms at \( m/z \) of analyte and \( m/z \) of IS (5 replicates for each sample) obtained. Using a calibration curve, the accuracy and precision can be obtained at the LLOQ. The %CV and %RE should be ≤± 20%.

2. **Calibration standards**
   
a. Matrix blank: matrix sample without IS
   
b. Zero standard: matrix sample with IS
   
c. 6 to 8 non-zero standards
   
d. The acceptance criteria (at least 75% of standards should meet the criteria):
      
      LLOQ standard ≤ 20% and all other standards ≤ 15%

3. **Upper limit of quantitation (ULOQ):** Although not required, the accuracy and precision may be determined in a similar manner as LLOQ but %CV and %RE should be ≤ 15%.

4. **Validation batches:** Analyzed at least 3 batches (i.e., calibration curves) for accuracy (%RE) and precision (%CV).

5. **QC samples**
a. QCs are added in multiples of three concentrations (low, mid, high) with 6 or a minimum of 5% of the total number of unknown samples in each analytical batch.

b. At least 67% (4 out of 6) of the QC samples should meet the acceptance criteria (LLOQ ≤ 20%, all others ≤ 15%); 33% of the QC samples (not all replicates at the same concentration) can be outside the acceptance criteria.

c. If there are more than 2 QC samples at a concentration, then 50% of QC samples at each concentration should meet the acceptance criteria.

6. **Preparation of calibration standards and QC samples:** Calibration standards and QC samples can be prepared from the same spiking stock solution if the solution stability and accuracy have been verified. A single source of matrix may also be used if selectivity has been verified.

7. **Positional differences:** During validation of the method, calibration standards and quality control samples are analyzed in a predefined order as follows:

a. One set of calibration standards is placed at the beginning of the run (front curve).

b. Then quality controls are analyzed and any other validation samples in the middle of the run, distributed randomly or placed in such a sequence as to help positional differences.

c. One set of calibration standards is analyzed at the end of the run (back curve).

d. A blank matrix sample or zero standard is placed after the high concentration sample (ULOQ) to assess carryover.
8. **Placement of samples:** Calibration curve samples, blanks, QCs, and study samples can be arranged as considered appropriate within the run, and support detection of assay drift over the run.

9. **Recovery**

   a. The extent of recovery of analyte and IS should be consistent, precise, and reproducible.

   b. Spiking method (can be used only for off-line extraction)

      i. Samples include a) Blank matrix + Analyte and IS

      b) Extracted blank matrix + Analyte and IS.

      ii. 5 replicates of QC samples at 3 concentration levels (low, mid, high) are determined.

      iii. A blank matrix sample or zero standard is placed after the high concentration sample (ULOQ) to assess *carryover*.

      iv. Peak areas (PA) of analyte and IS are obtained and recovery calculated using equation 1.4 -1.6.

\[
\text{Recovery}_{\text{analyte}} = \left[ \frac{\text{PA of Analyte in Matrix}}{\text{PA of Analyte in Extracted Matrix}} \right] \times 100\% \tag{1.4}
\]

\[
\text{Recovery}_{\text{IS}} = \left[ \frac{\text{PA of IS in Matrix}}{\text{PA of IS in Extracted Matrix}} \right] \times 100\% \tag{1.5}
\]
10. **Matrix effect**

a. An absolute MF or IS-normalized MF of about 1 is not necessary for a reliable bioanalytical assay. However, highly variable MF in individual subjects would be a cause for the lack of reproducibility of analysis. If IS is a heavy isotope, IS-normalized MF should be closed to 1.

b. The absolute MF (or IS-normalized MF) for 6 individual lots of matrix is determine and %CV should be less than 15%. If stable isotope IS is used, it is not necessary to determine the IS-normalized MF in 6 different lots.

c. Spiking method (can be used only for off-line extraction)
   
i. **Samples:**
   
   a) Extracted blank matrix + Analyte and IS
   
   b) Solvent + Analyte and IS.

   ii. 5 replicates of QC samples at 3 concentration levels (low, mid, high) are determined.

   iii. Peak area (PA) of analyte and IS are obtained

   iv. The MF is calculated using equation 1.1 - 1.3

11. **Accuracy and precision**
a. QC samples at a minimum of 3 concentrations (low, mid, and high) plus dilution QC.

b. **Low QC**: near the LLOQ (up to 3 x LLOQ).

c. **Mid QC**: middle of the range (at about the geometric mean of low and high QC concentration)

d. **High QC**: near the high end of the range, about 70% to 85% of ULOQ.

e. **Dilution QC**: sufficient to cover highest anticipated dilution.

f. **Intra-run accuracy and precision** at least 5 replicates at each concentration, the mean, SD, %CV, and %RE are determined (%CV and %RE ≤ 15%).

g. **Inter-run accuracy and precision** at least 5 parallels at each concentration, the mean, SD, %CV, and %RE are determined (%CV and %RE ≤ 15%).

12. **Stability**

a. **Stock solution**: minimum of 6 h at room temperature.

b. **Post-preparative (extracted samples/autosampler tray)**: Longest time from preparation through sample analysis. Assessed against fresh standards, except for autosampler re-injection reproducibility.

c. **Benchtop**: at ambient temperature (or temperature used for processing of samples) to cover the duration of time taken to extract the samples (typically ca. 4-24 h).
d. **Freeze-thaw:** QC samples at minimum of 2 concentrations (low, high), 3 cycles, completely thawed, refrozen at least 12 h between cycles, at anticipated temperature of sample storage.

e. **Long-term:** Covers longest time from collection to final analysis for any sample in study. 3 aliquots at low and high concentrations are analyzed with fresh standard curves and compare against intended (nominal) concentrations. Long-term stability can be completed post validation.

13. **Replicate analysis**

   a. In general, samples can be analyzed with a single determination without replicate analysis if the chromatographic assay method has acceptable variability as defined by the validation data.

   b. Duplicate or replicate analysis can be performed for a difficult procedure where high precision and accuracy may be difficult to obtain.

14. **Multiple analytes in a run:** Samples involving multiple analytes in a run should not be rejected based on the data from one analyte failing the acceptance criteria.

15. **Rejected run:** The data from rejected runs need not be documented, but the fact that a run was rejected and the reason for failure should be reported.

1.5 **LC-MS/MS method development workflow**
To develop a quantitative LC-MS/MS method, the following work flow could be utilized (Figure 18). First, the mass spectrometer (MS) is tuned by infusion or LC flow injection of the analytes of interest (i.e. drug compound or metabolite) and internal standard (IS) in the appropriate mobile phase to establish the detection parameters. The molecular ion spectrum is obtained to identify the compounds and then to enhance the specificity of the MS, multiple-reaction-monitoring (MRM) is used where by, the compounds undergo fragmentation to obtain the product ion spectra. Then, specific mass transitions i.e., parent/ product ion pairs are selected for quantitation of the analyte and IS.

There after, the LC method is developed by selecting the best conditions i.e., the mobile phase, column, flow rate, pH, etc, that provide sufficient separation and baseline resolution of the analyte from interfering components in the sample matrix. It’s important that the peak shapes are symmetrical and the retention times reproducible for accurate peak integration.

After the LC-MS/MS method has been established, a sample preparation method is developed to extract the analyte and IS from complex matrices (i.e., plant, plasma, urine etc.). The effectiveness of the sample preparation method to extract the analytes and minimize or eliminate the interferences in the sample matrices is evaluated by preliminary recovery and matrix effect studies. If the sample extraction method is not satisfactory other sample preparation techniques are tested as well as, the LC-MS/MS method is redeveloped or fine-tuned to provide maximum sensitivity and selectivity.

Last but not least the optimized method is validated according to the FDA guidance and once all the criteria’s are met the method is applied to real samples.
Figure 18: LC-MS/MS method work flow

1. Method Development
   - Optimize Mass Spectrometry (MS) (MRM transitions and detection parameters)
   - Optimize Liquid Chromatography (LC) (separation conditions)
   - Develop sample preparation method (extract analyte from complex matrices)
   - Evaluate LC-MS/MS method (preliminary recovery and matrix effect)

2. Method Validation (FDA guidance)

3. Method Application (animal study or patient samples)
1.6 Conclusion

This chapter introduces the importance of quantitative methods of analysis in the early stages of drug discovery and development, as well as, biomonitoring studies. Its focus is on development of LC-MS/MS methods for plant derived anticancer agents and synthetic estrogen. The capabilities of LC-MS/MS as the superior method of choice have been discussed as well as, sample preparation techniques that would be appropriate when analyzing complex matrices. An overview of stable isotope dilution (SID)- LC-MS/MS has been presented and the FDA requirements of newly developed LC-MS/MS method have been discussed in detail. Finally, a general work flow of LC-MS/MS method development has been summarized at end of the chapter.

1.7 References


[55] Bakhtiar R, Majumdar TK. Tracking problems and possible solutions in the quantitative determination of small molecule drugs and metabolites in biological fluids


CHAPTER II

QUANTITATIVE DETERMINATION OF (-)-SECURININE, A POTENTIAL ANTICANCER AGENT IN SECURINEGA SUFFRITICOSA PLANT BY LC-MS/MS

2.1 Introduction

Securinega suffruticosa (Figure 1) is a sub-tropical semi-shrub of the Euphorbiaceae subfamily that is widely distributed in north eastern parts of Asia [1]. It has mostly been used in traditional Chinese folk medicine as diuretics, antipyretics and for the treatment of hepatic disorders, the after-effects of infantile paralysis and against skin eruptions [2]. The main active constituent of this plant is securinine, which is the major alkaloid in the plant’s leaves and the minor alkaloid in the plant’s roots [3]. Securinine was first isolated from its natural source in 1956 and its structure (Figure 2) and absolute configuration were established by spectroscopic methods in 1962 then later confirmed 1965 by X-ray crystallography [4-6]. Securinine’s stereoisomers i.e., its
Figure 1: *Securinega suffruticosa* plant. (A) Mature leaves (B) Young leaves (C) Roots, (D) Stems (E) Bark and (F) Branches
Figure 2: Chemical structure of (-)securinine

(-)-Securinine (SE)
MW = 217.3

Figure 2: Chemical structure of (-)securinine
epimer at C-2; allosecurinine [7] and its enatiomers; virosecurinine [8] and viroallosecurinine [9] have also been isolated from natural plant sources and characterized [4-9].

(-)-Securine (SE) has been reported to have a wide range of pharmacological activities. It has been mainly used for the treatment of non-cancer related diseases such as poliomyelitis, aplastic anemia, multiple sclerosis [10, 11] and diseases related to the central nervous system (CNS) acting as a GABA receptor antagonist [12, 13]. Recently SE has been found to be a macrophage activator [14], and an inhibitor of parasitic (*Toxoplasma gondii*) proliferation [15], which means it could potentially be used in the treatment of infectious diseases. SE has also been found to have anticancer properties *i.e.*, induce apoptosis in human leukemia cells (HL-60) [16]; SW480 [17] and in p53-deficient colon cancer cells [18]; and acts as a myeloid differentiation inducing agent in acute myeloid leukemia with increased efficacy and low toxicity [19].

A rapid and reliable analytical method is required to ensure quality, efficacy and safety of SE herbal products. To support the pharmaceutical development and toxicological studies of SE, a sensitive and reliable analytical method is needed for quantitative analysis of the drug in *Securinega suffruticosa* plant. Based on a recent literature search, several analytical methods for SE determination in raw plant material have been described [20]. A majority of this methods are based on old and antiquated conventional methods *i.e.*, colorimetric [21], polarimetric [22], hydroxylamine-sulfanilic acid [23] and titrimetry [20]. The current analytical methods *i.e.*, HPLC-UV [24, 25], CE-UV [26] and GC-MS [27] are mainly used for analysis of biological samples and are limited in terms of selectivity for the determination of SE in raw plant material.
In this work, an LC-MS/MS method for quantitative determination of SE in *Securinega suffruticosa* has been developed. A liquid-liquid extraction (LLE) procedure using ethyl acetate was developed for sample preparation. Isocratic separation of SE was achieved on a Gemini®-Nx C\textsubscript{18} column using a 38% acetonitrile and 62% 20.0 mM ammonium acetate as the mobile phase. Quantification was carried out by positive electrospray tandem mass spectrometry (ESI\textsuperscript{+}–MS/MS) in multiple-reaction monitoring mode (MRM). The LC-MS/MS method developed has been validated and was applied to the quantitative analysis of the distribution and levels of SE in different parts of *S. suffruticosa* plant (i.e., leaves, roots, stems, bark and branches).

2.2 Experimental

2.2.1 Chemicals and solutions

(-)-Securinine or SE (C\textsubscript{13}H\textsubscript{15}NO\textsubscript{2}, CAS Registry Number: 5610-40-2) was obtained from LKT Laboratories (St. Paul, MN, USA). Ammonium acetate (NH\textsubscript{4}Ac), dimethyl sulfoxide (DMSO), ethyl acetate and ammonium hydroxide (NH\textsubscript{4}OH, 28-30 % wt NH\textsubscript{3}), were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade acetonitrile was from Pharmco-AAPER (Shelbyville, KY, USA). Deionized water was obtained from a Barnstead NANOpure\textsuperscript{TM} water purification system (Thermo Scientific, Waltham, MA, USA) with the resistance reading of 18.2 MΩ.
The stock standard solution of SE (18.0 mg mL\(^{-1}\)) was prepared by dissolving proper amounts of accurately weighed compound in a known volume of DMSO. The aliquots (20.0 μL) of the stock solutions were stored at -80°C prior to their use. SE working standard solutions (0.00, 10.0, 20.0, 30.0, 100, 200, 300, 1.00×10\(^3\), 2.00×10\(^3\), 3.00×10\(^3\), 1.00×10\(^4\) and 6.00×10\(^4\) ng mL\(^{-1}\)) were freshly prepared daily by serial dilution of the stock standard solution of SE with DMSO.

An ammonium acetate solution (0.100 M) was prepared by dissolving appropriate amounts of the buffer salt in a known volume of water. 20.0 mM ammonium acetate (pH 6.8) was prepared by a 1/10 dilution of the 0.100 M solution with water. The mobile phase for chromatographic separation was prepared by mixing 38% acetonitrile and 62% 20.0 mM ammonium acetate, pH 6.8.

### 2.2.2 Preparation of standard calibrators and quality controls (QCs)

SE standard calibrators (0.00, 0.500, 1.00, 5.00, 10.0, 50.0, 100 and 500 ng mL\(^{-1}\)), QCs (1.50, 15.0, 150 ng mL\(^{-1}\)) and dilution QCs (3.00 x 10\(^3\) ng mL\(^{-1}\)) were prepared by diluting every 50.00 μL of the aforementioned SE working standard solutions with 950.0 μL of the mobile phase in 1.50 mL microcentrifuge tubes (VWR, Radnor, PA, USA). The SE dilution QC samples (3.00 x 10\(^3\) ng mL\(^{-1}\), 10 μL) were diluted by a factor of 100 with 990 μL of mobile phase prior to sample analysis. The samples were vortex mixed using a VWR® Analog Multi-Tube Vortexer (VWR, Radnor, PA, USA) and transferred into auto-sampler vials for analysis by LC-MS/MS.
2.2.3 Plant material and the preparation of extracts

*Securinega suffruticosa* plant (Figure 1) was purchased from Glass Works (Stewart, OH) and grown in our laboratory at Cleveland State University. The leaves of *S. suffruticosa* were harvested, dried in a desiccator at room temperature in the dark and then finely powdered. Plant leaves were prepared by liquid-liquid extraction (LLE) as follows; approximately 20.0 mg powdered plant samples were placed into borosilicate glass tubes (16 mm x 125 mm, Fisher Scientific), and then basified with 1.00 mL of 25% NH$_4$OH and vortex-mixed for 1 min. The mixture was extracted three times with 5.00 mL of ethyl acetate each time. Following each extraction, the sample mixture was vortexed for 3 min and subsequently centrifuged for 5 min at 3800 × g (4 °C). The supernatant from each mixture was transferred into a clean borosilicate glass tube and then all the organic extracts were combined. 10 µL aliquots of the organic extract were diluted 100 fold with the mobile and vortex-mixed prior to LC–MS/MS.

