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Validation and comparison of three sample preparation techniques for quantitation of amobarbital, butalbital and phenobarbital in blood and urine using UFLC-MS/MS

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VALIDATION AND COMPARISON OF THREE SAMPLE PREPARATION TECHNIQUES FOR QUANTITATION OF AMOBARBITAL, BUTALBITAL AND PHENOBARBITAL IN BLOOD AND URINE USING UFLC-MS/MS

by

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ABSTRACT

This research study successfully completed three objectives: 1) validate liquid-liquid, supported-liquid, and solid-phase extractions for the quantitation of three barbiturates (amobarbital, butalbital, and phenobarbital) in blood and urine using liquid chromatography-tandem mass spectrometry; 2) to compare the efficiency and effectiveness among methods in accomplishing extraction of barbiturates under the laboratory setting at Boston University School of Medicine; and 3) to report all the analytical data to RTI International for interlaboratory comparison.

For the validation study, a six-point linear calibration model (20-2000 ng/mL) with inversely weighted concentration ($\frac{1}{x}$) was reproducible in all three sample preparation methods for both blood and urine with r^2 greater than or equal to 0.994. Bias and precision evaluated from three controls throughout the range of the curve were both within ±20% and ±20%CV, respectively. Neither carryover nor interference was observed. Detection limits were evaluated down to 5 ng/mL depending on the extraction procedure. Samples were able to be diluted up to 50 times prior to instrumental analysis. Samples were stable on autosampler at room temperature up to 72 hours after their initial analysis. Recovery of

barbiturates from blood and urine all ranged from 45% to 86%. The effect of ionization suppression or enhancement was found to have minimal impact on the validation.

For choosing the most suitable method quantifying barbiturates, efficiency and effectiveness were studied. Efficiency evaluates the time and ease of sample preparation required to prepare a sample for analysis. Supported-liquid extraction was found to be the most efficient method for extracting barbiturates as it required the least amount of time to perform and could be easily automated with minimal training. Effectiveness is an assessment of one's ability to selectively recover target analyte at a reasonably low concentration. By considering a method's recovery, extract cleanliness, detection limits, and reproducibility, liquid-liquid extraction was the best at quantifying barbiturates in blood and supported-liquid extraction was the most suitable method for extracting barbiturates from urine.

For interlaboratory comparison, all the data collected has been reported to RTI International. These findings can be used for examining the overall reliability and reproducibility of the validated methods. Results obtained can also be used to explore the possibility for streamlining sample preparation in the forensic laboratory, and hence reducing the case backlog.

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LIST OF ABBREVIATIONS

AAFS	American Academy of Forensic Sciences
ACN	Acetonitrile
Amo	Amobarbital
ASB	American Academy of Forensic Sciences Standards Board
BUMC	Boston University School of Medicine
Butal	Butalbital
CFS	Center of Forensic Sciences
CNS	Central Nervous System
CSA	Controlled Substances Act
DCM	Methylene Chloride
DEA	Drug Enforcement Administration
ESI	Electrospray Ionization
HLB	Hydrophilic-Lipophilic Balanced
H-bond	Hydrogen Bond
IS	Internal Standard
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography – Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
µL	Microliter

µg	Microgram
mg	Milligram
mL	Milliliter
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometer
m/z	mass-to-charge
Na_2HPO_4	Sodium Phosphate Dibasic Anhydrous
$NaH_2PO_4 \cdot H_2O$	Sodium Phosphate Monobasic Monohydrate
ng	Nanogram
Pheno	Phenobarbital
R	Correlation Coefficient
r^2	Coefficient of Determination
RTI	RTI International
Si-OH	Silanol
SLE	Supported-Liquid Extraction
SPE	Solid-Phase Extraction
SWGTOX	Scientific Working Group on Forensic Toxicology
T0	Time zero at initial analysis
T24	Twenty-four hours after initial analysis
T48	Forty-eight hours after initial analysis
T72	Seventy-two hours after initial analysis
UCT	United Chemical Technologies

UFLC	Ultra-Fast Liquid Chromatograph

1. INTRODUCTION

1.1 History, Discovery, and Current Trends

Barbiturates are a class of drugs that act as a nervous system (CNS) depressant. They were vastly used as sedative, hypnotic, anticonvulsant/ antiepileptic, and anesthetic drugs in the 20th century[1]. The discovery of this drug can be traced back to the synthesis of barbituric acid (or malonylurea) from malonic acid and urea in 1864 (Figure 1)[2] by Adolf von Baeyer. In 1905, Baeyer was awarded the Nobel Prize in Chemistry for his work on organic dyes and hydroaromatic compounds in 1905.[3] Barbituric acid itself has no medical value, yet, in the process of development during the early 20th century to synthesize compounds for medical use, modifications to the novel substance were extensive, thus leading to the start of the entire barbiturate era.

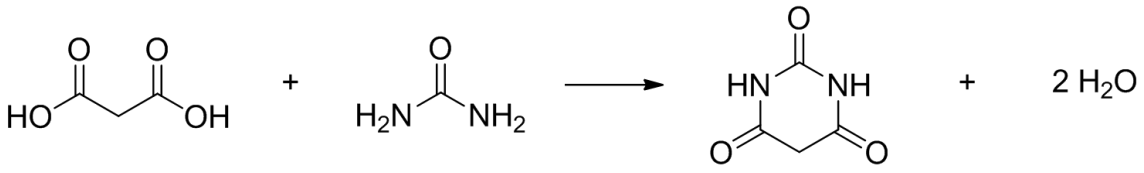


Figure 1. Synthesis of Barbituric Acid From Malonic Acid and Urea With the Loss of Water

The first barbiturate that made its way onto the market was 5,5-diethylbarbituric acid, also known as barbital or Veronal®.[1] It was found to have sedative, hypnotic, and anticonvulsant properties and was on clinical trial for the treatment of sleeping disorders in 1904.[1] However, phenobarbital, released in 1912 with the brand name Luminal®, was actually the first drug effective for psychiatric medication.[1] Compared to its predecessors, such as alcohol, paraldehyde, chloral hydrate, bromide or Veronal® , phenobarbital is more potent in inducing sleep, which let to its clinical prevalence of being used for insomnia or sleeping disorder therapy.[1] In addition, the sedative effect of phenobarbital also comes with an anticonvulsant action able to reduce the severity of seizures. As a result, phenobarbital became one of the first drugs for treating epilepsy in the early 20th century.[4]

The success of phenobarbital did not stop the development of new drugs. Research on creating new barbiturates by the slight modification of barbituric acid continued. In the early to mid-20th century, more than 2500 barbiturates were synthesized and approximately 50 of them made their way to the clinical market, including amobarbital, marketed as Amytal®, in 1923.[4] Between 1920 to the mid 1950s, with a peak during the 1940s, barbiturates were practically the only drug used as sedatives and hypnotics for clinical purposes.[1] Some of these compounds were also used for treating neurosis and psychosis.[1] Although the use of barbiturates was common, they did have some disadvantages. Psychiatrists or physicians at that time did not consider the adverse side effects that barbiturate medications might have, such as their synergistic effects, chance for addiction and overdose death.[1] In the mid-20th century, the lack of health policy and drug monitoring in early times led to a generation of barbiturate abuse.[4] This situation was alleviated by the

introduction of benzodiazepines in the 1950s, also a group of sedative and anticonvulsants but with a greater margin of safety.[4] With a safer substitute available, the gradual adoption of benzodiazepines for medication began, resulting in the continuous decline in the number of prescriptions for barbiturates over the mid to late-20th century.

Today, only 12 types of barbiturates are used for therapeutic purposes.[5] Phenobarbital, in particular, is still a prevalent option as a sedative for treating gastrointestinal and asthmatic functional disorders.[1] Although barbiturates are less commonly prescribed, they are still abused and detected in forensic cases. Detection methods for barbiturates is therefore necessary in the toxicology laboratories.

1.2 Recreational Use and Scheduling

Although barbiturates have been greatly replaced by benzodiazepines for therapeutic purposes, they are still a prevalent choice of abuse among the public. Barbiturates are mainly classified based on duration of action: long-acting, intermediate-acting, short-acting, and ultra-short acting (Table 1).[1] Recreational users usually prefer short or intermediate-acting barbiturates, such as amobarbital and secobarbital.[5] However, because of its small threshold between therapeutic and lethal dose, barbiturate overdose can occur. In response to this threat to public health, many barbiturates have been scheduled by the Drug Enforcement Administration (DEA) under the Controlled Substances Act (CSA).[5] Scheduling a drug under the CSA requires an evaluation of the drug according to three criteria: medical use, potential for abuse, and potential for psychological or physical dependence.[4] Since there are still 12 types of barbiturates used for medical purposes, they can only be scheduled within Schedule II to V due to their high potential for abuse and dependence. Currently, barbiturates are categorized as Schedule II, III, and IV depressants under the CSA (Table 1).[5] These schedules apply to all forms of barbiturates that exists regardless of whether they come in as pill, capsule, tablet, injectable solution or with other components.[5]

Table 1. Duration of Action and Scheduling for Common Barbiturates

Barbiturate	Brand/Trade Name	Duration of Action	Schedule
Phenobarbital	Luminal®	Long	IV
Amobarbital	Amytal®	Intermediate	II
Butalbital	Fiorinal®	Intermediate	III
Pentobarbital	Nembutal®	Short	II
Secobarbital	Seconal®	Short	II
Thiopental	Pentothal®	Ultrashort	III

1.3 Chemical Properties

1.3.1 Chemical Structures

Barbiturates are a modification of barbituric acid and have both hydrophilic and hydrophobic characteristics. Its hydrophilicity depends on the three carbonyl groups (-C=O) and the substituents (-H or -CH_3) at N1 and N3 positions, while additions at C5 position determine its hydrophobicity (Figure 2).[6] However, barbiturates are synthesized predominantly by having modifications at the barbituric acid C5 position at which the α-carbon has high tendency to release a proton (H^+) due to the resulting resonance stabilization by the aromatic conjugated system (Figure 3).[2] This reactive α-hydrogen allows various alkyl or aromatic additions to the barbituric acid, hence the formation of different barbiturates (Figure 3), including amobarbital (Amo), butalbital (Butal), and phenobarbital (Pheno) (Figure 4). Countless barbiturates have been created over the years using this process and the structure-activity relationship has therefore been extensively studied.[2] Different substituents at the C5 position can generate different actions. For example, increasing the length of the aliphatic chain (hydrophobicity) could increase the potency of barbiturate yet decrease its duration of activity; and extending the chain on substituent Rb over 7 carbons would make the barbiturate a CNS stimulant.[2]

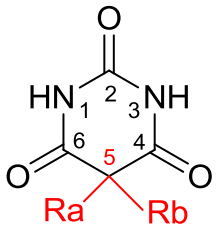


Figure 2. General Structure of Barbiturates (Ra, Rb = alkyl, allyl, or aromatic)

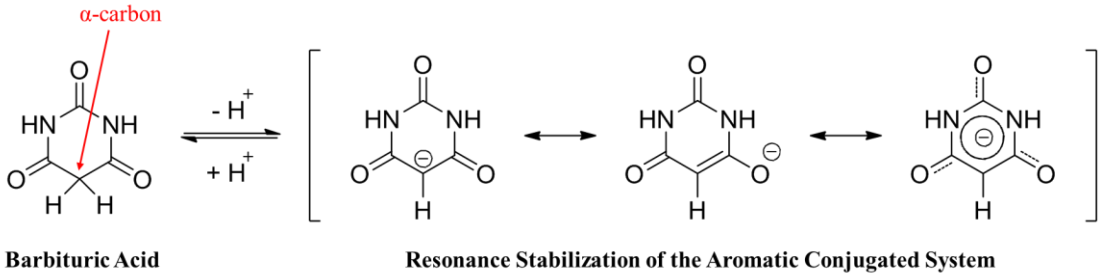


Figure 3. Reactive α-hydrogen on Barbituric Acid and Resonance Stabilization of the Resulting Aromatic Conjugated System

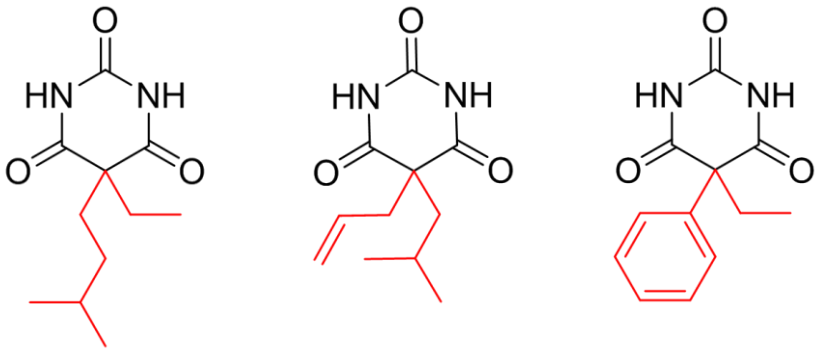


Figure 4. Structures of (A) Amobarbital, (B) Butalbital, and (C) Phenobarbital

1.3.2 Acidity

Barbiturates, unlike most of the modern drugs, are acidic. Its acidity originates from the proton (H^+) attached to the nitrogen atoms at positions 1 and 3 on the 2,4,6-pyrimidinetrione backbone.[6] The resonance stabilization when losing a proton makes them weak acids (Figure 5). Any substituent on a barbiturate disrupting this electron delocalizing system would decrease that acidity. The relative acidity of different barbiturates can therefore be ranked. Barbituric acid, despite the absence of medical value, is the most acidic compound among this category because it has no substituent. Addition at position C5 similar to those on amobarbital, butalbital, and phenobarbital (Figure 4) makes barbiturate a weaker acid because the alkyl group (electron-donating group) disrupts the electron delocalization by inductive effect. The same goes for the nitrogen atoms at position 1 and 3. Acidity decreases if one of the hydrogens connected to the nitrogen is substituted, while barbiturate is non-acidic if both the protons on the nitrogen atoms are eliminated.[6] The acidity also makes barbiturate possible to be dissolved in water. This can be done by first reacting acidic barbiturate with a strong base. The resulting, salt form is water-soluble.

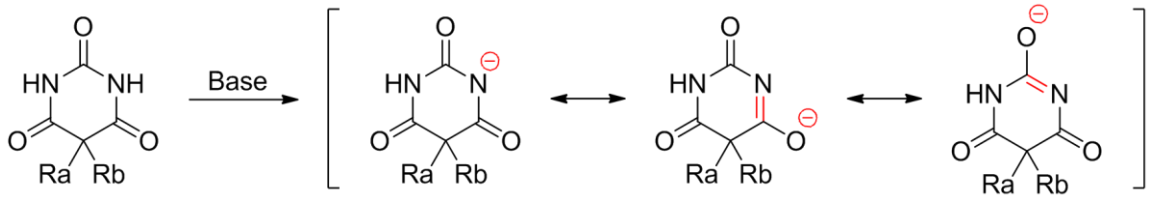


Figure 5. Resonance Stabilization of Barbiturate Conjugate Base

2. SAMPLE PREPARATION

Sample preparation is a common pretreatment step for instrumental analysis. It is optional in some disciplines or laboratories, where techniques such as the dilute-and-shoot method are used, yet in forensic toxicology, it is often an essential step for analysis. Forensic toxicology laboratories usually receive biological cases related to death investigations or other criminal events such as driving under the influence or drug facilitated sexual assault. To analyze drugs in biological matrices, direct injection of sample into the instrument will often not generate useful results because matrix components may interfere with analyte detection, resulting in low sensitivity and recovery of the target compound. It may also damage analytical instruments or contaminate instrument parts. Removal of biological matrices and other unwanted materials (i.e., obtaining clean target analyte) through sample preparation is therefore necessary for analyzing samples in most forensic toxicology cases.[7]

Various sample preparation techniques are available, ranging from non-selective methods providing less clean extract to highly selective technology offering great analyte recovery (Figure 6). Which technique to use is usually a choice of the laboratory but the rationale behind selection is always a consideration of three basic aspects: matrix composition, pH and pK_a, and polarity.