2.2.4 Instrumentation

The liquid chromatography tandem mass spectrometry system consisted of a Shimadzu Prominence UFLC system (Shimadzu, Columbia, MD, USA) with a system controller (CBM-20A), two binary pumps (LC-20AD), an online degasser (DGU20A3), and an AB Sciex API QTrap 3200 mass spectrometer (AB Sciex, Foster City, CA, USA) with a Turbo IonSpray ESI source. The mass spectrometer was connected to the LC-system outlet via high-pressure polyether ether ketone (PEEK) tubing (0.0625 in. o.d, x
The API QTrap 3200 mass spectrometer was operated in positive electrospray ionization (ESI⁺) mode. It was tuned by flow injection analysis of 1.00 x 10³ ng mL⁻¹ SE in the mobile phase for both the compound-dependent and the source-dependent parameters. The optimized detection parameters were as follows: curtain (CUR), 20 psi; collision-activated dissociation (CAD) gas, 5; nebulizer gas (GS1), 30 psi; turbo heater gas (GS2), 45 psi; Turbo IonSpray voltage (IS), 5500V; source temperature (TEM), 650°C. High purity nitrogen (99.99%) was used as the nebulizer, auxiliary, collision and curtain gases. The declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP), were set at 55, 5.0, 42 and 2.0 V respectively for SE. The multiple-reaction-monitoring (MRM) method was used for quantification of SE using mass transitions m/z 218.2 → 84.1. The dwell time for each transition was 805 ms and the mass resolutions (Q1 and Q3) were set to unit.

Analytical separation of SE was performed isocratically under ambient temperature on a reverse-phase Gemini Nx C₁₈ (2.1 mm i.d x 150 mm, 5 μm, 110 Å) column (Phenomenex, Torrance, CA, USA) with an inert stainless steel in-line filter (0.5 μm pore, 0.23-μL dead volume) from Upchurch Scientific (Oak Harbor, WA, USA) using the mobile phase at a flow rate of 0.200 mL min⁻¹. The injection volume of each
sample was 10.0 µL. Prior to sample analysis, the column was first equilibrated with the mobile phase using 20 times of the column volume.

2.2.6 Data acquisition and analysis

Data acquisition and peak integration were done using the AB Sciex Analyst® software (version 1.5.1) with IntelliQuan-MQII algorithm. The averaged peak area ratios of SE were plotted against the SE concentrations in standard calibrators for a linear regression equation using a weighting factor of $1/x$ (where $x$ is the nominal concentration of the standard calibrator). The SE alkaloid in *S. suffruticosa* plant was identified by comparison of the retention time (tR) and the MRM spectra, with those of the SE standard calibrators. The SE concentrations in the plant samples were back calculated with the peak area of SE, using the calibration equation and with dilution factor.

2.2.7 Recovery

The recovery studies were performed as follows; three different portions of *S.suffruticosa* plant leaves were first extracted using the LLE protocol described in section 2.2.3 to generate blank plant matrices. The basic aqueous layer (*i.e.*, blank plant matrix) was obtained from the LLE extract and an aliquot (25.0 µL) of each SE standard solutions at low-, mid- and high concentrations ($9.00 \times 10^4$ ng mL$^{-1}$, $9.00 \times 10^5$ ng mL$^{-1}$,
9.00 \times 10^6 \text{ ng mL}^{-1} \text{ mg mL}^{-1}) were spiked into the three blank plant matrices. Samples were vortex-mixed for 1 min and then extracted three times with 5.00 mL of ethyl acetate each time, then centrifuged for 5 min at 3800 \times g (4 ^\circ \text{C}) as described in section 2.2.3. Aliquots (10 \mu L) of the organic extract (supernatant) were diluted 100 fold with the mobile, vortexed and analyzed by LC–MS/MS. The recoveries were determined by comparing the peak areas of SE in the standard calibrators with those of the corresponding samples prepared by spiking SE to the blank plant matrix before LLE.

\subsection*{2.2.8 Application of the method}

The feasibility of the LC-MS/MS method developed was evaluated by the analysis of the distribution and content of SE in different parts of S.suffruticosa plant. The plant leaves (young and mature leaves), stems, roots, bark and branches were harvested and dried in a desiccator at room temperature in the dark. Prior to analysis, the plant samples were finely powdered and prepared by the LLE procedure described, then analyzed (n=5) by the LC-MS/MS method.

\subsection*{2.3 Results and discussion}
2.3.1 Mass spectrometric detection

Based on the chemical structure (Figure 2), SE readily acquires a proton than lose one in electrospray ionization. Hence the positive electrospray ionization mode was used in this work. As shown in Figure 3A (full scan spectrum), SE produced a protonated molecular ion [M+H]⁺ at m/z 218.2. Upon fragmentation by collision with ultra-high purity helium gas in the mass spectrometer, this molecular ion produced a major fragment at m/z 84.1 (Figure 3B). Therefore, the mass transitions m/z 218.2 → 84.1 was chosen for quantification of SE in multiple-reaction-monitoring (MRM) mode.

2.3.2 Chromatographic separation

In this work, the Waters Xterra® RP-18 (2.1 mm x 50mm, 3.5μm) and the Phenomnex Gemini Nx C₁₈ (2.1 mm i.d x 150mm, 5μm,) were tested for optimal separation of SE in S.suffruticosa plant. Both columns were suitable for the separation of SE; however, Gemini Nx C₁₈ was chosen for subsequent study because it gave higher signal response for SE.

The composition of the mobile phase used for separation was also optimized to improve LC separation and enhance MS sensitivity. Acetonitrile was selected as the organic modifier due to the greater solvent strength, and higher signal response in mass spectrometry than methanol; and ammonium acetate was used as the buffer salt because it found to suppress the formation of sodium ion adducts and resulted in greater
Figure 3: The full scan mass spectra of SE and its major product ions.
detection signal than ammonium formate. Working at this pH, SE (p<sub>ka</sub> = 8.29 ±0.20) was protonated and could be retained on the column; however, some peak tailing was observed. Therefore, to obtain symmetrical peak shapes, the ionic strength of the buffer was optimized. 5mM, 10mM and 20mM ammonium acetate were evaluated and 20mM was selected because it resulted in a greater improvement in the peak shape, reduced peak tailing and did not compromise the mass spectrometry detection.

For this work, the optimized mobile phase composition consisted of 38 % ACN and 62% 20 mM ammonium acetate, pH 6.8. Using this mobile phase, separation of SE was achieved on the Gemini Nx C<sub>18</sub> with retention time of 5.1 min (Figure 4).

### 2.3.3 Extraction of SE from <i>S.suffruticosa</i> leaves

A liquid-liquid extraction (LLE) method was developed for sample clean up prior to analysis with LC-MS/MS. SE being a weak acid in solution it was necessary to make it neutral for easy extraction into the organic phase. In this method the plant samples were rendered alkaline by adding a base. The alkalinization buffer and pH was found to affect the extraction of SE from the <i>S.suffruticosa</i> plant and therefore was optimized. Saturated ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>) pH 10.2, 0.05% and 25% NH<sub>4</sub>OH were evaluated and the latter base was used in this work, because it enabled efficient extraction of SE from <i>S.suffruticosa</i> plant samples.
Figure 4: The mass chromatograms of SE standards (A) double blank (no SE detected) (B) lowest standard calibrator (LLOQ, 0.500 ng mL\(^{-1}\)) (C) highest standard calibrator (ULOQ, 500 ng mL\(^{-1}\)).
Various organic solvents such as dichloromethane (DCM), chloroform, methyl tert-butyl ether (MTBE) and ethyl acetate (EAC) were tested for use in the LLE method. DCM and chloroform were prone to form emulsions during the extraction process and resulted in poor or incomplete analyte extraction. The extraction recovery of MTBE was found to be lower than all the other solvents. LLE performed using EAC with 25 % NH₄OH resulted in high extraction efficiencies and therefore was chosen for this work.

Finally, the effect of extraction times was investigated by running four consecutive extractions on the same sample. To evaluate the repeatability of the extraction procedure, two replicates were performed. As shown in TABLE I, three extraction cycles were sufficient to completely extract SE from *S.suffruticosa* plant, therefore, in this work, plant samples were extracted three times with ethyl acetate in the LLE method developed.

### 2.3.4 Method validation

The method developed was validated in terms of linear response, lower limit of quantitation (LLOQ), recovery, accuracy and precision.
TABLE I: THE EFFECT OF EXTRACTION TIMES (N=3)

<table>
<thead>
<tr>
<th>Extraction time</th>
<th>Sample 1 Measured [SE] ± SD (ng mL⁻¹)</th>
<th>Sample 2 Measured [SE] ± SD (ng mL⁻¹)</th>
<th>Average [SE] ± SD (ng mL⁻¹)</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>237 ± 1</td>
<td>240 ± 2</td>
<td>239 ± 2</td>
<td>90.2</td>
</tr>
<tr>
<td>2</td>
<td>22.8 ± 0.1</td>
<td>24.3 ± 0.2</td>
<td>23.6 ± 1.1</td>
<td>8.92</td>
</tr>
<tr>
<td>3</td>
<td>2.20 ± 0.10</td>
<td>2.24 ± 0.15</td>
<td>2.22 ± 0.03</td>
<td>0.840</td>
</tr>
<tr>
<td>4</td>
<td>0.242 ± 0.035</td>
<td>0.222 ± 0.026</td>
<td>0.232 ± 0.014</td>
<td>0.051</td>
</tr>
</tbody>
</table>
2.3.4.1 Linearity and lower limit of quantitation (LLOQ)

The calibration curves for SE were constructed using seven non-zero standard calibrators (*i.e.*, 0.500, 1.00, 5.00, 10.0, 50.0, 100 and 500 ng mL\(^{-1}\)), and a double-blank (without SE). The linear calibration range of 0.500-500 ng mL\(^{-1}\) was established by plotting the mean peak areas of SE against the nominal concentration of SE in the mobile phase. The calibration equation derived from five validation batches of calibrators using a 1/x weighted least-square linear regression was \(Y=1946(\pm32) + 225(\pm64), r^2= 0.9997 \) (±0.0002). The accuracy and precision of the standard calibrators were \(\leq \pm 16\) and \(\leq 7\) respectively (TABLE II).

In this work, the LLOQ of the method was defined by the lowest concentration on the calibration curve (0.500 ng mL\(^{-1}\)) with a limit of accuracy and precision within \(\pm 20\%\) and 20\% respectively. It was confirmed by analyzing six replicate measurement of SE at LLOQ, independent of the calibration standards. The mean accuracy and mean precision of the method at LLOQ were acceptable with \(\leq \pm 2\%\) of the relative error (RE) and \(\leq 8\%\) of the coefficient of variation (CV) (data not shown).
TABLE II: Accuracy and precision of SE calibrators (five validation batches).

<table>
<thead>
<tr>
<th>Nominal [SE] (ng mL(^{-1}))</th>
<th>Measured [SE] (ng mL(^{-1}))</th>
<th>SD (ng mL(^{-1}))</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.500</td>
<td>0.422</td>
<td>0.036</td>
<td>-16</td>
<td>7</td>
</tr>
<tr>
<td>1.00</td>
<td>1.0</td>
<td>0.1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>5.00</td>
<td>5.0</td>
<td>0.2</td>
<td>0.3</td>
<td>4</td>
</tr>
<tr>
<td>10.0</td>
<td>10.9</td>
<td>0.4</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>50.0</td>
<td>50.2</td>
<td>2</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>105</td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>500</td>
<td>494</td>
<td>6</td>
<td>-1</td>
<td>1</td>
</tr>
</tbody>
</table>

\(\text{%RE} = \left(\frac{\text{measured} - \text{nominal}}{\text{nominal}}\right) \times 100\%; \text{%CV} = \left(\frac{\text{SD}}{\text{mean}}\right) \times 100\%\).
2.3.4.2 Recovery

The absolute recovery of the method was determined in low-, mid- and high- QC controls (1.50, 15.0, 150 ng mL\(^{-1}\)) by comparing the mean peak areas of SE in the corresponding QC standard samples (n=5) with those of SE in the spiked in blank plant matrix before LLE. TABLE III shows the mean absolute recoveries for SE were consistent and ranged from 100-106%.

2.3.4.3 Accuracy and precision

The intra-run accuracy and precision were determined by five replicate measurements of each QCs (1.50, 15.0, 150 ng mL\(^{-1}\)) and dilution QCs (3.00 x 10\(^3\) ng mL\(^{-1}\)) in the same validation batch. The inter-run accuracy and precision was determined by five parallel measurements of five identical sets of each QC samples in five different validation batches. The accuracy was expressed as percent relative error (%RE) and the precision expressed as coefficient variation (% CV). As shown in TABLE IV the intra-run accuracy and precisions of all the QCs samples were \(\leq \pm 3\%\) and \(\leq 3\%\) and the inter-run accuracy and precisions were \(\leq \pm 7\%\) and \(\leq 10\%\) respectively. These results indicate that this method has adequate reproducibility and accuracy.
### TABLE III: RECOVERY OF SE FROM *S. SUFFRUTICOSA* PLANT MATRIX (N = 5)

<table>
<thead>
<tr>
<th>Nominal [SE] (ng mL(^{-1}))</th>
<th>PA(_{SE}) in extracted matrix ± SD (x10^4)</th>
<th>PA(_{SE}) in mobile phase ± SD (x10^4)</th>
<th>Recovery(_{SE}) ± SD (%) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>0.317± 0.01</td>
<td>0.316± 0.006</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>15.0</td>
<td>3.06 ± 0.06</td>
<td>2.94 ± 0.02</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>150</td>
<td>31.8 ± 0.3</td>
<td>29.9 ± 0.2</td>
<td>106 ± 1</td>
</tr>
</tbody>
</table>

\(^a\)Recovery of SE = (PA of SE in extracted plant matrix / PA of SE in mobile phase) \times 100%

PA = Mean peak area.
TABLE IV: INTRA- AND INTER-RUN ACCURACY AND PRECISION

<table>
<thead>
<tr>
<th>Nominal [SE] (ng mL⁻¹)</th>
<th>Measured [SE] (ng mL⁻¹)</th>
<th>SD (ng mL⁻¹)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>1.5</td>
<td>0.1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>15.0</td>
<td>15.1</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>150</td>
<td>146</td>
<td>1</td>
<td>-3</td>
<td>1</td>
</tr>
<tr>
<td>3.00 x10⁻³</td>
<td>3.03 x10⁻³</td>
<td>7</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nominal [SE] (ng mL⁻¹)</th>
<th>Measured [SE] (ng mL⁻¹)</th>
<th>SD (ng mL⁻¹)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>1.6</td>
<td>0.1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>15.0</td>
<td>15.7</td>
<td>1.6</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>150</td>
<td>161</td>
<td>14</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>3.00 x10⁻³</td>
<td>3.16 x10⁻³</td>
<td>2.3 x10⁻²</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

a Determined by five replicate measurements of each QC sample within a validation batch.
b Determined by nine parallel measurements of three identical QC samples at each concentration over three validation batches.
c The dilution QC was measured by a 100 times dilution.
2.3.5 Method application

The developed LC-MS/MS method was applied to the quantitative analysis of the distribution and levels of SE in different parts of *S. suffruticosa* plant (*i.e.*, leaves, roots, stems, bark and branches) (Figure 1). The five plant samples were collected and extracted by the LLE procedure described. The diluted plant samples, along with standard calibrators, QCs and dilution QCs were analyzed (*n*=5) by the LC-MS/MS method. The resultant mass chromatograms are shown in Figure 5. SE was separated from the complex plant matrix and detected in *Securinega suffruticosa* plant extracts with a retention time (tR) of 5.1 min.