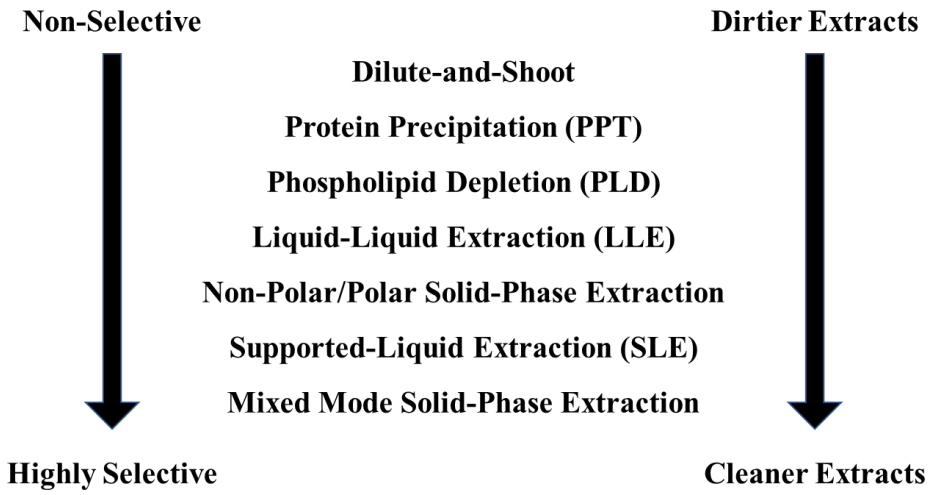


Figure 6. Selectivity and Cleanliness of Various Sample Preparation Techniques[7]

2.1 Matrix Compositions

To extract target analyte(s) out of the biological matrix, knowledge of the composition of each matrix is essential. Human blood and urine are the two most commonly encountered biological matrices in forensic toxicology casework. Blood is a composition of cellular elements (~45%) and plasma (~55%). Cellular elements consist of red blood cells (erythrocytes), white blood cells (leukocytes), and platelets, while plasma contains mainly water (~92%), salts, plasma proteins and other materials transported by blood, such as nutrients, wastes, respiratory gases, and hormones (Figure 7).[7] Urine is composed of mostly water (~95%) with other metabolic by-products, such as urea, uric acid, creatinine, and various electrolytes (Figure 8).[7] Since blood and urine are mainly water, while most of the drugs of abuse today are organic compounds,[2] the selection of proper sample preparation technique will need to take this aqueous-organic relationship between the biological specimen and the target analyte into account.

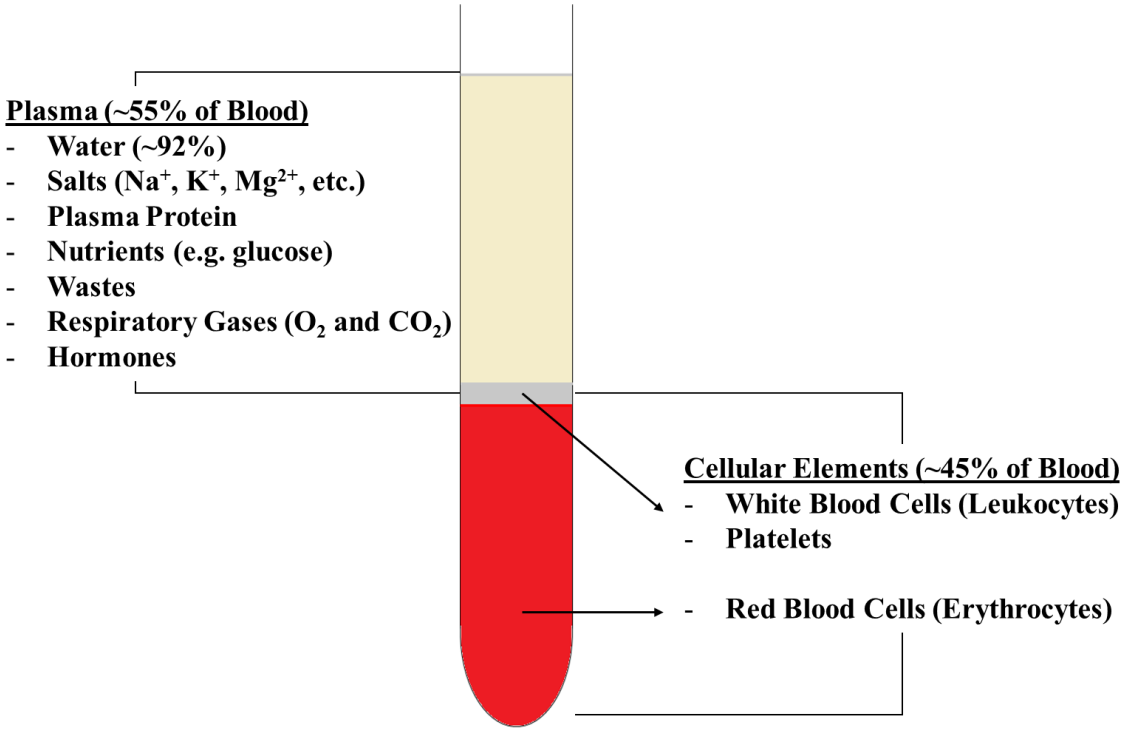


Figure 7. Composition of Human Blood

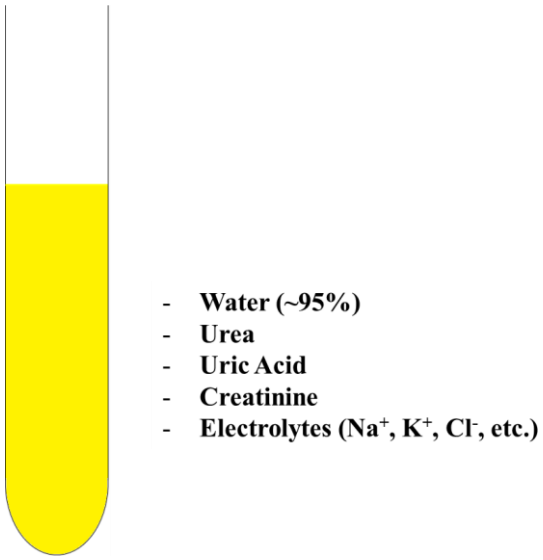


Figure 8. Composition of Human Urine

2.2 pH and pK_a

Drugs of abuse can be acidic, neutral, or basic. Their acidity (or basicity) may be described by a drug's pK_a values. Acidic drugs have low pK_a values while basic drugs have high pK_a (low pK_b) values. The pK_a value of a drug also determines the ionization state of the drug under different pH conditions according to the Henderson-Hasselbalch equation:

$$For\ weakly\ acidic\ drug\ (HA)\ (\mathrm{HA} \rightleftharpoons \mathrm{H^+} + \mathrm{A^-}):\ pH = pK_a + log\frac{[A^+]}{[HA]}$$

$$For\ weakly\ basic\ drug\ (B)\ (\mathrm{BH^+} \rightleftharpoons \mathrm{B} + \mathrm{H^+}):\ pH = pK_a + log\frac{[B]}{[BH^+]}$$

A drug is 50% ionized (or dissociated) and 50% non-ionized (or neutral) when the pH of its surrounding environment equals to the pK_a of the drug. Altering each pH unit of the environment will affect the concentration ratio of the ionized and non-ionized drugs by a factor of 10. A two-unit difference between environmental pH and drug's pK_a will lead to an almost 100% ionization or neutralization of the drug depending on its acidity (Table 2) or basicity (Table 3). The pK_a of drug is therefore a crucial factor to be considered when choosing a sample preparation method because proper pretreatment can determine the ionization state of a target drug, and hence its efficiency of separating analyte(s) from other materials during extraction.

Table 2. Change of Medium pH in Relation to the Percent Ionization of Weakly Acidic Drug

Medium pH	$[A^-]/[HA]$	Percent of Ionized Acidic Drug (%)	Percent of Unionized Acidic Drug (%)
$pK_a + 2$	100:1	~99	~1
$pK_a + 1$	10:1	90	10
pK_a	1:1	50	50
$pK_a - 1$	1:10	10	90
$pK_a - 2$	1:100	~1	~99

Table 3. Change of Medium pH in Relation to the Percent Ionization of Weakly Basic Drug

Medium pH	$[B]/[BH^+]$	Percent of Ionized Basic Drug (%)	Percent of Unionized Basic Drug (%)
$pK_a + 2$	1:100	~1	~99
$pK_a + 1$	1:10	10	90
pK_a	1:1	50	50
$pK_a - 1$	10:1	90	10
$pK_a - 2$	1100:1	~99	~1

2.3 Polarity

Nearly all the drugs encountered today, as mentioned in section 2.1, are organic compounds.[2] Their chemical structures vary with different functional groups and thus possess different degree of polarity (Figure 9). Polar compounds have a higher affinity towards polar medium over the non-polar medium due to the stronger intermolecular force (dipole-dipole interaction) existing in between. Compounds with functional groups containing electronegative atoms (e.g., oxygen, nitrogen) can even form hydrogen bonds (H-bond) with other compounds in an aqueous environment. The polarity of an analyte is therefore another important factor to be considered when selecting a sample preparation method as it affects the extraction efficiency of that drug from the sample mixture.

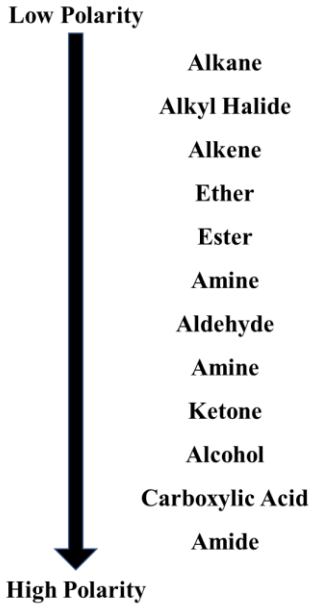


Figure 9. Polarity of Different Functional Groups of Organic Compounds

3. RESEARCH BACKGROUND AND OBJECTIVE

Drug-related death is currently one of the biggest challenges faced by the United States. Requests for toxicological analysis arrive every day at forensic laboratories. With limited resources and analysts, cases received by a laboratory are usually not handled immediately but stored prior to analysis. As time goes by, more cases would be backlogged, hence creating a burden on the laboratory. To help tackle the backlog issue, outsourcing part of the forensic analysis to private laboratories or hiring more analysts are two possible solutions. Other means include utilizing efficient sample preparation methods or employing state-of-the-art instrumentation.

Sample preparation methods in particular, have been researched and improved by manufacturers all along. Novel separation techniques emerging in recent years have been proven to offer greater sensitivity and faster analytical processes while maintaining analyte integrity. However, even with the potential to reduce case backlogs, many forensic laboratories are still reluctant to shift from traditional techniques to new methods. Common reasons for not initiating the change include the lack of time and resources to validate and optimize new method(s); the performance uncertainty of a new method for a specific drug or matrix; as well as the favor of traditional methods due to easily accessible literature evaluating protocols and effectiveness, despite their lengthy and time-consuming procedures. In response to these hindrances of change, Center of Forensic Sciences (CFS) at RTI International (RTI) has initiated a collaborative project with Boston University School of Medicine (BUMC) to help streamline sample preparation, and hence potentially help to reduce laboratory backlog.[8]

The purpose of this collaborative project was to evaluate various sample preparation methods for the extraction of common drugs in different biological matrices. Liquid-liquid extraction (LLE), solid-phase extraction (SPE), and supported-liquid extraction (SLE) were chosen to compare the efficiency between traditional (LLE and SPE) and novel (SLE) extraction methods. The capability of a method to perform multi-analyte extraction is also worth consideration as often only small sample volumes are available for analysis. A minimum of 25 drugs and metabolites were therefore chosen from eight common drug classes for evaluation, i.e. sympathomimetic amines, antidepressants, barbiturates, designer drugs, hallucinogens, opiates, and miscellaneous, such as cocaine and PCP. Blood, urine, and oral fluid were selected as targeted matrices as they are the most commonly encountered biological matrices in forensic toxicology.

Despite having the same analyte and testing method, discrepancy among analytical results always exist between laboratories. The variability can be attributed to the difference in operating conditions (e.g., instrumentation) or the involvement of human variables/inconsistency (e.g., manual extraction steps), etc. To take these interlaboratory differences into account, a comparative project between two laboratories was proposed and this was the purpose of the collaboration between RTI and BUMC. Each laboratory developed and validated sample preparation techniques for a selected group of drugs and matrices previously mentioned. In addition to the evaluation of method performance within one laboratory, analytical data collected from both laboratories will be used collaboratively to examine the methods' overall reliability and reproducibility. Results obtained can be

used to explore the possibility for streamlining sample preparation methods in forensic laboratories, and hence potentially reducing case backlogs.

This research study constitutes part of the BUMC collaborative project with RTI. The objectives of this specific study are to validate three different sample preparation methods (LLE, SLE, and SPE) for the quantitation of three barbiturates (amobarbital, butalbital, and phenobarbital) in blood and urine using liquid chromatography-tandem mass spectrometry (LC-MS/MS); to compare the efficiency and effectiveness between methods in accomplishing extraction of barbiturates under the laboratory setting at BUMC; and to report all the analytical data to RTI for interlaboratory comparison.

4. MATERIALS AND METHODS

4.1 Chemicals and Reagents

Amobarbital (Amo), Butalbital (Butal), Phenobarbital (Pheno) and their respective deuterated internal standards (IS) (Amo-d_5, Butal-d_5, and Pheno-d_5) were purchased from Cerilliant Inc. (Austin, TX, USA). LC-MS grade methanol was purchased from Fisher Chemical (Waltham, MA, USA). Standards came in ampules at 1 mg/mL, while internal standards were at 100 µg/mL. Stock solution 1 was made up in methanol with all three barbiturates evaluated at 100,000 ng/mL. Stock solution 2 was made up in methanol with all three barbiturates evaluated at 10,000 ng/mL. Working calibration solutions 1-6 were made up in methanol using stock solutions 1 and 2 at 200, 500, 1500, 5,000, 10,000, and 20,000 ng/mL. Working control solutions (low, medium, high) were made up in methanol using stock solution 1 at 600, 9,500, and 18,000 ng/mL. Working IS solution was made up in methanol with all deuterated barbiturates at 5,000 ng/mL. Blank human blood was obtained from Boston Medical Center (Boston, MA, USA), Biological Specialty Corporation (Colmar, PA, USA), Equitech Enterprises Inc. (Kerrville, TX, USA) and UTAK Laboratories Inc. (Valencia, CA, USA). Human urine was obtained from donation through approved Institutional Review Board protocols (Boston University School of Medicine, Boston, MA, USA). LC-MS grade hexane, ethyl acetate, methylene chloride (DCM), and acetonitrile (ACN) were obtained from Fisher Chemical (Waltham, MA, USA). Formic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate dibasic anhydrous (Na_2HPO_4) and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) were obtained from Acros Organics (Pittsburgh, PA, USA). Ammonium

acetate was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water used was purified using Milli-Q® Type 1 Ultrapure Water Systems from MilliporeSigma (Burlington, MA, USA).