As shown in the chromatogram of the plant extract (Figure 5), an additional peak was observed at a tR of 2.9 min. The same product ions from MS/MS as SE and closely eluted retention times, suggest that it could be an epimeric isomer of SE. Several stereoisomeric alkaloids of SE have been isolated in the plants of *Securinega* species, and according Saito et.al [3], the leaves of *S. suffruticosa* contain SE as the major alkaloid and its epimer at C-2; allosecurinine (alloSE) as the minor alkaloid. Whereas, the roots of the plant contain alloSE as the major alkaloid and SE as the minor alkaloid [3]. We thus hypothesize that the peak observed at tR of 2.9 min with the mass transition *m/z* 218.2 → 84.1 is possibly alloSE.

Since the chemical and physiological properties of the epimeric pairs are similar, the SE standard calibration curve was used to quantify both SE and alloSE in
Figure 5: The mass chromatograms (A) Mature leaves (B) Young leaves (C) Roots, (D) Stems (E) Bark and (F) Branches.
S. suffruticosa plant. The concentrations of these analytes in the plant samples were back calculated with the peak areas of SE, using the calibration equation and with dilution factors. The results were reported as milligram (mg)/gram (g) dry plant material (TABLE V).

A great variation in the distribution of SE and alloSE in the different parts of *S. suffruticosa* was observed. The SE content in the plant decreases in the order of; mature leaves > young leaves > roots > stems > bark > branches and that of alloSE in the order; roots > stems > mature leaves > branches and not detected (ND) in the young leaves and bark. It was noted that the older the leaves were, the more they contained SE. The highest content of SE was found in plant leaves with a concentration of 7.44 ± 0.05 mg/g of dry plant material which corresponds to 0.744% (wt/wt) ± 0.005 whereas, the highest content of alloSE was found in the roots with a concentration of 8.43 ± 0.06 mg/g plant which corresponds to 0.843% (wt/wt) ± 0.006. These results are in good agreement with levels of SE in the *S. suffruticosa* plant reported in the literature of 0.3 - 0.90% [1].

### 2.4 Conclusions

An LC-MS/MS method for the quantitative determination of SE in *Securinega suffruticosa* plant has been developed and validated. In this work, SE alkaloid was isolate from its natural plant material by a liquid-liquid extraction protocol and then separated by a Gemini®-Nx C_{18} prior to detection by tandem MS/MS. This method is fast, highly
<table>
<thead>
<tr>
<th>Securinega suffruticosa</th>
<th>[SE] (mg g(^{-1}) plant)</th>
<th>[AlloSE] (mg g(^{-1}) plant)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature leaves</td>
<td>7.44 ± 0.05</td>
<td>0.139 ± 0.004</td>
</tr>
<tr>
<td>Young leaves</td>
<td>5.06 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>Roots</td>
<td>2.23 ± 0.01</td>
<td>8.43 ± 0.06</td>
</tr>
<tr>
<td>Stems</td>
<td>1.21 ± 0.01</td>
<td>1.67 ± 0.04</td>
</tr>
<tr>
<td>Bark</td>
<td>0.335 ± 0.005</td>
<td>ND</td>
</tr>
<tr>
<td>Branches</td>
<td>0.238 ± 0.004</td>
<td>0.115 ± 0.003</td>
</tr>
</tbody>
</table>

\(^a\) ND = not detected
selective, and offers good analyte recoveries, precision and accuracy. The method developed was applied to the quantitative analysis of the distribution and levels of SE in different parts of *S. suffruticosa* plant (*i.e.*, leaves, stems, roots, bark and branches) and can be useful as a routine quality control assay for the determination of SE and its stereoisomers in raw plant material and herbal products.

### 2.5 References


CHAPTER III

DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR QUANTITATIVE DETERMINATION OF (-)-SECURININE IN MOUSE PLASMA

3.1 Introduction

Securinine is the major alkaloid found in the plant leaves of Securinega suffruticosa, a sub-tropical semi-shrub that has been used in traditional Chinese folk medicine [1, 2]. (-)-Securinine (SE) has been reported to have a wide range of pharmacological activities such as antagonist of γ-GABA\textsubscript{A} receptor [3, 4], therapeutic agent for the treatment of sequela of poliomyelitis, amyotrophic lateral sclerosis (ALS) and aplastic anemia [5, 6], and macrophage activator against Coxiella burnetii [7], or an inhibitor of parasitic (Toxoplasma gondii) proliferation [8]. Furthermore, SE has shown
anticancer activity such as inducing apoptosis in various human cell lines including HL-60 [9]; SW480 [10]; p53-deficient colon cancer cells [11] and promote differentiation in several acute myeloid leukemia (AML) cells lines ((i.e., HL-60, THP-1 and OCI-AML3) and cells from primary leukemic patients [12]. Moreover, SE was found to synergize with other differentiation agents (i.e., all-trans retinoic acid, decitabine and 1, 25-dihydroxyvitamin D3) which are currently used for treatment of AML; hence it could be used to enhance the clinical activity and reduce the toxicity of these agents [12].

To support the preclinical and clinical studies of SE, a quantitative analytical method is needed for the measurement of the drug in biological sample. Based on a recent Sci-finder® scholar database search, the current analytical methods for SE analysis are mainly qualitative which include HPLC-UV [13, 14], and CE-UV [15]. The only quantitative assay reported is a GC-MS [16] method which lacks adequate sensitivity for biological samples and has not been validated.

In this work, an LC-MS/MS method for quantitative determination of SE in mouse plasma has been developed. (+)-Norsecurinine (norSE) was used as the internal standard (IS). A salting-out assisted liquid-liquid extraction (SALLE) procedure using 2.00 M ammonium acetate (volatile salt) was developed for sample preparation. Isocratic separation of SE and the IS was achieved on a reverse-phase C_{18} column using a 40% acetonitrile and 60% 10.0 mM ammonium acetate as the mobile phase. Quantification was carried out by positive electrospray tandem mass spectrometry (ESI^+–MS/MS) in multiple reaction monitoring mode (MRM). The LC-MS/MS method developed for SE analysis has been validated in mouse plasma according to the guidance for industry on bioanalytical method validation by the US Food and Drug Administration (US-FDA) [17]
and the subsequent 2006 white paper of bioanalytical method validation workshop [18,19], and applied to the measurement of SE in mouse plasma samples from an animal study.

3.2 Experimental

3.2.1 Chemicals and solutions

(-)-Securinine or SE (C_{13}H_{15}NO_{2}, CAS No. 5610-40-2) was obtained from LKT Laboratories (St. Paul, MN, USA). (+)-Norsecurinine or Norse (C_{12}H_{13}NO_{2}, CAS No. 25472-13-3) was from Ryan Scientific (Mount Pleasant, SC, USA). Ammonium acetate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was from Pharmco-AAPER (Shelbyville, KY, USA). Six pooled blank mouse plasma (Na citrated) with specified lot numbers (1H1453-02, 1R11-0823a, 1R11-0823b, 1H1453-01, 1R11-MS-Nac-0811 and 1R10-NSA-02) were purchased from Innovative Research (Novi, MI, USA). Deionized water was obtained from a Barnstead NANOpure™ water purification system (Thermo Scientific, Waltham, MA, USA) with the resistance reading of 18.2 MΩ.

Stock standard solutions of SE (2.00 mg mL^{-1}) and norSE (1.20 mg mL^{-1}) were prepared individually by dissolving proper amount of each accurately weighed compound
in a known volume of DMSO. Aliquots (20.0 µL) of the each stock solution were stored at -80°C prior to use. SE working standard solutions (12.0, 24.0, 36.0, 120, 240, 360, 1.20 x 10³, 2.40 x 10³, 1.08 x 10⁴, 1.20 x 10⁴ and 3.60 x 10⁴ ng mL⁻¹) were prepared daily by serial dilution of the stock standard solution of SE with DMSO. Working IS solution (1.20 x 10³ ng mL⁻¹) was prepared by three sequential dilutions (1/10) of the stock IS solution in DMSO.

Ammonium acetate solutions (0.100 M and 2.00 M) were prepared by dissolving appropriate amounts of the buffer salt in known volumes of water. 10.0 mM ammonium acetate (pH 6.8) was prepared by a 1/10 dilution of the 0.100 M solution with water. The chromatographic mobile phase consisting of 40% acetonitrile and 60% 10.0 mM ammonium acetate was prepared by mixing 200 mL of acetonitrile with 300 mL of 10.0 mM ammonium acetate.

### 3.2.2 Preparation of plasma calibrators and quality controls (QCs)

SE plasma calibrators (0.600, 1.20, 6.00, 12.0, 60.0, 120 and 600 ng mL⁻¹), QCs (1.80, 18.0, 540 ng mL⁻¹) and dilution QC (1.80 x 10³ ng mL⁻¹) were prepared by diluting every 50.0 µL of the aforementioned SE working standard solutions with 950 µL of pooled blank mouse plasma. SE plasma zero calibrator (0.000 ng mL⁻¹) was prepared by diluting 50.0 µL of DMSO with 950 µL of pooled blank mouse plasma. Aliquots (100.0 µL) of plasma calibrators, QCs and dilution QC were kept in 1.50 mL microcentrifuge tubes (VWR, Radnor, PA, USA) and stored at -80°C before use.)
3.2.3 Preparation of plasma samples

Plasma calibrators, QCs, dilution QC and the mouse plasma samples from animal study were thawed at room temperature. Prior to the sample extraction by SALLE, the mouse plasma samples and dilution QC were subjected to 10-time dilution using pooled blank mouse plasma, then 5.00 μL of the working IS solution \((1.20 \times 10^3 \text{ ng mL}^{-1})\) was added to each 100 μL of plasma sample except the double blank where 5.00 μL of DMSO was added. After vortex mixing, 600 μL of cold acetonitrile (-20 °C) and 100 μL of 2.00 M ammonium acetate were added sequentially to each plasma sample. The resultant mixture was vortexed for 3 s, and then centrifuged at 15,000 × g and 4 °C for 10 min. After centrifugation, 200 μL aliquot of supernatant was diluted 1:1 with 10.0 mM ammonium acetate (pH 6.8) and used directly for LC–MS/MS analysis.

3.2.4 LC-MS/MS system

The LC-MS/MS instrumentation used for this work consisted of a Shimadzu Prominence UFLC system (Shimadzu, Columbia, MD, USA) and an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Foster City, CA, USA). The UFLC system included a system controller (CBM-20A), two binary pumps (LC-20AD), a temperature-controlled autosampler (SIL 20AH\text{\textregistered}) and an online degasser (DGU20A\textsubscript{3}), and the mass spectrometer came with a Turbo IonSpray source. The UFLC system outlet was connected to the mass spectrometer via high-pressure polyether ether ketone (PEEK) tubing (0.0625 in. o.d. x 0.0100 in. i.d.).
3.2.5 Chromatographic separation

The chromatographic separation was carried out isocratically under ambient temperature on a reverse-phase Gemini Nx C\textsubscript{18} (2.1 mm i.d x 150 mm, 5 μm, 110 Å) column (Phenomenex, Torrance, CA, USA) with an inline VHP filter (0.5 μm, stainless steel) from Upchurch Scientific (Oak Harbor, WA, USA). The sample injection volume was 10.0 μL and the mobile phase flow rate was 0.200 mL min\textsuperscript{-1}. Prior to sample analysis, the column was first equilibrated with at least 20 column volumes of the mobile phase at a flow rate of 0.200 mL min\textsuperscript{-1}.

3.2.6 Mass spectrometric detection

AB Sciex QTrap 5500 tandem mass spectrometer was operated in positive electrospray ionization (ESI+) mode. It was tuned by flow injection analysis of SE (250 ng mL\textsuperscript{-1}) and norSE (500 ng mL\textsuperscript{-1}) in the mobile phase for both compound-dependent and source-dependent parameters. The optimized ionization parameters were as follows: curtain (CUR), 20 psi; collision-activated dissociation (CAD) gas, medium; nebulizer gas (GS1), 40 psi; turbo heater gas (GS2), 25 psi; ion spray voltage (IS), 5500V; source temperature (TEM), 300 °C. High purity nitrogen (99.99%) was used as the nebulizer, auxiliary, collision and curtain gases. The declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were set at 65, 6.0, 30 and 8.0 V for SE and 80, 7.0, 35, 6.0 V for norSE. Quantification was performed by multiple-reaction-monitoring (MRM) mode with mass transitions $m/z$ 218.1 $\rightarrow$ 84.1 for
SE, and $m/z$ 204.1 $\rightarrow$ 70.2 for norSE. The dwell time for each transition was 300 ms and the mass resolutions (Q1 and Q3) were set at unit.

3.2.7 Data acquisition and analysis

Data acquisition and peak integration were carried out by the AB Sciex Analyst® software (version 1.5.2). The linear regression calibration equation was obtained by plotting the mean-peak-area ratios of SE plasma calibrators to those of the IS versus the concentrations of SE calibrators using a weighting factor of $1/x$ (where $x$ is the nominal concentration of a plasma calibrator).

3.2.8 Stability study

The stability of SE was investigated using the SE stock solutions (2.00 mg mL$^{-1}$), low and high plasma QC samples (1.80, 540 ng mL$^{-1}$), and the stability of norSE was also determined separately using the norSE stock solution (1.20 mg mL$^{-1}$) and a plasma sample at a concentration of 60.0 ng mL$^{-1}$. In the latter studies, SE was used as the IS for norSE.

Stabilities of stock solution and mouse plasma samples were assessed by short-term (6 and 24 h) standing on bench-top at 23°C and in the auto sampler at 4°C (post preparative); freeze-and-thaw cycles; and long-term storage at -20 and -80°C. The
stabilities of SE and norSE were determined by comparing the mean-peak-area ratios of analyte to the IS in the test sample to those of freshly prepared samples, and expressed as percentages.

3.2.9 Animal study

The LC-MS/MS method developed was applied to the measurement of SE concentrations in the following animal study which had been approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC). BALB/c mice were obtained from Jackson laboratories (Bar Harbor, Maine, USA). The mice were randomly housed, and had an average body weight of 25 g at the time of SE injections. The injectable SE (1 mg mL\(^{-1}\)) was prepared in 10% DMSO aqueous solution which was given to mice as a single-bolus intraperitoneal (i.p.) injection at a dose of 4.00 mg kg\(^{-1}\). The mouse blood samples were collected via ocular puncture into 1.5 mL microcentrifuge tubes containing heparin as anti-coagulant at the following time points: 0 (pre-dose), 1.5, 3, 7, 9, 15, 30 and 120 min. For the pre-dose sample, the mouse was injected with only 10% DMSO aqueous solution without SE. One whole blood sample (ca. 100 µL) was drawn from a single mouse at each time point, and was placed on ice immediately. The whole blood samples were processed within 15 min of collection by centrifugation at 1000 x g and 4 °C for 10 min. The harvested plasma samples (ca. 50 µL each) were stored at -80°C until analysis.
3.3 Results and discussion

3.3.1 Method development

3.3.1.1 Internal standard (IS)

Due structural analog of SE was used as IS since stable heavy isotope of SE was not available. Initially, several custom-synthesized SE analogs were tested; however, most of the analogs chosen contained trace amount of SE due to the use of SE as precursor in synthetic routes. NorSE was eventually chosen since it was free of SE. NorSE is structurally similar to SE (Figure 1), but differs only by one \(-\text{CH}_2\) unit, which has a pyrrolidinly instead of piperidinyl ring.

3.3.1.2 Analyte solubility

According to the Sci-Finder\textsuperscript{®} Scholar database search, the log \(P\) values of SE and norSE are -0.969 and -1.497 respectively. These compounds should be hydrophilic rather than hydrophobic. However, we found them to be difficult to dissolve either aqueous solution or plasma directly, which is probably attributed to their large rigid ring.
Figure 1: Chemical structures of SE and the internal standard (IS).

(-)-Securinine (SE) (monoisotopic mass = 217.1)  (+)-Norsecurinine (norSE) (monoisotopic mass = 203.1)
structures. Although ethanol can dissolve these compounds, it can cause protein precipitation in plasma, resulting in poor analytical reproducibility. The suitable solvent to dissolve both SE and norSE is DMSO. In this work, the SE and norSE working standard solutions were prepared in 100% DMSO, which was diluted to ≤10% DMSO in plasma calibrators and QC samples to ensure no protein precipitation from plasma matrices [20].

3.3.1.3 Mass spectrometric detection

Due to the chemical structures of SE and norSE (IS), they are more readily to acquire protons than lose one in electrospray ionization. Hence, the positive electrospray ionization mode was used in this work.

As shown in Figure 2A and 2C (full scan spectra), SE and norSE produced protonated molecular ions [M+H]⁺ at m/z 218.1 and m/z 204, respectively. These molecular ions produced major fragments at m/z 84.1 and m/z 70.2 (Figure 2B and 2D). Therefore, the mass transitions of m/z 218.1 → 84.2 for SE and m/z 204.1 → 70.2 for the IS were chosen for quantification.

The assignment of the SE and norSE (IS) fragments was done by ACD/MS Fragmenter software (ACD Labs Toronto, Canada), and later confirmed by the experimental data.
Figure 2: The mass spectra of the precursor and product ions of SE and norSE (IS). (A) the molecular ion of SE; (B) the major fragment of SE; (C) the molecular ion of norSE; and (D) the major fragment of norSE.
3.3.1.4 Chromatographic separation

In this work, reverse phase-liquid chromatography (RP-LC) columns such as Waters Xterra® RP-18 and Phenomnex Gemini Nx C_{18} (Phenomnex, Torrance, California) columns were tested for separation of SE and norSE. Both columns were suitable for the separation of these compounds; however, Gemini Nx C_{18} was chosen for subsequent study because it gave higher signal intensity.

The composition of the mobile phase used for separation was first optimized. Acetonitrile was chosen as the organic solvent due to the greater solvent strength, and higher signal response in mass spectrometry than those of methanol; and ammonium acetate (10.0 mM) was used as the buffer salt because it suppressed the formation of sodium ion adducts and reduced peak tailing. Since pH value of a mobile phase can influence the retention of analytes containing amine groups in RP-LC, the optimal pH value was also determined in this work. The optimum separation of SE and norSE was obtained with ammonium acetate at pH 6.8. Under this pH, SE (pKa = 8.29 ±0.20) and norSE (pKa = 8.78 ±0.20) were protonated and could be separated with baseline resolution.

The percent content of acetonitrile in the mobile phase was investigated. Initially 70% acetonitrile (ACN) was used; although it produced shorter retention times for the analytes it caused co-elution of endogenous lipids, and resulted in significant ion
suppression (Figure 3A). It was identified by our experiment the lipid co-eluted was 1-stearyl-rac-1glycerol (monoacylglycerol) [21]. To resolve this problem, the percent content of ACN was reduced to 40% where no apparent 1-stearyl-rac-1glycerol was observed (Figure 3B).

For this work, the optimized mobile phase composition consisted of 40% ACN and 60% 10 mM ammonium acetate, pH 6.8. Using this mobile phase, separation of SE and norSE was achieved on the Gemini Nx C_{18} with retention times of 5.5 min and 2.6 min for SE and norSE respectively (Figure 4).

3.3.1.5 Preparation of plasma samples

In this work, liquid–liquid extraction (LLE) was first examined for sample preparation. In detail, 100 μL of plasma sample was first diluted with 0.05% ammonium aqueous solution to deprotonate the analytes, then 1.00 mL of ethyl acetate was added. The organic layer was transferred into borosilicate glass tubes (12 mm x 75 mm, VWR) and dried under nitrogen (10 psi) by TurboVap LV evaporator (Zymark, Hopkinton, Massachusetts, USA) at 40 °C for 30 minutes, and reconstituted in the mobile phase for analysis. Due to the high volatilities of SE and norSE, cross-vial contamination was observed. Further the recoveries of analytes were low and the matrix effect was high by LLE. To overcome these problems, a salting-out assisted liquid-liquid extraction (SALLE) protocol was later adopted for plasma sample preparation [22, 23], where a concentrated volatile salt (i.e., 2.00 M ammonium acetate) with a cold organic solvent
Figure 3: The effect of the percent organic additive in the mobile phase on the separation of SE from lipid interference. (A) 70% ACN and 30% buffer (10 mM ammonium acetate pH 6.8) (B) 40% ACN and 60% buffer (10 mM ammonium acetate pH 6.8). Top chromatograms shows the MRM mass transitions of SE, and the bottom chromatograms shows the MRM mass transitions of 1-Stearyl-rac-1-glycerol (monoacyl glycerol).
(i.e., acetonitrile) were added to promote protein precipitation and induce phase separation. The analytes extracted were determined by LC-MS/MS after a direct dilution of the organic phase with 10.0 mM ammonium acetate (pH 6.8) buffer (at 1:1 ratio). The SALLE protocol was found to be effective in removal of matrix interference and producing good analyte recoveries (see data in sections 3.3.2.2 and 3.3.2.3).

### 3.3.2 Method validation

The method developed was validated in mouse plasma according to the US-FDA guidance for industry on bioanalytical method validation [17] and the subsequent 2006 white paper of bioanalytical method validation workshop [18,19] in terms of selectivity, lower limit of quantitation (LLOQ), linear response range, recovery, matrix effect, accuracy and precision, as well as stability for both short-term sample processing and long-term sample storage.

#### 3.3.2.1 Selectivity and lower limit of detection (LLOQ)

The selectivity of the method was evaluated by comparing the mass chromatograms of six lots of blank plasma samples with those of the spiked plasma calibrator at the LLOQ. There were no endogenous interference observed at the retention
times and mass transitions of SE and the IS (Figure 4A). Furthermore, there was no interference from the IS at the retention time and m/z of SE (Figure 4B).

The LLOQ of the method was defined by lowest concentration on the calibration curve (0.600 ng mL$^{-1}$) (Figure 4C). As shown in TABLE I, the accuracy expressed as percent error (%RE) and precision as correlation of variation (%CV) were $\leq \pm 7\%$ and $\leq 5\%$ respectively based on five replicates measurements in six lots of blank plasmas. Since the acceptable accuracy (%RE) and precision (%CV) were $\leq \pm 20\%$ and 20%, these results imply that the actual LLOQ of the method may be lower than 0.600 ng mL$^{-1}$.

### 3.3.2.2 Matrix effect

Matrix effect was assessed by the matrix factor (MF), in six independent lots of mouse plasmas. The absolute MF was determined by comparing the mean peak area of SE and IS in the spiked plasma matrix after SALLE ($n = 5$) with those of SE and IS in the mobile phase at low- and high- QC concentrations (1.80 and 540 ng mL$^{-1}$). The IS normalized MF was determined by MF of SE over those of the IS. As shown in TABLE II, the absolute MFs of SE and IS ranged from 0.99-1.15 and 1.01-1.10 respectively. The IS normalized MFs were in the range of 0.92-1.07. These results indicate that the plasma matrix effect was negligible in this method.
Figure 4: The representative mass chromatograms of SE and the IS in mouse plasma: (A) double blank plasma (with neither SE nor IS); (B) single blank plasma (with IS only, 60 ng mL\(^{-1}\)); and (C) at LLOQ (0.600 ng mL\(^{-1}\); IS, 60 ng mL\(^{-1}\)).
**TABLE I: Accuracy and precision of SE at LLOQ in six different lots of mouse plasma**

<table>
<thead>
<tr>
<th>Plasma matrix</th>
<th>Nominal [SE](ng mL(^{-1}))</th>
<th>Mean measured [SE](ng mL(^{-1}))</th>
<th>SD(ng mL(^{-1}))</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot 1</td>
<td>0.600</td>
<td>0.61</td>
<td>0.02</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Lot 2</td>
<td>0.600</td>
<td>0.58</td>
<td>0.03</td>
<td>-3</td>
<td>5</td>
</tr>
<tr>
<td>Lot 3</td>
<td>0.600</td>
<td>0.57</td>
<td>0.01</td>
<td>-5</td>
<td>2</td>
</tr>
<tr>
<td>Lot 4</td>
<td>0.600</td>
<td>0.61</td>
<td>0.02</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Lot 5</td>
<td>0.600</td>
<td>0.56</td>
<td>0.02</td>
<td>-7</td>
<td>4</td>
</tr>
<tr>
<td>Lot 6</td>
<td>0.600</td>
<td>0.56</td>
<td>0.03</td>
<td>-7</td>
<td>5</td>
</tr>
</tbody>
</table>

Each datum point calculated by five replicate measurements

\%RE =\[(\text{measured} - \text{nominal})/\text{nominal}] \times 100\% \; \%CV = (\text{SD/mean}) \times 100\%.
<table>
<thead>
<tr>
<th>Plasma matrix</th>
<th>[SE] (ng mL(^{-1}))</th>
<th>MF(_{SE}) ± SD(^a)</th>
<th>MF(_{IS}) ± SD(^b)</th>
<th>IS Normalized MF ± SD(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot 1</td>
<td>1.80</td>
<td>1.03 ± 0.02</td>
<td>1.04 ± 0.04</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>540</td>
<td>1.05 ± 0.05</td>
<td>1.09 ± 0.05</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td>Lot 2</td>
<td>1.80</td>
<td>1.02 ± 0.03</td>
<td>1.04 ± 0.02</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>540</td>
<td>1.01 ± 0.05</td>
<td>1.10 ± 0.06</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td>Lot 3</td>
<td>1.80</td>
<td>1.03 ± 0.03</td>
<td>1.03 ± 0.04</td>
<td>0.99 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>540</td>
<td>1.03 ± 0.03</td>
<td>1.03 ± 0.04</td>
<td>0.99 ± 0.02</td>
</tr>
<tr>
<td>Lot 4</td>
<td>1.80</td>
<td>1.05 ± 0.03</td>
<td>1.05 ± 0.03</td>
<td>1.01 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>540</td>
<td>0.99 ± 0.06</td>
<td>1.06 ± 0.03</td>
<td>0.93 ± 0.07</td>
</tr>
<tr>
<td>Lot 5</td>
<td>1.80</td>
<td>1.01 ± 0.01</td>
<td>1.09 ± 0.02</td>
<td>0.92 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>540</td>
<td>1.02 ± 0.01</td>
<td>1.01 ± 0.08</td>
<td>1.01 ± 0.09</td>
</tr>
<tr>
<td>Lot 6</td>
<td>1.80</td>
<td>1.15 ± 0.04</td>
<td>1.08 ± 0.02</td>
<td>1.07 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>540</td>
<td>1.02 ± 0.01</td>
<td>1.02 ± 0.06</td>
<td>1.00 ± 0.05</td>
</tr>
</tbody>
</table>

\(^a\) MF\(_{SE}\) = (mean peak area of SE in extracted plasma matrix)/ (mean peak area of SE in mobile phase).

\(^b\) MF\(_{IS}\) = (mean peak area of IS in extracted plasma matrix)/ (mean peak area of IS in mobile phase).

\(^c\) IS normalized MF = MF\(_{SE}\)/MF\(_{IS}\).
3.3.2.3 Analyte recovery by SALLE

The absolute recoveries were determined by comparing the mean peak area of SE and the IS in the corresponding QC plasma samples (n = 5) with those of SE and IS in the spiked plasma matrix after SALLE at low-, mid- and high- QC concentrations (1.80, 18.0, 540 ng mL$^{-1}$). The IS normalized recoveries were determined by the recoveries of SE over those of the IS. As shown in TABLE III, the mean absolute recoveries for SE and the IS ranged 79-86% and 79%- 80%, respectively; and the mean IS normalized recoveries ranged 99%-109%.

3.3.2.4 Carryover

The carryover of the analytes was assessed with five batches of validation calibrators by injecting plasma blank control immediately after the injection of the plasma calibrator at the highest concentration (600 ng mL$^{-1}$). There were no analyte peaks observed using 90% acetonitrile as the wash solvent. Hence, sample carryover was not a problem in this method.
**TABLE III: RECOVERY OF SE IN POOLED MOUSE PLASMA (N = 5)**

<table>
<thead>
<tr>
<th>[SE] (ng mL$^{-1}$)</th>
<th>Recovery$_{SE}$ ± SD (%)</th>
<th>Recovery$_{IS}$ ± SD (%)</th>
<th>IS Normalized Recovery ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.80</td>
<td>86 ± 3</td>
<td>79 ± 2</td>
<td>109 ± 2</td>
</tr>
<tr>
<td>18.0</td>
<td>84 ± 3</td>
<td>79 ± 1</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>540</td>
<td>79 ± 2</td>
<td>80 ± 3</td>
<td>99 ± 2</td>
</tr>
</tbody>
</table>

PA = Mean peak area.
Recovery of SE = (PA of SE in plasma matrix / PA of SE in extracted plasma matrix) x 100%.
Recovery of IS = (PA of IS in plasma matrix / PA of IS in extracted plasma matrix) x 100%.
IS normalized Recovery = (recovery of SE / recovery of the IS) x 100%.
3.3.2.5 Accuracy and precision

The intra-run accuracy and precision were determined by five replicate measurements of each low-, mid- and high- QC samples (1.80, 18.0, 540 ng mL\(^{-1}\)) as well as dilution QC 1.80 x 10\(^3\) ng mL\(^{-1}\) in the same validation batch. The inter-run accuracy and precision was determined by five parallel measurements of five identical sets of each QC samples over five different validation batches. As shown in TABLE IV, the intra-run accuracy and precisions of all the QCs samples were ≤ ± 6% and ≤ 5%, and the inter-run accuracy and precisions were ≤ ± 6% and ≤ 6% respectively. These results indicate that this method has adequate accuracy and precision.