4.2 Liquid-Liquid Extraction (LLE)

4.2.1 Principle

Liquid-liquid extraction is one of the traditional means used in chemistry to separate a target analyte from a mixture. It works by the partitioning of compounds in a mixture into two immiscible liquid phases based on their affinity or interaction with each phase. This process is usually enhanced by shaking or rocking the vessel to create larger surface area for reaching partition equilibrium. A successful LLE will have only the target analyte partitioned into one phase and the rest of the mixture into another phase. The two solvent phases appear as immiscible layers and based on their densities, the layer containing the target drug is separated out. In forensic toxicology, the most common scenario involving compound separation is the extraction of target drug from a biological matrix for analysis. Since most drugs are organic and biological matrices contain mainly aqueous ingredients as mentioned in Section 2.1, an aqueous-organic LLE can be used. It can be done by mixing the sample mixture with an aqueous phase, followed by the addition of organic solvent in which protein or cellular elements are insoluble.

To facilitate the partitioning of a drug from the aqueous layer into organic solvent, the non-ionic neutral form of drug is desired. This can be accomplished by having a buffer as the aqueous phase and adjusting its pH two units lower (higher) than the pKa of the acidic (basic) drug. If a target drug is neutral in nature, it can be extracted in all pH conditions (from 0 to 14).[2] However, the extract may demand a further cleaning step as it is isolated passively by first removing the acid and base fractions of the mixture. The LLE design under this situation might rely more on the drug's polarity. In this study,

amobarbital, butalbital, and phenobarbital are the target analytes. They are weakly acidic drugs and have pKa values between 7 to 9 (Figure 4). Phosphate buffer at pH 5 was therefore used as the aqueous phase to convert all ionized drug to their non-ionized form, hence ready the analytes for being extracted into the organic phase.

As mentioned in Section 2.3, most drugs possess different degrees of polarity. Adjusting the polarity of organic solvent in LLE can therefore improve the partition specificity, resulting in the extraction of the target drug out of the aqueous layer. This can be done by mixing a non-polar organic solvent, such as hexane, with other polar organic solvents that have the ability to donate (e.g., chloroform) or accept (e.g., diethyl ether) hydrogen in a H-bond. Alcohols (e.g. isopropanol, butanol) are a common choice for this practice as they can form H-bonds in either case. The proportion of each organic solvent in the mix is actually flexible. The ratio of solvents can be adjusted according to the polarity of the target compound. For example, 1-5% of isopropanol in hexane is effective in extracting phenothiazine from blood due to its chemical structure.[2] In this study, 50% of ethyl acetate by volume in hexane was chosen to extract barbiturates. This was used because the oxygen atoms on ethyl acetate are able to form H-bonds with the hydrogen atoms at positions N1 and N3 on barbiturate (Figure 10).[2] This can facilitate the partitioning of barbiturate from biological matrix to the organic layer.

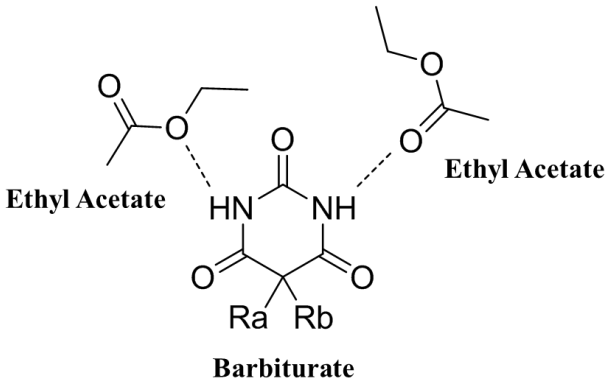


Figure 10. H-Bond Interactions Between Ethyl Acetate and Barbiturate

4.2.2 Procedure

To 200 µL of drug free blood or urine in a 7 mL screw top vial, 20 µL of IS was added (except for the double blank; sample with blank matrix only), followed by 0.5 mL of pH 5 phosphate buffer into all samples. After vortexing, 1.5 mL of extraction solvent (Hexane:Ethyl Acetate 50:50) was added into the solution. The vial was then capped, rocked for 10 minutes, and centrifuged at 3500 rpm for 5 minutes. The top organic layer was then transferred into a 15 mL test tube and was evaporated to dryness at 40ºC. Dried sample was reconstituted in 100 µL of mobile phase (0.1% formic acid in water) for LC-MS/MS analysis.

4.3 Supported-Liquid Extraction (SLE)

4.3.1 Principle

Supported-liquid extraction is an emerging sample preparation technique analogous to aqueous-organic LLE. Similar to LLE, the separation works by the partitioning of target analyte and sample mixture into two immiscible phases.[9] However, unlike increasing the partition interface by shaking or rocking in LLE, the contact surface area in SLE is enhanced by dispersing the liquid aqueous phase over a modified diatomaceous earth-based material in a cartridge. This material is designed with a network of pores. It is inert to sample and solvent, yet can retain the aqueous phase as small droplets and facilitate the formation of aqueous layer all over. When applying immiscible organic solvent into the cartridge, it flows through the material and comes in contact with the aqueous phase droplets on the surface. Partitioning of the target analyte and sample mixture between two phases therefore occurs over a large surface area supported by the material. Further addition of organic solvent through the sorbent bed allows partitioning to happen again, hence imitating a repeat LLE and maximizing the extraction.[9]

The extraction of a drug from biological matrix with SLE starts with buffering the sample to ensure the unionized form of the drug is achieved. The sample in aqueous buffer solution is then loaded into the cartridge and is waited to spread over the modified diatomaceous earth-based material. Applying organic solvent similar to that used in LLE, in which the protein and cellular elements of the matrix are insoluble, the drug can be eluted into a collection vessel for instrumental analysis (Figure 11). In this study, ammonium acetate buffer (100 mM) at pH 5 was used to convert weakly acidic amobarbital, butalbital,

and phenobarbital (pK_a values between 7 to 9) into their unionized forms. DCM was chosen as the elution solvent because its chlorine atoms can form H-bonds with the hydrogen atoms at positions N1 and N3 on barbiturate (Figure 12),[2] therefore facilitating the partition of barbiturate into the organic phase.