3.3.2.6 Linearity

The calibration curves of SE in mouse plasma were constructed using a double blank (with neither SE nor IS), a single blank (zero calibrator, with IS) and seven non-zero SE plasma calibrators (0.600, 1.20, 6.00, 12.0, 60.0, 120 and 600 ng mL\(^{-1}\)), with the concentration of the IS at 60.0 ng mL\(^{-1}\). The mean peak area ratios of SE to IS were plotted against the nominal concentration of SE. The calibration equation derived from five validation batches of calibrators using a 1/x weighted least-square linear regression was \(Y = 0.135 \pm 0.009 \times + 0.009 \pm 0.008\). A linear range of over
TABLE IV: **Intra- and Inter-run Accuracy and Precision of SE in Pooled Mouse Plasma (N = 5)**

<table>
<thead>
<tr>
<th>Nominal [SE] (ng mL⁻¹)</th>
<th>Measured [SE] (ng mL⁻¹)</th>
<th>SD (ng mL⁻¹)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.80</td>
<td>1.8</td>
<td>0.1</td>
<td>0.0</td>
<td>6</td>
</tr>
<tr>
<td>18.0</td>
<td>17.0</td>
<td>0.5</td>
<td>-6</td>
<td>3</td>
</tr>
<tr>
<td>540</td>
<td>545</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1.80 x10³[^c]</td>
<td>1.75 x10³[^c]</td>
<td>8 x10⁻¹</td>
<td>-3</td>
<td>5</td>
</tr>
</tbody>
</table>

**Intra-Run[^a]**

<table>
<thead>
<tr>
<th>Nominal [SE] (ng mL⁻¹)</th>
<th>Measured [SE] (ng mL⁻¹)</th>
<th>SD (ng mL⁻¹)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.80</td>
<td>1.7</td>
<td>0.1</td>
<td>-6</td>
<td>6</td>
</tr>
<tr>
<td>18.0</td>
<td>17.0</td>
<td>0.4</td>
<td>-6</td>
<td>2</td>
</tr>
<tr>
<td>540</td>
<td>549</td>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1.80 x10³[^c]</td>
<td>1.91 x10³[^c]</td>
<td>7 x10⁻¹</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

[^a]: Determined by five replicate measurements of each QC sample within a validation batch.

[^b]: Determined by five parallel measurements of five identical QC samples at each concentration over five validation batches.

[^c]: The dilution QC was measured after 10 times dilution with the pooled blank plasma.
three orders of magnitude (0.600-600 ng mL\(^{-1}\)) was obtained with a high correlation coefficient (\(r = 1.00\)) and the accuracy and precision of plasma calibrators were \(\leq \pm 3\) and \(\leq 6\), respectively (TABLE V).

### 3.3.2.7 Stability study

The stabilities of SE and norSE under various storage conditions were tested. As shown in TABLE VI, the recoveries for SE stock solutions (23°C) at 6 and 24h time periods were 97-104% and at long term storage (-80°C, 5 months) it was 105%. The recoveries for SE in mouse plasma at 6 and 24h time periods were 94-101% at the bench-top (23°C), and 100-104% in the auto sampler (4°C). The recoveries of 3 freeze-and-thaw cycles (-20 to 23°C) and the long-term storage at -80°C (30 days) were 97-99% and 97-99%, respectively. However, the recovery of long term storage at -20°C (30 days) was low at 76-79%. Therefore, -80°C should be used for long term storage and plasma sample analysis should be done within 24h timeframe.

The recoveries of the IS under various conditions were in the range of 96-105%, indicating no significant loss was observed under the experimental conditions.
TABLE V: ACCURACY AND PRECISION OF SE PLASMA CALIBRATORS OVER FIVE VALIDATION BATCHES.

<table>
<thead>
<tr>
<th>Nominal [SE] (ng mL⁻¹)</th>
<th>Measured [SE] (ng mL⁻¹)</th>
<th>SD (ng mL⁻¹)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.600</td>
<td>0.59</td>
<td>0.02</td>
<td>-2</td>
<td>3</td>
</tr>
<tr>
<td>1.20</td>
<td>1.20</td>
<td>0.06</td>
<td>0.0</td>
<td>5</td>
</tr>
<tr>
<td>6.00</td>
<td>6.00</td>
<td>0.3</td>
<td>0.0</td>
<td>5</td>
</tr>
<tr>
<td>12.0</td>
<td>11.9</td>
<td>0.7</td>
<td>-1</td>
<td>6</td>
</tr>
<tr>
<td>60.0</td>
<td>62.0</td>
<td>4.0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>120</td>
<td>120</td>
<td>5</td>
<td>0.0</td>
<td>4</td>
</tr>
<tr>
<td>600</td>
<td>597</td>
<td>7</td>
<td>-0.5</td>
<td>1</td>
</tr>
</tbody>
</table>
TABLE VI: Stability of SE and IS in mouse plasma samples under various conditions.

<table>
<thead>
<tr>
<th>Test conditions</th>
<th>Temperature (°C)</th>
<th>SE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recovery ± SD (%) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>Stock solution</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Freezer (5 months)</td>
<td>-80</td>
<td>Stock solution</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>Low QC</td>
<td>100 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>Autosampler</td>
<td>4</td>
<td>Low QC</td>
<td>100 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>3 Freeze-thaw cycles</td>
<td>-20 to 23</td>
<td>Low QC</td>
<td>97 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>99 ± 4</td>
</tr>
<tr>
<td>Long-term (30 days)</td>
<td>-20</td>
<td>Low QC</td>
<td>76 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>Long-term (30 days)</td>
<td>-80</td>
<td>Low QC</td>
<td>99 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>97 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test conditions</th>
<th>Temperature (°C)</th>
<th>NorSE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Recovery ± SD (%) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>Stock solution</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>Plasma sample</td>
<td>102 ± 6</td>
</tr>
<tr>
<td>Autosampler</td>
<td>4</td>
<td>Plasma sample</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>3 Freeze-thaw cycles</td>
<td>-20 to 23</td>
<td>Plasma sample</td>
<td>96 ± 5</td>
</tr>
</tbody>
</table>

<sup>a</sup> The concentration of SE stock solution was 2.00 mg mL<sup>-1</sup> which was measured by serial dilution to 1.80 ng mL<sup>-1</sup> in mobile phase. The concentrations of SE in the low and high QC were 1.80 and 540 ng mL<sup>-1</sup> respectively. The concentration of NorSE stock solution was 1.20 mg mL<sup>-1</sup> which was measured by serial dilution to 60.0 ng mL<sup>-1</sup> in both the mobile phase and plasma sample.
3.3.3 Method application

The validated LC-MS/MS method was applied to the measurement of plasma concentrations of SE in BALB/C mice. The animal samples were collected and analyzed by the procedure described in the experimental section. The representative mass chromatograms of SE in mouse plasma from the animal study are shown in Figure 5. The concentration of SE in each mouse sample was back calculated using the peak-area ratio of SE to that of the IS from the calibration equation with the proper dilution factor. Figure 6 shows the mean SE plasma concentration-time profile in mice (n = 3) after a single bolus i.p. injection of 4 mg kg\(^{-1}\) SE, which demonstrated the applicability of the method for pharmacokinetic study in mice.

3.4 Conclusions

An LC-MS/MS method for quantitative determination of securinine (SE) in mouse plasma has been developed and validated. The method uses salting-out assisted liquid-liquid extraction (SALLE) procedure for sample preparation, reverse phase-liquid chromatography (RP-LC) for separation and tandem mass spectrometry for detection.
Figure 5: The representative mass chromatograms of animal study: (A) predosed mouse plasma (with IS, 60 ng mL$^{-1}$); (B) plasma sample collected at 3 min after i.p. injection (with IS, 60 ng mL$^{-1}$); and (C) plasma sample collected 30 min after i.p. injection (with IS, 60 ng mL$^{-1}$).
Figure 6: The mean concentration-time profile of SE in mouse plasma (n = 3) after single i.p. bolus injection at the dose of 4 mg kg$^{-1}$.
The method has good analyte selectivity, wide linear calibration range, and high accuracy and precisions, as well as the stability of routine analysis. It has been applied to the measurement of SE in mouse plasma samples; therefore, maybe be useful for the pharmacological study of SE in mice.

3.5 References


4.1 Introduction

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA) is an industrial chemical, that is widely used to make polycarbonate plastics and epoxy resins which are found in baby bottles, reusable water bottles, food containers as protective coatings, food and beverage cans as linings; and in dental composite fillings and sealants[1-3], among other applications. The prevalent use of BPA-containing plastics in consumer products, frequently exposes humans to this compound via ingestion of trace amounts that leach into the food and beverages, as the BPA polymers are hydrolyzed at high temperature or under acidic/basic conditions [3-5].
BPA is a synthetic estrogen, which mimics the endogenous female hormone action and interferes with the endocrine function [6, 7]. As a known endocrine-disrupting compound (EDC), the adverse physiological effects of BPA are subjects of ongoing clinical studies. Nevertheless, BPA has been associated with altered behavior in children [8], whereas in adults; increased risk of diabetes [9] heart disease[9], obesity [10], as well as, endocrine related dysfunctions i.e., disrupt thyroid hormone action [11, 12], block testosterone synthesis [13] and proliferation of human breast cancer cells [13, 14] cells and prostate cancer cells [15] etc.

Therefore, in the recent years, studies have focused extensively on biological monitoring of BPA human exposure, potential toxicity and risk assessment by quantifying BPA concentrations in biological matrices [16, 17]. However, the measurement of BPA in biological samples often produces false results due to fact that BPA is readily available in laboratory environment and reagents [18, 19]. In humans, BPA is rapidly metabolized in the liver to bisphenol A β-D-glucuronide (BPA-G) and is eliminated via urine (Figure 1) [20-22]. Since BPA-G does not bind to estrogen receptor and does not exist in the environment without metabolism, it can therefore be used as a biomarker to assess human exposure to BPA. Moreover, due to the rapid metabolism, the levels of urinary metabolites can be measured more precisely than those of the parent compound (i.e., BPA) and also the risk of sample contamination is reduced [23, 24].

The existing analytical methods for the measurement of BPA-G include: ELISA [25, 26], HPLC-FL [27], LC-ECD [26, 28], RIA [24] and LC-MS/MS [29-34]. However, there several limitations of this methods when analyzing biological samples, such as; 1)
Figure 1: BPA biotransformation in humans
use of solvent rather than blank urine as sample matrix for calibration [33]; 2) labor intensive sample preparation (i.e., solid phase extraction [30, 33, 35], enzymatic hydrolysis [31, 32] and chemical derivatization [34]; and 3) potential cross reactivity of the antibody with structurally related compounds [24, 25] which often result in bias results.

Therefore, in this work, a stable isotope dilution liquid chromatography tandem mass spectrometry (SID-LC-MS/MS) method has been developed. Deuterated BPA-G ($d_6$-BPA-G) was used as a surrogate standard and carbon-13 labeled BPA-G ($^{13}C_{12}$-BPA-G) as the internal standard (IS). A liquid-liquid extraction procedure using phosphoric acid (15%) and ethyl acetate was developed for urine sample preparation. Isocratic separation of BPA-G, $d_6$-BPA-G and the IS from urine matrix was achieved on a Waters X-select HSS T3 column (2.1 mm i.d x 100 mm, 2.5 μm) column with 17% acetonitrile and 83% 5.00 mM ammonium acetate, pH 7.3 at a flow rate of 0.580 mL min$^{-1}$ in less than 6.5 min. Quantification was carried out by negative electrospray tandem mass spectrometry (ESI–MS/MS) in multiple-reaction monitoring mode (MRM). The SID-LC-MS/MS method developed has been validated according to the US Food and Drug Administration (USFDA) guidance for bioanalytical method validation [36-38] and applied to the measurement of BPA-G concentration in urine samples of 40 patients.

4.2 Experimental

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4.2.1 Chemicals and solutions

Bisphenol A β-D-glucuronide (BPA-G, $C_{21}H_{24}O_8$, MW, 404.41), Bisphenol A- (rings-$^{13}\text{C}_{12}$) β-D-glucuronide ($^{13}\text{C}_{12}$-BPA-G, $^{13}\text{C}_{12}C_9H_{24}O_8$, MW, 416.32), creatinine, ammonium acetate (NH$_4$Ac) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bisphenol A-$d_6$ β-D-Glucuronide ($d_6$-BPA-G, $C_{21}H_{18}D_6O_8$, MW 410.45) used as the internal standard (IS), was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). Creatinine-$N$-methyl-$D_3$ ($d_3$-creatinine) was purchased from C/D/N Isotope Inc. (Pointe-Claire, Quebec, Canada) and HPLC-grade acetonitrile was from Pharmco-AAPER (Shelbyville, KY, USA). HPLC-grade water, ACS-grade o-phosphoric acid (85 wt. %), ACS-grade ammonium hydroxide (28-30 w/w %) and ethyl acetate were purchased from Fisher Scientific (Pittsburg, PA, USA).

The standard stock solutions of BPA-G, $d_6$-BPA-G and $^{13}\text{C}_{12}$-BPA-G were prepared in DMSO at a concentration of (100 µg mL$^{-1}$) and aliquots (20.0 µL) of the stock solutions were stored at -20°C prior to their use. The standard working solutions of $d_6$-BPA-G (0.00, 10.0, 20.0, 30.0, 100, 200, 600, 1.00×$10^3$, 2.00×$10^3$, 9.00×$10^3$, 1.00×$10^4$ and 3.00×$10^4$ ng mL$^{-1}$) were freshly prepared daily by serial dilution of the standard stock solutions with DMSO. The working internal standard solution of $^{13}\text{C}_{12}$-BPA-G (400 ng mL$^{-1}$) was prepared by serial dilution of the stock IS solution in DMSO.

The stock solution of ammonium acetate (100 mM, pH 6.8) was prepared by dissolving appropriate amounts of buffer salt in a known volume of HPLC-water. The working solution of ammonium acetate (5.00 mM, pH 7.3) was prepared by a 1/20
dilution of the 100 mM ammonium acetate with HPLC-water and the pH adjusted with ammonium hydroxide. The mobile phase for liquid chromatographic separation consisted of 17% acetonitrile and 83% 5.00 mM ammonium acetate, pH 7.3.

4.2.2 Urine samples

Human urine was obtained from several healthy donors at Cleveland State University. Samples were collected in sterile polypropylene containers and stored at -20°C prior to analysis. Since BPA-G is endogenous, it’s difficult to obtain a real blank urine matrix; therefore, urine samples were first screened for background concentrations BPA-G. Individual samples were analyzed by LC-MS/MS and the urine sample containing no detectable BPA-G was used as the blank urine matrix. Six lots of urine, assigned as lot A, B, C, D, E, and F were chosen from six different donors. Urine specimen from 20 newly diagnosed breast cancer patients were collected at University Hospitals Case Medical Center (UHCMC). Samples were obtained from patients at a clinic visit prior to initiation of radiation therapy or chemotherapy. Control samples were collected from 20 women undergoing routine screening mammography at UHCMC whose mammograms were negative and had no prior history of cancer. All specimens were collected in standard urine collection cups and were aliquoted the same day into 10 mL BPA-free storage containers. Specimens were then frozen at -80°C until analysis.
4.2.3 Preparation of urine calibrators and quality controls (QCs)

$d_6$-BPA-G urine calibrators (0.00, 0.500, 1.00, 5.00, 10.0, 50.0, 100, 500 ng mL$^{-1}$) were prepared by diluting every 10.00 µL of the corresponding $d_6$-BPA-G working standard solutions with 190.0 µL of blank urine in borosilicate glass tubes (13 mm x 100 mm, Fisher Scientific). The $d_6$-BPA-G urine QCs (1.50, 30.0 and 450 ng mL$^{-1}$) and dilution QC (1.50 x 10$^3$ ng mL$^{-1}$) were prepared in the same manner as that of the urine calibrators. 200-µL aliquots of urine calibrators and QCs were kept at -20°C before use in capped borosilicate glass tubes.

4.2.4 Sample preparation by liquid-liquid extraction (LLE)

The urine samples (i.e., urine calibrators, QCs, dilution QC and patient samples) were thawed at room temperature unassisted. Prior to sample preparation by liquid-liquid extraction (LLE), the $d_6$-BPA-G dilution QC samples (1.50 x 10$^3$ ng mL$^{-1}$, 20 µL) were diluted by a factor of 10 with 180 µL blank urine. To each aliquot of 200.0 µL urine sample, 10.00 µL of the IS working solution (400 ng mL$^{-1}$) was added except for the double blank where 10.00 µL of DMSO was added and vortex-mixed for 3 s using a VWR® Analog Multi-Tube Vortexer (VWR, Radnor, PA, USA). The samples were acidified with 200 µL of 15% (v/v) phosphoric acid, vortex-mixed for 30 s and extracted with 2.00 mL of ethyl acetate. After vortexing for 3 min, the mixture was centrifuged at
3800 x g for 10 min, then 1700 µL of the organic phase was transferred into a fresh borosilicate glass tube and dried under nitrogen (12 psi) in turbovap LV evaporator (Biotage, Charlotte, NC) at 40°C for 30 min. The residue was reconstituted in 170 µL of solution containing 10% acetonitrile and 90% of 5.00 mM ammonium acetate (pH 7.3) before LC-MS/MS analysis.

4.2.5 Instrumentation

The liquid chromatography tandem mass spectrometry (LC-MS/MS) system consisted of a Shimadzu Prominence UFLC system (Shimadzu, Columbia, MD, USA) equipped with a system controller (CBM-20A), two binary (LC-20AD) pumps, a temperature controlled (SIL 20AH_) autosampler, an online (DGU20A3) degasser, coupled to an AB Sciex QTRAP 5500 mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a Turbo IonSpray ESI source which was used for mass analysis and detection. The mass spectrometer was connected to the LC-system outlet via high-pressure polyether ether ketone (PEEK) tubing (0.0625 in., o.d. x 0.0050 in., i.d) designed for use in UPLC applications. During the first few minutes the effluent flow was diverted to waste to prevent contamination of the MS.

4.2.6 Liquid chromatography
The chromatographic separation was carried out at ambient temperature by isocratic elution using 17% acetonitrile and 83% 5.00 mM ammonium acetate, pH 7.3 at a flow rate of 0.580 mL min\(^{-1}\) on a Water X-Select™ HSS T3 C\(_{18}\) (2.1 mm i.d x 100 mm, 2.5 μm,) column (Waters, Milford, MA, USA) preceded by a Water X-Select™ HSS T3 C\(_{18}\) (2.1 mm i.d x 5 mm, 2.5 μm,) Vanguard pre-column and an inert stainless steel in-line filter (0.5 μm pore, 0.23 μL dead volume) from Upchurch Scientific (Oak Harbor, WA, USA). The injection volume of each sample was 20.0 μL. Prior to sample analysis, the column was first equilibrated with the mobile phase using 20 times of the column volume.

### 4.2.7 Mass spectrometry

The AB Sciex QTrap 5500 tandem mass spectrometer was operated in negative electrospray ionization (ESI\(^{-}\)) mode. It was tuned by flow injection of individual standard compounds (\textit{i.e.}, 500 ng mL\(^{-1}\) BPA-G, 500 ng mL\(^{-1}\) \(d_6\)-BPA-G and 500 ng mL\(^{-1}\) IS) in the mobile phase for the ionization parameters. The source-dependent ionization parameters were as follows: curtain (CUR), 20 psi; collision-activated dissociation (CAD) gas, medium; nebulizer gas (GS1), 70 psi; turbo heater gas (GS2), 65 psi; Turbo IonSpray voltage (IS), -3200 V; source temperature (TEM), 550°C. High purity nitrogen was used as the nebulizer, auxiliary, collision and curtain gases. The optimized compound-dependent ionization parameters are summarized in TABLE I. Quantification was
<table>
<thead>
<tr>
<th>MS/MS parameters</th>
<th>BPA-G</th>
<th>$d_6$-BPA-G</th>
<th>$^{13}C_{12}$-BPA-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1 Mass (Da)</td>
<td>403.1</td>
<td>409.1</td>
<td>415.1</td>
</tr>
<tr>
<td>Q3 Mass (Da)</td>
<td>227.0</td>
<td>233.1</td>
<td>113.0</td>
</tr>
<tr>
<td><strong>Source-Dependent parameters (V)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Declustering potential (DP)</td>
<td>-100</td>
<td>-90</td>
<td>-90</td>
</tr>
<tr>
<td>Entrance potential (EP)</td>
<td>-12</td>
<td>-12</td>
<td>-11</td>
</tr>
<tr>
<td>Collision energy (CE)</td>
<td>-36</td>
<td>-38</td>
<td>-40</td>
</tr>
<tr>
<td>Collision cell exit potential (CXP)</td>
<td>-15</td>
<td>-15</td>
<td>-18</td>
</tr>
</tbody>
</table>
performed by multiple-reaction-monitoring (MRM) mode and the mass transitions of \( m/z \) 403.1 → 227.1 for BPA-G, \( m/z \) 409.1 → 233.1 for \( d_6 \)-BPA-G and \( m/z \) 415.1 → 113.0 for the IS were monitored. The dwell time for each transition was 533 ms and the instrument (Q1 and Q3) was operated in unit mass resolution.

4.2.8 Stability

The stability study was performed on \( d_6 \)-BPA-G stock solution (100 µg mL\(^{-1}\)) and in human urine using low and high QC samples (1.50, 450 ng mL\(^{-1}\)) prepared as described in section 4.2.4 except the IS (20.0 ng mL\(^{-1}\)) was added prior to sample preparation. Short-term stabilities were assessed for 6h and 24h on the bench-top at 23°C, in the auto sampler (post preparative) at 4°C and by three freeze and thaw cycles where the samples were frozen at -20°C and -80°C for at least 24h and thawed at 23°C (room temperature) unassisted. Long-term stability was assessed for \( d_6 \)-BPA-G urine samples at -20°C and -80°C for 30 days.

4.2.9 Application of the method

The method developed was applied to the measurement of the BPA-G concentrations in 40 patients’ urine samples. Prior to analysis, the urine specimen were thawed at room temperature and 200.0 µL aliquots were placed in borosilicate glass
tubes, capped and kept at -20°C along with the urine calibrators. The urine samples were thawed at room temperature and prepared along with urine calibrators (i.e., one double blank, one single blank and seven none-zero calibrators) and QCs at low-, med- and high-concentrations (i.e., 1.50, 30.0 and 450 ng mL\(^{-1}\)). The samples were prepared by LLE as (section 4.2.4), and analyzed by the SID-LC-MS/MS method. The creatinine concentration of each patient urine sample was measured using LC-MS/MS method developed.

### 4.2.10 Analysis of creatinine by LC-MS/MS

The standard stock solutions (1 mg mL\(^{-1}\)) of creatinine and its deuterated IS (\(d_3\)-creatinine) were prepared by dissolving appropriate amounts of the compound in a known volume of HPLC-water. The stock solutions were stored at -20°C prior to their use. The standard working solutions of Creatinine (0.00, 0.50, 2.00, 30.0, 100, 200, 300, 1.00×10\(^3\), 2.00×10\(^3\), 3.00×10\(^3\), 1.00×10\(^4\) ng mL\(^{-1}\)) were freshly prepared daily by serial dilution of the standard stock solutions with HPLC-water. The IS working solution (500 ng mL\(^{-1}\), \(d_3\)-creatinine) was prepared by serial dilution of the stock IS solution in HPLC-water.

Creatinine standard calibrators (0.00, 0.50, 1.00, 5.00, 10.0, 50.0, 100 and 500 ng mL\(^{-1}\)) were prepared by diluting every 50.00 µL of the corresponding creatinine working standard solutions with 950.0 µL of the mobile phase in 1.50 mL microcentrifuge tubes (VWR, Radnor, PA, USA). The creatinine solution QCs (1.50,
15.0 and 150 ng mL\(^{-1}\)) were prepared in the same manner as that of the calibrators. To each standard calibrator and QCs sample, 50 μL of IS working solution (500 ng mL\(^{-1}\), \(d_3\)-creatinine) was added and the samples were capped, vortex mixed for 30s and transferred into autosampler vial for LC-MS/MS analysis.

The 40 patient urine samples were thawed at room temperature, vortex mixed and diluted 400 fold with the mobile phase. An aliquot (10uL) of each sample diluent was mixed with 990.0 μL of the mobile phase and 50 μL of the IS working solution (500 ng mL\(^{-1}\), \(d_3\)-creatinine) in capped 1.5-mL microcentrifuge tubes. The urine samples were vortex mixed for 30s and analyzed by LC-MS/MS along with creatinine standard calibrators and QCs samples.

The AB Sciex QTrap 5500 tandem mass spectrometer was operated in positive electrospray ionization (ESI) mode. It was tuned by flow injection analysis of 500 ng mL\(^{-1}\) creatinine and IS in the mobile phase for both the compound-dependent and the source-dependent parameters. The optimized ionization parameters were as follows: curtain (CUR), 25 psi; collision-activated dissociation (CAD) gas, medium; nebulizer gas (GS1), 65 psi; turbo heater gas (GS2), 65 psi; Turbo IonSpray voltage (IS), 3800V; source temperature (TEM), 550°C. High purity nitrogen (99.99%) was used as the nebulizer, auxilliary, collision and curtain gases. The declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP), were set at 40, 13.8, 27 and 18.0 V, and 50, 6.6, 28, 7.0 V for creatinine and the IS respectively. Quantitative analysis was performed by multiple-reaction-monitoring (MRM) mode with mass transitions \(m/z\) 114.0 → 44.1 for creatinine and \(m/z\) 117.0 →
47.1 for the IS. The dwell time for each transition was 800 ms and the mass resolutions (Q1 and Q3) were set to unit.

The chromatographic separation of creatinine and IS was achieved by isocratic elution on the Water X-Select™ HSS T3 C$_{18}$ column with a mobile phase consisting of 12% acetonitrile and 88% 10.00 mM ammonium acetate, pH 6.8 at a flow rate of 0.300 mL min$^{-1}$. The injection volume of each sample (the standard or diluted samples) was 10.0 μL and the column was maintained at ambient temperature. Prior to analysis, the column was first equilibrated with the mobile phase at a flow rate 0.3 mL min$^{-1}$ with 20 times the column volume.

4.2.11 Data acquisition and analysis

Data was acquired by the AB Sciex Analyst® software (version 1.6.1) and processed by the AB Sciex MultiQuant™ Software. Quantitation of BPA-G was based on stable isotope ratio i.e., deuterated BPA-G ($d_6$-BPA-G) was used as the calibration standard and carbon-13 labeled BPA-G ($^{13}$C$_{12}$-BPA-G) as the internal standard (IS). The averaged peak area ratios of $d_6$-BPA-G to the IS were plotted against the $d_6$-BPA-G concentrations in urine calibrators for a linear regression equation using a weighting factor of $1/x$ (where x is the concentration of the calibrator). The BPA-G concentration in a patient’s samples was calculated by the MultiQuant™ Software using the peak area ratio of $d_6$-BPA-G to that of the IS and the calibration equation.
4.3 Results and discussion

4.3.1 Method development

4.3.1.1 Matrix and ideal isotope calibration

Due to the widespread use of plastic in consumer products and food containers, BPA-G is readily detectable in human urine. Therefore, it’s difficult to acquire blank urine matrices for quantitative method development. The current analytical methods available use solvents (i.e., aqueous or organic) rather than blank urine as sample matrix for calibration [33]; which is not a “true” calibration. A few of the methods report the use of one stable isotope as an internal standard (IS) i.e., BPAG-$d_6$ [31], BPAG-$d_{14}$ and BPAG-$d_{16}$ [39], where BPAG is measured relative to the heavy isotope IS.

In this work, two stable heavy isotope standards are used i.e., deuterated BPAG ($d_6$-BPAG) is used as the calibration standard and carbon-13 labeled BPAG ($^{13}$C$_{12}$-BPAG) is used as the internal standard (IS) for specific and accurate measurement of BPAG in human urine. These stable heavy isotopes have the same physico-chemical properties as BPAG but different molecular masses. Due to their low natural abundance, they are
not detectable in blank human urine, hence; solve the pre-existing problem of endogenous BPA-G in urine calibration matrices.

**4.3.1.2 Mass spectrometric detection**

Based on the chemical structures of BPA-G, \textit{d}_6\text{-BPA-G} and \textit{^{13}C}_{12}\text{-BPA-G} (IS) (Figure 2), these compounds are more readily to lose a proton than acquire one in electrospray ionization; therefore, the negative electrospray ionization mode was used in this work. As shown in Figure 3A, 3C and 3E. BPA-G was deprotonated and produced a molecular ion \([\text{BPA-G-H}]^-\) at \textit{m/z} 403.2, \textit{d}_6\text{-BPA-G} produced a molecular ion \([\text{d}_6\text{-BPA-G -H}]^-\) at \textit{m/z} 409.1 and the IS produced a molecular ion \([\text{IS-H}]^-\) at \textit{m/z} 415.1. These molecular ions were fragmented in the collision cell with high purity nitrogen gas, and resulted in the product ion spectra shown in Figure 3B, 3D and 3F).

The predominant product fragments for BPA-G, \textit{d}_6\text{-BPA-G} and the IS predicted by ACD/MS Fragmenter software (ACD Labs Toronto, Canada), and confirmed by the experimental data were \textit{m/z} 227.0, \textit{m/z} 233.1 and \textit{m/z} 239.1 respectively. However, interference was observed in the mass transitions of \textit{m/z} 415.1 \rightarrow 239.1 for the IS, therefore a different product ion for the IS was selected. Hence, the mass transitions of \textit{m/z} 403.1 \rightarrow 227.0 for BPA-G, \textit{m/z} 409.1 \rightarrow 233.1 for BPA-G and \textit{m/z} 415.1 \rightarrow 113.0 for the IS were chosen for quantification by multiple-reaction-monitoring (MRM) mode (Figure 4).
Figure 2: Chemical structures of BPA-G, $d_6$-BPA-G and $^{13}C_{12}$-BPA-G (IS).

Bisphenol A $\beta$-D-glucuronide (BPA-G)  
(monoisotopic mass = 404.1)

Bisphenol A $d_6$ $\beta$-D-Glucuronide ($d_6$-BPA-G)  
(monoisotopic mass = 410.2)

Bisphenol A-(rings-$^{13}C_{12}$) $\beta$-D-glucuronide  
($^{13}C_{12}$-BPA-G, Internal Standard)  
(monoisotopic mass = 416.2)
Figure 3: The full scan mass spectra of BPA-G, d6-BPA-G and IS and their major product ions.
Figure 4: The proposed major fragments of BPA-G, $d_6$-BPA-G and IS
4.3.1.3 Chromatographic separation

Separation of BPA-G, $d_6$-BPA-G and IS was performed on reverse phase-liquid chromatography (RP-LC) columns (*i.e.*, Waters Atlantis® T3 and Water X-Select™ HSS T3 C$_{18}$) suitable for polar compounds. The Water X-Select™ HSS T3 C$_{18}$ was chosen for method development because it not only gave a higher signal intensity and better separation of the analytes from interfering urine components, but also symmetrical peak shapes without the need of adding ion pairing agents in the mobile phase.