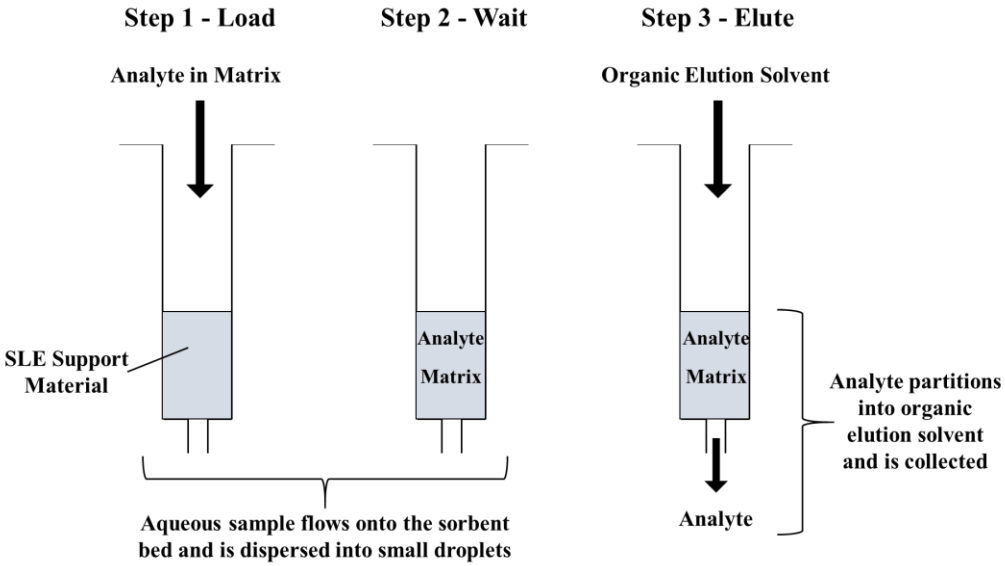
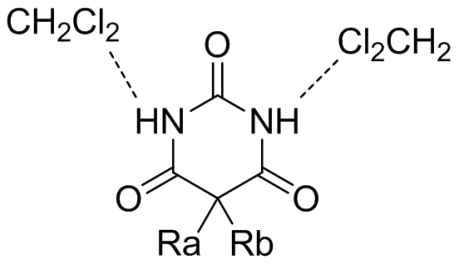


Figure 11. Load-Wait-Elute Procedure for SLE[9]



Barbiturate

Figure 12. H-Bond Interactions Between DCM and Barbiturate

4.3.2 Procedure

To 300 µL of blank blood or urine in a 6 mL test tube, 20 µL of IS was added (except for the double blank), followed by 300 µL of pH 5 ammonium acetate buffer (100 mM) into all samples. After vortexing, sample was loaded into a Biotage ISOLUTE® SLE+ cartridge (1 mL sample volume) (Uppsala, Sweden) for extraction. A pulse of pressure was applied on the cartridge to initiate flow. After absorbing for 5 minutes, the sample was eluted by gravity into a 15 mL test tube with 2.5 mL of DCM. Elution was performed again into the same test tube after waiting for another 5 minutes. The extraction was then completed by applying a pulse of pressure on the cartridge. Sample collected in the test tube was evaporated to dryness at 40ºC. Dried sample was reconstituted in 100 µL of mobile phase (0.1% formic acid in water) for LC-MS/MS analysis.

4.4 Solid-Phase Extraction (SPE)

4.4.1 Principle

Solid-phase extraction, technically also known as liquid-solid phase extraction, is one of the most frequently used sample preparation methods today in the analytical laboratory. It originated from column chromatography that utilized the interaction with packing materials in a column for characterization.[2] However, instead of using a large, tall glass column for chromatography, SPE utilizes a small cartridge or column packed with a specifically designed sorbent for separation. The goal of SPE is to first retain target analyte in cartridge; then remove unwanted components in the mixture; and later recover and concentrate target analyte by eluting through the column with a little solvent.[2] The entire extraction will be most effective if the proper sorbent material chosen.

Common sorbents include bonded silica material and polymer. Bonded silica sorbent is made by joining the silicon atom or silanol group (Si-OH) on the surface of a refined silica particle covalently to selectively active groups.[11] Polymer packing material, on the other hand, does not contain silica but of hydrocarbons incorporated with selectively active groups. Active groups are functional groups manufactured on a sorbent surface that selectively interact with a target analyte and hence influence retention as the analyte passes through the cartridge. These groups be hydrocarbon chain (e.g., C2, C4, C8, C18, phenyl group, etc.) for the extraction of non-polar analytes via non-polar intermolecular force, hydroxyl group (e.g., silanol or diol group) for retaining polar analytes,[10] or anion/cation exchange site for ionized analytes.[2] Anion exchange sites usually constitute positively charged quaternary amines for the retention, and hence the extraction, of ionized acidic

analytes (negatively charged), while cation exchange sites are usually composed of negatively charged sulfonic acid or carboxylic acid for the extraction of ionized basic compounds (positively charged).[10] In fact, sorbent materials (for both bonded silica and polymer) can possess more than one selectively active groups and when they do, they are regarded as mixed mode sorbents offering primary and secondary interactions with the sample.[7] For example, a polymer sorbent can have a long hydrocarbon chain on one end and an anion exchange site on the other end, allowing it to extract acidic yet non-polar analytes from the sample mixture.[10] What sorbent type used depends on the chemical properties of the analyte of interest and in forensic toxicology, drugs of abuse in biological matrix are common targets. Since most of the drugs, as mentioned in Sections 2.2 and 2.3, possess certain degrees of acidity (or basicity) and polarity at the same time, SPE cartridges containing a mixed mode sorbent is a popular choice for many forensic toxicology laboratories.

Regardless of the sorbent type used, many of the sorbents in SPE cartridges are extremely hydrophobic due to their silica or hydrocarbon-based composition. Target analytes, which are usually in aqueous phase (polar) after sample pretreatment, will not be retained in the sorbent at all even with the selective active groups available on the sorbent surface. Application of water-miscible organic solvent, such as methanol or ACN, into the cartridge to generate an interface for the hydrophobic sorbent and hydrophilic sample to interact is therefore essential.[11] This constitutes the first step of the general five-step SPE procedure: condition, equilibrate, load, wash, and elute. The second step is to equilibrate the cartridge with sample-miscible (water-miscible) buffer. It is to ensure the active groups

on the sorbent surface are at desired state (e.g. ionization state of ion exchange site) for the retention of the target analyte and the release of unwanted materials. After the buffer has passed through and without drying the sorbent, similarly buffered pretreated sample can be loaded onto the cartridge. With the interaction between the active groups and the sample occurring over the sorbent, washing solvents can be used to selectively remove the undesired materials while leaving the target analyte unaltered. Once the contaminants are washed off, the target analyte can be separated by elution into a collection vessel. The elution solvent used in this final step should be able to specifically disrupt the retention interaction between the sorbent active groups and the target analyte, but also retain the unwanted material which may have endured the washing step.[11]

Although this five-step procedure is the general practice for SPE, some SPE cartridges neither require conditioning nor equilibrating steps, allowing a three-step or less SPE procedure. These cartridges are usually water-wettable, meaning in addition to the extremely hydrophobic structure and selective active groups, the sorbent incorporates hydrophilic groups as well. This hydrophilic group functions the same as the water-miscible organic solvent added during the normal conditioning step, thus permitting interaction between hydrophobic sorbent and hydrophilic sample without conditioning or equilibrating the cartridge. Since the sorbent surface is already active with the water-wettable cartridge, avoiding drying the sorbent bed during sample preparation is no longer a concern.[11]

The Waters (Milford, MA, USA) hydrophilic-lipophilic balanced (HLB) cartridge is one example of a water-wettable SPE cartridge. The sorbent is composed of hydrophobic

(lipophilic) polymers with hydrophilic groups on the surface (Figure 13),[12] thus allowing the elimination of cartridge conditioning and equilibration (3-step procedure) steps. In addition to the 3-step procedure, this cartridge can also be used for a 2-step cleanup according to the manufacturer's protocol (Figure 14).[13] During the 2-step extraction, pretreated sample is first loaded into the cartridge. Target analyte is then eluted into a collection vessel while leaving the unwanted materials on the sorbent. In this study, a 2-step procedure with an HLB cartridge was adopted for the extraction of barbiturates, yet two modifications were made. The first change was the addition of elution solvent (ACN) into the sample during pretreatment, thus combining the load and elution steps. The second change was the addition of methanol and DI water conditioning steps to facilitate the extraction.