The composition of the mobile phase used for isocratic elution was optimized. A significantly higher signal response and better peaks shapes where obtained with acetonitrile than with methanol, therefore acetonitrile was chosen as the organic solvent. The use of buffer salt was also evaluated and the addition of ammonium acetate (5.00 mM) improved separation by decreasing the background signal; however, higher amounts of the salt were found to dramatically reduce the sensitivity. The effect of the pH of the mobile phase on the analyte separation and detection was also investigated and ammonia was tested as an additive. While minimal improvement in the signal intensity of the analytes was obtained, a greater decrease in the background noise was achieved. In this work, optimal separation and detection of BPA-G, $d_6$-BPA-G and IS was obtained with 5.00 ammonium acetate at pH 7.3. Under this pH the analytes were sufficiently deprotonated (glucuronide pKa = 3.1-3.2) and could be detected in ESI–MS/MS.
4.3.1.4 Resolution of matrix interference

The use of Water X-Select™ HSS T3 C\textsubscript{18} (2.1 mm i.d x 5 mm, 2.5 μm,) for separation and 75% acetonitrile and 25% 5 mM ammonium acetate (pH 7.3) as mobile phase was first evaluated. Although shorter retention times were observed for BPA-G and \textit{d}_{6}-BPA-G, severe matrix interference was encountered in the urine matrix (Figure 5A). The endogenous compounds co-eluted at the same retention time (t\textsubscript{R}) \textit{i.e.}, 3.5 min as the analytes and produced a similar product fragment of \textit{m/z} 227.1 with BPA-G and \textit{m/z} 233.1 with \textit{d}_{6}-BPA-G in tandem mass spectrometer. It was identified that the glucuronide conjugates of some isoflavones, \textit{i.e.}, resveratrol which are present in the human diet, have a molecular weight identical to BPA-G and similar lipophilicity (Figure 6) and could possibly interfere with MS analysis of BPA-G.

To minimize the matrix interference, different product ions for quantitation of BPA-G and \textit{d}_{6}-BPA-G were examined; however, this reduced the sensitivity of detection significantly and the interference was still observed. Therefore, separation was further optimized and the percent content of acetonitrile in the mobile phase was reduced to 20%, where baseline resolution of BPA-G (t\textsubscript{R} = 8.7 min) and \textit{d}_{6}-BPA-G (t\textsubscript{R} = 8.4 min) from the matrix interference was obtained (Figure 5B).

Lastly, the RP-LC was further fine-tuned by varying the percent composition of the organic solvent (acetonitrile), along with other parameters such as flow rate, column temperature and the sample injection volume to achieve fast and efficient separation conditions. The optimal separation of the analytes was achieved under ambient
Figure 5: The effect of the organic percent additive in the mobile phase on the separation of BPA-G and $d_6$-BPA-G from matrix interference. (A) 25% ACN 75% Buffer (5.00 mM ammonium acetate, pH 7.3) (B) 19.4% ACN 80.6% Buffer (5.00 mM ammonium acetate, pH 7.3).
Figure 6: The logD comparison of BPA-G and resveratrol
temperature on a Waters X-select HSS T3 column with retention times of 6.2 min for BPA-G, 6.1 min for $d_6$-BPA-G and 6.2 min for the IS, using a mobile phase consisting of 17% acetonitrile and 83% 5.00 mM ammonium acetate, pH 7.3 at a flow rate of 0.580 mL min$^{-1}$ with sample injection volume of 20.0 µL. Under these conditions, the previously observed interference was completely separated and resolved from the analytes (Figure 7). Therefore, during the first few minutes of SID-LC-MS/MS analysis, the effluent flow was diverted to waste to prevent contamination of the MS; hence the spike observed at tR=5.5 minutes is caused by switching the effluent flow from waste to the LC-MS/MS.

4.3.1.5 Sample preparation by LLE

In order to minimize the matrix interference due to the presence of co-eluting endogenous compounds in urine samples, two sample preparation techniques i.e., solid phase extraction (SPE) and liquid–liquid extraction (LLE) methods were evaluated. First, Strata X (C18) and Strata A-X (strong anion) SPE cartridges (Phenomenex, Torrance, California, USA) were tested. Although sufficient recoveries (>80%) were obtained on both cartridges, the recoveries were inconsistent and resulted in poor reproducibility. Moreover, SPE method used did not completely eliminate the matrix interference observed. Therefore, LLE using organic solvents i.e., methyl-t-butyl ether
Figure 7: The mass chromatograms (A) double blank-1 (BPA-G is readily detected) (B) double blank-2 (neither BPA-G, d6-BPA-G nor IS detected) (C) single blank (IS only, 20 ng mL\(^{-1}\)) (D) lowest urine calibrator (LLOQ, 0.500 ng mL\(^{-1}\)).
(MTBE), ethyl acetate and MTBE: ethyl acetate (1:1) in combination with various acids (i.e., 4%, 10% and 15% phosphoric acid and 1M HCL) was investigated. Significant ion suppression was observed when MTBE was used; therefore, it was abandoned. Urine samples acidified with 4% and 10% phosphoric acid, followed by extraction with ethyl acetate were found to have low recoveries whereas, those acidified with 1M HCL and 15% phosphoric resulted in high extraction efficiencies. In this work, ethyl acetate was chosen as the organic solvent for extraction in combination with 15% phosphoric acid due to the high signal response and low matrix interference, with matrix factors close to one (see section 4.3.2.2).

4.3.1.6 Creatinine normalization

Creatinine correction was performed to eliminate any effects of physiological urine dilution or concentration on patients’ samples. Creatinine is a breakdown product of creatinine phosphate, produced primarily in the muscle and excreted in urine. The rate of creatinine production is relatively constant, therefore, it’s used in urinary assays to normalize various metabolite concentrations [40, 41].

In this work, an LC-MS/MS method was developed to analyze creatinine in patients’ urine samples, because the commonly used methods (i.e., enzymatic and colorimetric assays) lack analytical selectivity. Creatinine and $d_3$-creatinine (IS) readily acquire a proton than lose one in electrospray ionization; therefore the positive mode was utilized in this work. As shown in (Figure 8A and 8C) creatinine produced
Figure 8: The full scan mass spectra of creatinine and IS and their major product ions.
a protonated molecular ion \([\text{Creatinine} + \text{H}]^+\) at m/z 114.0 and IS (\(d_3\)- creatinine) produced a protonated molecular ion \([\text{IS} + \text{H}]^+\) at m/z 117.0. Fragmentation of these molecular ions produced a major product ion at m/z 44.1 for creatinine and m/z 47.1 for the IS (Figure 8B and 8D). The mass transitions chosen for quantification in multiple-reaction-monitoring (MRM) were of m/z 114.0 → 44.1 and m/z 117.0 → 47.1 for the creatinine and IS respectively. Using a mobile phase of 12% acetonitrile and 88% 10.00 mM ammonium acetate, pH 6.8, at a flow rate of 0.300 mL min\(^{-1}\), chromatographic separation of creatinine and the IS was achieved on a Waters X-select HSS T3 column (2.1 mm i.d x 100 mm, 2.5 μm) in less than 2 min (Figure 9).

To determine the creatinine concentration in patient samples, standard calibration curves of creatinine in the mobile phase were constructed using the ABSciex MultiQuant™ Software by plotting the mean peak area ratios of creatinine to the IS (\(d_3\)- creatinine) against the creatinine concentration in standard calibrators for a linear regression equation (Figure 10). The calibration equation obtained using a 1/x weighted least-square linear regression was \(Y = 0.0250 x + 0.029\), \(r = 0.999\). A linearity of over three orders of magnitude \(i.e., 0.500 - 500 \text{ ng mL}^{-1}\) was obtained. The concentrations of creatinine in patient urine samples were back calculated with the peak area ratios of creatinine to those of the IS, using the calibration equation and dilution factor. In this study the BPA-G concentration in patient samples were normalized by the amount of creatinine in each sample and reported as creatinine normalized BPA-G concentrations.
Figure 9: The mass chromatograms of A) creatinine standard calibrator (50 ng mL\(^{-1}\)) and IS (25 ng mL\(^{-1}\)) B) patient urine sample after dilution (1.56 mg mL\(^{-1}\) creatinine) and IS (25 ng mL\(^{-1}\)).
"Linear" Regression ("1 / x" weighting): $y = 0.025 x + 0.0292$ ($r = 0.999$)

Figure 10: Standard calibration curve for creatinine.
4.3.2 Method validation

The method developed was validated in mouse plasma according to the US Food and Drug Administration (USFDA) guidelines [36-38] in terms of selectivity, lower limit of quantitation (LLOQ), linear response, recovery, matrix effect, accuracy, precision and stability for both short-term samples processing and long-term sample storage.

4.3.2.1 Selectivity and lower limit of quantitation (LLOQ)

The selectivity of the method was evaluated by comparing the chromatograms of six different lots of urine samples with the corresponding \( d_6 \)-BPA-G spiked urine matrices. BPA-G is readily detected in blank human urine (Figure 7A). In this work, human urine containing no detectable BPA-G was used as the blank urine matrix, and no endogenous interferences from the urine matrix were observed in the double blank at the same retention time and \( m/z \) of BPA-G, \( d_6 \)-BPA-G and IS (Figure 7B). In addition, no interference or cross-talk from the \( ^{13}C_{12} \)-BPAG (IS) was observed in the urine matrix at the retention time and \( m/z \) of BPA-G and \( d_6 \)-BPA-G (Figure 7C).

In this work, the LLOQ of the method was defined by lowest concentration on the calibration curve (0.500 ng mL\(^{-1}\)) with a limit of accuracy and precision within ± 20% and 20% respectively (Figure 7D). It was determined by analyzing five replicates of the
\(d_6\)-BPA-G with IS at LLOQ in six sources of human urine matrices, independent of the urine calibrators. The mean accuracy and mean precision of the method at LLOQ (TABLE II) were acceptable with \(\leq \pm 12\%\) of the relative error (RE) and \(\leq 6\%\) of the coefficient of variation (CV).

### 4.3.2.2 Matrix effect

The matrix effect was assessed by the matrix factor (MF). The absolute MF was determined in low-, med- and high-QC controls (1.50, 30.0 and 450 ng mL\(^{-1}\)) by comparing the peak area ratios of \(d_6\)-BPA-G to the IS in the spiked urine matrix after LLE \(n = 5\) with those prepared in the mobile phase. The IS normalized MF was determined by MF of \(d_6\)-BPA-G over the MF of the IS. As shown in TABLE III, the absolute MFs of \(d_6\)-BPA-G and IS ranged from 0.95-1.03 and 0.96-0.97 respectively. The IS normalized MFs were in the range of 0.98-1.06. These results revealed that the urine matrix effect was in the magnitude of -2% to 6% (<\(\pm 15\%\)), hence it was not significant and could be neglected.
TABLE II: ACCURACY AND PRECISION OF $d_6$-BPA-G AT LLOQ IN SIX DIFFERENT LOTS OF HUMAN URINE

<table>
<thead>
<tr>
<th>Urine matrix</th>
<th>Nominal $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>Mean measured $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>SD (ng mL$^{-1}$)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot A</td>
<td>0.500</td>
<td>0.52</td>
<td>0.09</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Lot B</td>
<td>0.500</td>
<td>0.47</td>
<td>0.03</td>
<td>-6</td>
<td>4</td>
</tr>
<tr>
<td>Lot C</td>
<td>0.500</td>
<td>0.44</td>
<td>0.01</td>
<td>-12</td>
<td>1</td>
</tr>
<tr>
<td>Lot D</td>
<td>0.500</td>
<td>0.45</td>
<td>0.04</td>
<td>-10</td>
<td>6</td>
</tr>
<tr>
<td>Lot E</td>
<td>0.500</td>
<td>0.47</td>
<td>0.01</td>
<td>-6</td>
<td>2</td>
</tr>
<tr>
<td>Lot F</td>
<td>0.500</td>
<td>0.47</td>
<td>0.01</td>
<td>-6</td>
<td>1</td>
</tr>
</tbody>
</table>

Each datum point calculated by five replicate measurements

%RE = [measured − nominal / nominal] × 100%; %CV = (SD/mean) × 100%.
TABLE III: MATRIX EFFECT OF \( d_6\)-BPA-G IN SIX INDIVIDUAL LOTS OF HUMAN URINE (\( N = 5 \))

<table>
<thead>
<tr>
<th>( [d_6\text{-BPA-G}] ) (ng mL(^{-1}))</th>
<th>( \text{MF}_{d_6\text{-BPA-G}} \ ± \ SD^a )</th>
<th>( \text{MF}_{\text{IS}} \ ± \ SD^b )</th>
<th>IS Normalized ( \text{MF} \ ± \ SD )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>0.95 ± 0.04</td>
<td>0.96 ± 0.01</td>
<td>0.98 ± 0.04</td>
</tr>
<tr>
<td>30.0</td>
<td>0.99 ± 0.01</td>
<td>0.96 ± 0.02</td>
<td>1.03 ± 0.02</td>
</tr>
<tr>
<td>450</td>
<td>1.03 ± 0.01</td>
<td>0.97 ± 0.03</td>
<td>1.06 ± 0.02</td>
</tr>
</tbody>
</table>

PA = Mean peak area.

\(^a\) \( \text{MF}_{d_6\text{-BPA-G}} = (\text{PA of } d_6\text{-BPA-G in extracted urine matrix})/ (\text{PA of } d_6\text{-BPA-G in mobile phase}) \).

\(^b\) \( \text{MF}_{\text{IS}} = (\text{PA of IS in extracted urine matrix})/ (\text{PA of IS in mobile phase}) \).

\(^c\) IS normalized \( \text{MF} = \text{MF}_{d_6\text{-BPA-G}}/\text{MF}_{\text{IS}} \).
4.3.2.3 Recovery

The absolute recovery of LLE was determined in low-, mid- and high- QC controls (1.50, 30.0, 450 ng mL$^{-1}$) by comparing the mean peak area of $d_6$-BPA-G and IS in the corresponding QC urine samples (n=5) with those of $d_6$-BPA-G and IS in the spiked in urine matrix after LLE. The IS normalized recovery was determined by the recovery of $d_6$-BPA-G over the recovery of the IS. TABLE IV shows the mean absolute recoveries for $d_6$-BPA-G ranged from 101-103% and 104% - 107% for the IS and the mean IS normalized recoveries obtained were 97%.

4.3.2.4 Carry over

Carry-over was assessed in each validation batch of calibrators by analysis of the standard calibrator with the highest concentration (500ng mL$^{-1}$) followed by an extracted blank urine sample. In this assay, no peak was observed in the plasma double blank at the retention time of BPA-G or $d_6$-BPA-G using 90% acetonitrile as the wash solvent. Hence no carry-over was observed in this work.
TABLE IV:  RECOVERY OF D6-BPA-G IN URINE MATRIX (N = 5)

<table>
<thead>
<tr>
<th>[d₆-BPA-G] (ng mL⁻¹)</th>
<th>Recovery\textsubscript{d₆-BPA-G} ± SD (%)</th>
<th>Recovery\textsubscript{IS} ± SD (%)</th>
<th>IS Normalized Recovery ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>102 ± 2</td>
<td>105 ± 3</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>30.0</td>
<td>101 ± 2</td>
<td>104 ± 1</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>450</td>
<td>103 ± 1</td>
<td>107 ± 2</td>
<td>97 ± 2</td>
</tr>
</tbody>
</table>

PA = Mean peak area.

Recovery of d₆-BPA-G = (PA of d₆-BPA-G in urine matrix / PA of d₆-BPA-G in extracted urine matrix) x 100%.

Recovery of IS = (PA of IS in urine matrix / PA of IS in extracted urine matrix) x 100%.

IS normalized Recovery = (recovery of d₆-BPA-G / recovery of the IS) x 100%.
4.3.2.5 Accuracy and precision

The intra-run accuracy and precision were determined by five replicate measurements of each low-, mid- and high- QC samples (1.50, 30.0, 450 ng mL$^{-1}$) as well as dilution QC 1.50 x 10$^3$ ng mL$^{-1}$) in the same validation batch. The accuracy was expressed as percent relative error (%RE) and the precision expressed as coefficient variation (% CV). The inter-run accuracy and precision was determined by five parallel measurements of five identical sets of each QC samples in five different validation batches. As shown in TABLE V the intra-run accuracy and precisions of all the QCs samples were ≤ ± 7% and ≤ 3% and the inter-run accuracy and precisions were ≤ ± 10% and ≤ 4% respectively. These results indicate that this method has adequate reproducibility and accuracy.

4.3.2.6 Linearity

The calibration curves of $d_6$-BPA-G in urine were constructed using a double blank (with neither BPA-G, $d_6$-BPA-G nor IS), a single blank (zero calibrator, with IS only) and seven non-zero $d_6$-BPA-G urine calibrators (0.00, 0.500, 1.00, 5.00, 10.0, 50.0,100, and 500 ng mL$^{-1}$) with the concentration of the IS at 10.0 ng mL$^{-1}$. The mean peak area ratios of $d_6$-BPA-G to the IS were plotted against the nominal concentration of $d_6$-BPA-G in urine. The calibration equation derived from five batches of calibration
<table>
<thead>
<tr>
<th>Nominal $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>Measured $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>SD (ng mL$^{-1}$)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>1.47</td>
<td>0.06</td>
<td>-2</td>
<td>3</td>
</tr>
<tr>
<td>30.0</td>
<td>32.1</td>
<td>0.7</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>450</td>
<td>481</td>
<td>7</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>$1.50 \times 10^3$</td>
<td>$1.44 \times 10^3$</td>
<td>$1.0 \times 10^1$</td>
<td>-4</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nominal $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>Measured $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>SD (ng mL$^{-1}$)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>1.39</td>
<td>0.06</td>
<td>-8</td>
<td>4</td>
</tr>
<tr>
<td>30.0</td>
<td>29.6</td>
<td>0.5</td>
<td>-1</td>
<td>2</td>
</tr>
<tr>
<td>450</td>
<td>409</td>
<td>2</td>
<td>-9</td>
<td>1</td>
</tr>
<tr>
<td>$1.50 \times 10^3$</td>
<td>$1.35 \times 10^3$</td>
<td>$1.0 \times 10^1$</td>
<td>-10</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ Determined by five replicate measurements of each QC sample within a validation batch.

$^b$ Determined by five parallel measurements of five identical QC samples at each concentration over five validation batches.

$^c$ The dilution QC was measured by a 10 times dilution.
curves using a 1/x weighted least-square linear regression was \( Y = 0.0652 (\pm 0.0028) x + 0.0282(\pm 0.0097) \), \( r = 9997 (\pm 0.0004) \). A linearity of over three orders of magnitude (0.500-500 ng mL\(^{-1}\)) was obtained in urine with high correlation coefficient. The accuracy and precision of \( d_6\)-BPA-G urine calibrators were \( \leq \pm 12 \) and \( \leq 13 \) respectively (TABLE VI).

### 4.3.2.7 Stability study

The short term stability of \( d_6\)-BPA-G in human urine was tested under various storage conditions and was determined by comparing the mean peak-area ratios of \( d_6\)-BPA-G to the IS in the test sample to those of freshly prepared samples and expressed as a percent recovery.

As shown in TABLE VII, the recoveries for BPA-G stock solutions (23°C) at 6 and 24h time period were 95-98%. The recoveries for BPA-G in urine at 6 and 24h time period were 97-101% and 98-104%, for the bench-top (23°C) and the auto sampler (4°C) conditions respectively. The three freeze-and-thaw cycles (-20 to 23°C and -80 to 23°C) were in the range of 93-104%. The recovery at long term storage (-20°C and -80°C, 30 days) were 98-101%. These studies indicate that \( d_6\)-BPA-G is stable under the experimental conditions tested and so is BPA-G, since no significant loss or degradation was observed.
TABLE VI: ACCURACY AND PRECISION OF $d_6$-BPA-G URINE CALIBRATORS OVER FIVE VALIDATION BATCHES.

<table>
<thead>
<tr>
<th>Nominal $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>Measured $[d_6$-BPA-G] (ng mL$^{-1}$)</th>
<th>SD (ng mL$^{-1}$)</th>
<th>%RE</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.500</td>
<td>0.560</td>
<td>0.120</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>1.00</td>
<td>1.1</td>
<td>0.1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>5.00</td>
<td>5.2</td>
<td>0.4</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>10.0</td>
<td>10.5</td>
<td>0.6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>50.0</td>
<td>51.0</td>
<td>2.0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>102</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>500</td>
<td>490</td>
<td>19</td>
<td>-2</td>
<td>4</td>
</tr>
<tr>
<td>Test conditions</td>
<td>Temperature (°C)</td>
<td>$d_6$-BPA-G$^a$</td>
<td>Recovery ± SD (%) (n = 3)</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------</td>
<td>----------------</td>
<td>-------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>Stock solution</td>
<td>98 ± 2</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Bench-top</td>
<td>23</td>
<td>Low QC</td>
<td>99 ± 4</td>
<td>97 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>104 ± 2</td>
<td>104 ± 1</td>
</tr>
<tr>
<td>Autosampler</td>
<td>4</td>
<td>Low QC</td>
<td>98 ± 3</td>
<td>99 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>104 ± 2</td>
<td>104 ± 1</td>
</tr>
<tr>
<td>3 Freeze-thaw cycles</td>
<td>-20 to 23</td>
<td>Low QC</td>
<td></td>
<td>93 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td></td>
<td>104 ± 2</td>
</tr>
<tr>
<td>3 Freeze-thaw cycles</td>
<td>-80 to 23</td>
<td>Low QC</td>
<td></td>
<td>96 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td></td>
<td>101 ± 1</td>
</tr>
<tr>
<td>Long-term (30 days)</td>
<td>-20</td>
<td>Low QC</td>
<td>100 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>98 ± 1</td>
<td></td>
</tr>
<tr>
<td>Long-term (30 days)</td>
<td>-80</td>
<td>Low QC</td>
<td>101 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>High QC</td>
<td>99 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The concentration of $d_6$-BPA-G stock solution was 100 µg mL$^{-1}$ which was measured by serial dilution to 1.50 ng mL$^{-1}$ in mobile phase. The concentrations of $d_6$-BPA-G in the low and high QCs were 1.50 and 450 ng mL$^{-1}$ respectively.
4.3.2.8 Method application

The applicability of the validated stable isotope dilution LC-MS/MS method was demonstrated by analysis of BPA-G concentrations in 40 patients’ samples to assess variability within this patient population. Prior to analysis, the patients’ urine samples were thawed at room temperature. The urine samples, along with urine calibrators and QCs were prepared by the procedure mentioned in section 4.2.4, and analyzed by the LC-MS/MS method developed. The concentrations of $d_6$-BPA-G in patients’ urine samples were back calculated with the peak area ratios of $d_6$-BPA-G to those of the IS using the calibration equation. Figure 11 shows examples of SID-LC-MS/MS mass chromatograms corresponding to patients’ urine samples with no BPA-G detected (Figure 11A) and with 12.2 g mL$^{-1}$ BPA-G detected (Figure 11B).

The concentrations of creatinine in patient urine samples were determined by the LC-MS/MS method developed (see section 4.2.10) and the BPA-G measured from each patient’s sample was normalized by the amount of creatinine in each sample. The BPA-G concentrations in patient samples were reported as µg g$^{-1}$ of creatinine (i.e., creatinine normalized BPA-G concentrations). BPA-G was detected and quantified in 65% (n = 13/20) of the analyzed samples with creatinine-normalized BPA-G concentrations ranging from ND (not detected) to 7.82 µg g$^{-1}$ of creatinine (TABLE VIII). This method is useful for the evaluation and assessment of human exposure to BPA.
Figure 11: Mass chromatogram of patients (A) P1 urine sample, with neither BPA-G nor $d_6$-BPA-G detected and IS (20 ng mL$^{-1}$) (B) P10 urine sample with 12.2 ng mL$^{-1}$ BPA-G detected, no $d_6$-BPA-G detected and IS (20 ng mL$^{-1}$).
<table>
<thead>
<tr>
<th>Patient #</th>
<th>Sample ID</th>
<th>Measured [BPA-G] (ng mL(^{-1}))</th>
<th>Measured [Creatinine] (mg mL(^{-1}))</th>
<th>Creatinine-Corrected [BPA-G] µg g(^{-1}) Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>2155</td>
<td>ND</td>
<td>0.597</td>
<td>ND</td>
</tr>
<tr>
<td>P2</td>
<td>2156</td>
<td>0.534</td>
<td>0.968</td>
<td>0.552</td>
</tr>
<tr>
<td>P3</td>
<td>2162</td>
<td>ND</td>
<td>0.194</td>
<td>ND</td>
</tr>
<tr>
<td>P4</td>
<td>2165</td>
<td>ND</td>
<td>1.40</td>
<td>ND</td>
</tr>
<tr>
<td>P5</td>
<td>2171</td>
<td>6.07</td>
<td>2.85</td>
<td>2.13</td>
</tr>
<tr>
<td>P6</td>
<td>2178</td>
<td>1.56</td>
<td>1.79</td>
<td>0.870</td>
</tr>
<tr>
<td>P7</td>
<td>2179</td>
<td>ND</td>
<td>0.889</td>
<td>ND</td>
</tr>
<tr>
<td>P8</td>
<td>2180</td>
<td>ND</td>
<td>0.861</td>
<td>ND</td>
</tr>
<tr>
<td>P9</td>
<td>2181</td>
<td>1.02</td>
<td>1.72</td>
<td>0.592</td>
</tr>
<tr>
<td>P10</td>
<td>2182</td>
<td>12.2</td>
<td>1.56</td>
<td>7.82</td>
</tr>
<tr>
<td>P11</td>
<td>2187</td>
<td>2.03</td>
<td>1.33</td>
<td>1.53</td>
</tr>
<tr>
<td>P12</td>
<td>2186</td>
<td>0.815</td>
<td>2.08</td>
<td>0.391</td>
</tr>
<tr>
<td>P13</td>
<td>4000</td>
<td>5.57</td>
<td>0.972</td>
<td>5.73</td>
</tr>
<tr>
<td>P14</td>
<td>4037</td>
<td>ND</td>
<td>0.565</td>
<td>ND</td>
</tr>
<tr>
<td>P15</td>
<td>4041</td>
<td>4.97</td>
<td>2.45</td>
<td>2.03</td>
</tr>
<tr>
<td>P16</td>
<td>4079</td>
<td>4.76</td>
<td>3.12</td>
<td>1.52</td>
</tr>
<tr>
<td>P17</td>
<td>4088</td>
<td>2.02</td>
<td>2.16</td>
<td>0.934</td>
</tr>
<tr>
<td>P18</td>
<td>4090</td>
<td>ND</td>
<td>0.682</td>
<td>ND</td>
</tr>
<tr>
<td>P19</td>
<td>4091</td>
<td>1.63</td>
<td>0.789</td>
<td>2.06</td>
</tr>
<tr>
<td>P20</td>
<td>4093</td>
<td>5.44</td>
<td>1.32</td>
<td>4.13</td>
</tr>
<tr>
<td>P21</td>
<td>3939</td>
<td>0.531</td>
<td>1.73</td>
<td>0.307</td>
</tr>
<tr>
<td>P22</td>
<td>3924</td>
<td>5.25</td>
<td>3.33</td>
<td>1.58</td>
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<td></td>
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<tr>
<td>---</td>
<td>-----</td>
<td>---</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>P23</td>
<td>3906</td>
<td>ND</td>
<td>2.52</td>
<td>ND</td>
</tr>
<tr>
<td>P24</td>
<td>3904</td>
<td>ND</td>
<td>0.707</td>
<td>ND</td>
</tr>
<tr>
<td>P25</td>
<td>3900</td>
<td>1.01</td>
<td>1.56</td>
<td>0.652</td>
</tr>
<tr>
<td>P26</td>
<td>3899</td>
<td>0.972</td>
<td>0.477</td>
<td>2.04</td>
</tr>
<tr>
<td>P27</td>
<td>3898</td>
<td>2.84</td>
<td>1.83</td>
<td>1.55</td>
</tr>
<tr>
<td>P28</td>
<td>3895</td>
<td>0.616</td>
<td>1.95</td>
<td>0.316</td>
</tr>
<tr>
<td>P29</td>
<td>4042</td>
<td>ND</td>
<td>0.190</td>
<td>ND</td>
</tr>
<tr>
<td>P30</td>
<td>4027</td>
<td>0.728</td>
<td>0.817</td>
<td>0.891</td>
</tr>
<tr>
<td>P31</td>
<td>4019</td>
<td>ND</td>
<td>2.50</td>
<td>ND</td>
</tr>
<tr>
<td>P32</td>
<td>4003</td>
<td>ND</td>
<td>0.867</td>
<td>ND</td>
</tr>
<tr>
<td>P33</td>
<td>3999</td>
<td>0.677</td>
<td>1.04</td>
<td>0.654</td>
</tr>
<tr>
<td>P34</td>
<td>3996</td>
<td>ND</td>
<td>0.807</td>
<td>ND</td>
</tr>
<tr>
<td>P35</td>
<td>3976</td>
<td>0.962</td>
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4.4 Conclusions

A stable isotope dilution LC-MS/MS method for specific and accurate quantitative measurement of BPA-G in human urine has been developed and validated. The method uses deuterated BPA-G ($d_6$-BPA-G) as the calibration standard and carbon-13 labeled BPA-G ($^{13}$C$_{12}$-BPA-G) as the internal standard (IS). A simple liquid-liquid extraction protocol was used for efficient urine samples preparation, reverse phase-liquid chromatography (RP-LC) for separation and tandem mass spectrometry for detection. The method has high analyte selectivity and sensitivity with an LLOQ of 0.500 ng mL$^{-1}$ in urine matrix. It has good accuracy and precisions as well as the stability of routine analysis. This method was successfully applied to the measurement of BPA-G concentrations in urine samples of 40 patients and may be useful in biomonitoring and toxic kinetics studies of BPA.

4.5 References


[31] Kosarac I, Kubwabo C, Lalonde K, Foster W. A novel method for the quantitative determination of free and conjugated bisphenol A in human maternal and umbilical cord