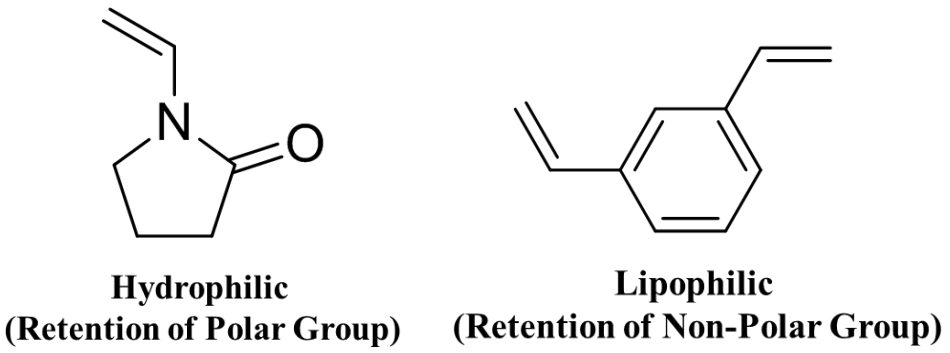


Figure 13. Structures of Hydrophilic-Lipophilic Balanced (HLB) Copolymers[12]

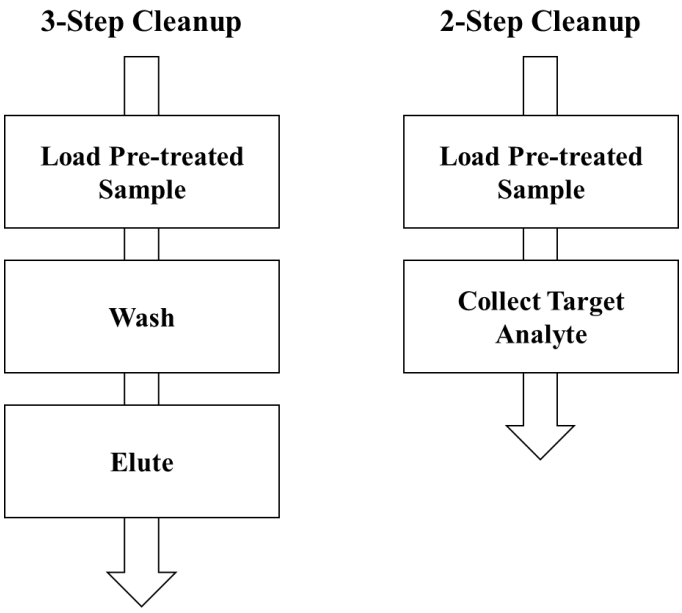


Figure 14. Three-Step and Two-Step Procedures Waters HLB Cartridge[13]

4.4.2 Procedure

To 200 µL of blank blood or urine in a 6 mL test tube, 20 µL of IS was added (except for double blank), followed by 1 mL of cold ACN into all samples. After vortexing, the samples were centrifuged at 4000 rpm for 5 minutes. Waters Oasis PRiME HLB cartridge (3cc, 60 mg) in a United Chemical Technologies (UCT) (Bristol, PA, USA) positive pressure manifold was utilized for extraction. The cartridge was conditioned by methanol (2 mL) and deionized water (2 mL). To the conditioned columns, sample was loaded and allowed to pass through by gravity, followed by positive pressure at 1 to 2 mL/min, and quick full vacuum. Sample was collected in a 15 mL test tube and was evaporated to dryness at 40ºC. Dried sample was reconstituted in 100 µL of mobile phase (0.1% formic acid in water) for LC-MS/MS analysis.

4.5 Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

4.5.1 Principle

Chromatography is a method using two phases, i.e. stationary phase and mobile phase, to separate components in a mixture. Liquid chromatography (LC) means liquid mobile phase is utilized for separation as well as identification.[2] There are four basic components in an LC system. They are the solvent reservoirs, the pumps, the sample injector, and the LC column. The pumps are used to deliver mobile phase stored in solvent reservoirs and the sample from injector to the LC column.

The LC column is where separation occurs and is packed with materials acting as the stationary phase. Stationary phase is usually relatively non-polar (while mobile phase is relatively polar) as most of the LC systems are operated in reversed phase. When the solvents (mobile phase) containing the sample mixture pass through the LC column (stationary phase), separation of the mixture begins. The entire separation works based on the differential affinity of target analyte towards the mobile phase and the stationary phase. If one component has higher affinity towards the mobile phase over the stationary phase, it will be eluted out of the column earlier and vice versa. The total time for an analyte to be retained in the column before being eluted out for detection is called the retention time. Retention time is usually specific to a particular analyte under the same chromatographic conditions. Identification using LC is therefore theoretically available.[14]

For each component in a mixture to be eluted out of the LC column at a specific time, they have to be effectively resolved from one another to a certain degree. Apart from using a LC column with suitable packing material or having a guard column to minimize

contaminants, effective separation can also be accomplished by having appropriate elution conditions. Isocratic elution and gradient elution are the two commonly used elution types for LC.[14] Isocratic elution means only one solvent or a single composition of solvent mixture is used as mobile phase, while gradient elution is the use of two or more solvents with gradual change in composition over the course of a method. The latter is often preferred as mobile phase polarity can be adjusted easily by changing the composition of solvents. This allows fine separation of mixture components based on polarity to happen without the need to alter the LC column. Separation efficiency is therefore greatly enhanced.

Mass spectrometry (MS) is a technique used to characterize and identify sample components in a mixture. The MS system consists of three major components: ion source, mass analyzer, and ion detector. When a sample is carried into the MS system, it is first fragmented and ionized by the ion source. Charged or ionized sample fragments will then be transferred to the mass analyzer where unique ions are selected and detected based on their mass-to-charge (m/z) ratio. A quadrupole mass spectrometer is the most common mass analyzer used for this purpose. It filters the fragment ions by altering the direct current and radio frequency voltages across four conduction rods. Only fragments with selected m/z ratio are allowed to reach the ion detector for detection. Since each component in a sample will have a unique fragmentation pattern under a specific MS condition, identification or characterization can be made by examining the resulting ionized fragments in the mass spectrum.[14]

Electrospray ionization (ESI) is one of the common ion sources used to produce ions from liquid sample prior to entry into MS from LC when these two analytical

techniques are coupled together. ESI works by charging the liquid sample either positively or negatively during nebulization so that when undergoing evaporation, a sample will be fragmented automatically due to the extensive increase in charge density. Since ESI is a soft ionization technique, most of the samples will remain intact without fragmentation after ionization. Tandem mass spectrometers (MS/MS) are common mass analyzers used in conjunction with LC, thus forming LC-MS/MS. In MS/MS, there are three quadrupole mass spectrometers. After receiving the ionized samples from the ion source, the first quadrupole detects the parent transition by isolating ions of interest for further fragmentation. Further fragmentation happens in the second quadrupole, also called the collision chamber. Ionized sample fragments will then be transferred into the third quadrupole and the ion detector for analysis. Ions selected by the first quadrupole are called precursor ions, while ions being analyzed at the third quadrupole are the product ions.[14] With the advantage of triple quadrupoles, multiple precursor ions from a sample and their respective product ions can be targeted and monitored, i.e. multiple reaction monitoring (MRM). This allows the identification of specific analytes from an unknown sample. Overall, LC-MS/MS is able to separate and identify target analyte from a sample. With the use of a calibration curve, controls and internal standards, LC-MS/MS can also be used for quantitation.

4.5.2 Hardware and Software

All analyses were conducted using a Waters XBridge™ C18 LC column (3.5 µm particle size; 50 mm length; 2.1 mm internal diameter) on a Shimadzu Ultra-Fast Liquid Chromatograph (UFLC) coupled to a SCIEX 4000 QTRAP® MS/MS. The Shimadzu UFLC system consisted of a DGU-20A3R degassing unit, two LC-20AD pumps, a SIL-20ACHT auto-sampler, a CTO-20A column oven, and a CBM-20A LC system controller (Kyoto, Japan). The SCIEX 4000 QTRAP® system contained a hybrid triple quadrupole/linear ion trap tandem mass spectrometry with a Turbo V™ source for ESI (Framingham, MA, USA). Data was collected by Analyst® Software (version 1.6.2), while statistical analysis was performed using MultiQuant™ Software (version 3.0.5373.0).

4.5.3 Parameters

Sample injection volume was 10 µL (Table 4) with the flow rate maintained at 0.6 mL/minute. Column oven temperature was set at 40ºC throughout the analysis. The total sample run time was 6.5 minutes. Two mobile phases were used in this study and they were 0.1% of formic acid in water (mobile phase A) and 0.1% of formic acid in ACN (mobile phase B). Gradient elution was utilized during the analysis, starting with 90% mobile phase A from the beginning to 90% mobile phase B at the end (Table 5). Approximate retention times for each barbiturate and their respective IS are showed in Figure 15. All samples passing through the liquid chromatography system were then ionized in negative ESI mode

and detected by the tandem mass spectrometer in MRM scanning mode. The precursor and product (quantifying and qualifying) ions for each barbiturate and IS are listed in Table 6.

Table 4. Auto-Sampler (SIL-20ACHT) Parameters

Rinsing Volume	1000 µL
Rinsing Speed	35 µL/second
Rinse Dip Time	5 seconds
Needle Stroke	52 mm
Sampling Speed	3.0 µL/second
Purge Time	25 minutes
Cooler Temperature	15°C
Injection Volume	10 µL

Table 5. Time Program for Gradient Elution

Time (minute)	**Mobile Phase A (%)**	**Mobile Phase B (%)**
0.01	90	10
4	30	70
5	10	90
5.5	10	90
6.01	90	10
6.5	Stop	

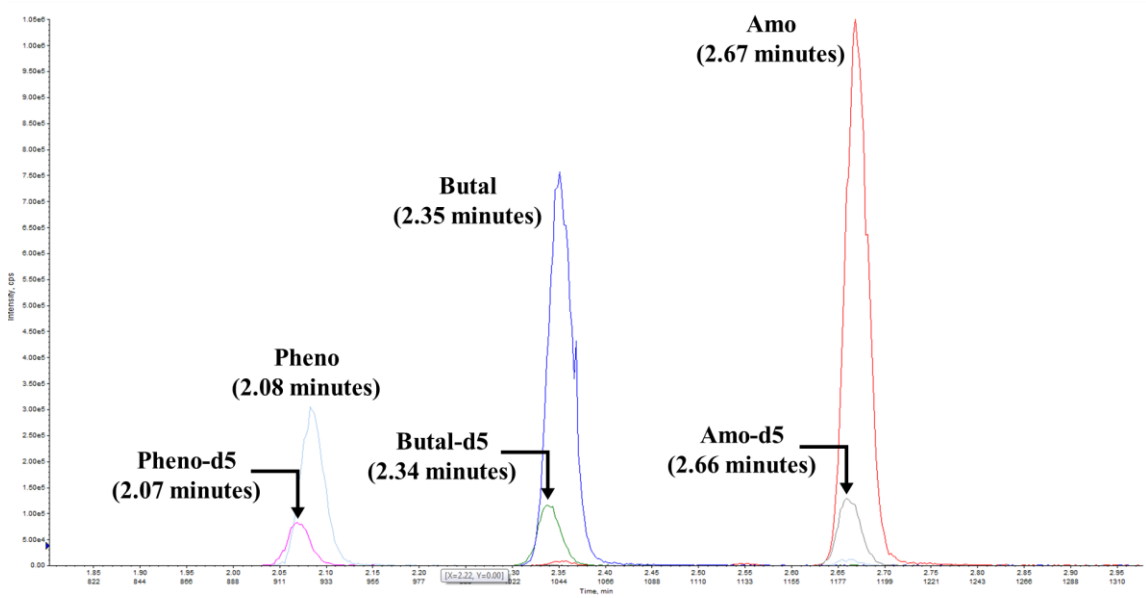


Figure 15. Total Ion Chromatogram Showing the Approximate Retention Times for Each Barbiturate and Respective Internal Standard

Table 6. Precursor and Product (Quantifying and Qualifying) Ions for Each Barbiturates and IS in MRM Mode

Barbiturate and IS	Precursor Ions	Product Ions (Quantifying)	Product Ions (Qualifying)
Amobarbital	224.9	42.2	182.0
Amobarbital-d_5	229.9	42.0	187.0
Butalbital	222.9	42.2	180.1
Butalbital-d_5	228.0	42.0	184.9
Pentobarbital	225.0	42.0	181.7
Pentobarbital-d_5	230.1	42.0	186.9

5. METHOD VALIDATION

Method validation involves a set of experimental procedures performed to objectively demonstrate that a particular analytical method is valid and is fit for its purpose under normal laboratory conditions. It is also conducted to identify a method's limitations before implementation of the actual casework analysis. Validation of a method is needed when a laboratory 1) intends to employ a new analytical method; 2) modifies an existing method for improvement; 3) evaluates equivalent performance between existing and new methods; and 4) needs to demonstrate an existing method is meeting newly establish criteria.[15] The guidelines for validating an analytical method varies among different disciplines. In forensic toxicology, laboratories generally follow the American Academy of Forensic Sciences (AAFS) Standards Board (ASB) standard, which was originally drafted by the Scientific Working Group on Forensic Toxicology (SWGTOX)[16] for method validation. This standard illustrates the parameters required for validation for each kind of analytical technique, as well as the minimum criteria for each validation parameter to be acceptable. As mentioned in section 3, the purpose of this study is to validate sample preparation methods for the quantitation of barbiturates in LC-MS/MS. The abilities of each sample preparation technique to extract and facilitate the detection and quantification of barbiturates need to be demonstrated. According to the ASB standard, ten parameters are required to be assessed to validate a quantitative method. They are calibration model, carryover, limit of detection (LOD), limit of quantification (LOQ), bias and precision, dilution integrity, stability, interference studies, ionization suppression or enhancement, and recovery.[15]

5.1 Calibration Model

Calibration model of a method is the working range of concentration in which a target analyte can be detected and quantified based on its response ratio (area of analyte over area of IS). It is evaluated by having at least six calibrators, preferably matrix-matched, spanning across different non-zero concentrations over five separate runs. For each calibrator and control, the ion ratio (area of qualifying ion over area of quantifying ion) has to be within ±20% of the average ion ratio;[17] and the calculated concentration against the calibration curve have to be within ±20% of the expected value. Coefficient of determination (r^2), the square of correlation coefficient (r), and standardized residual plots are also the parameters to assess the calibration model. Any concentration calculated that lies outside ±3 standard deviation of the expected value will be treated as an outlier. In this situation, a transformation of variables, and hence the calibration model to other regression models maybe needed.

In this study, a six-point linear calibration model with inversely weighted concentration ($\frac{1}{x}$) was desired. Matrix-matched calibrators at 20, 50, 150, 500, 1000 and 2000 ng/mL; and controls at 60, 950 and 1800 ng/mL were prepared from working calibration solutions and working control solutions, respectively, for evaluation. Internal standard at 500 ng/mL prepared from working IS solution was also included in both calibrators and controls.

5.2 Carryover

Carryover means the appearance of preceding sample response on subsequent sample detection during instrumental analysis. It will lead to inaccurate results and therefore needs to be assessed. Carryover is usually assessed by evaluating a blank matrix sample immediately after a sample fortified at a high concentration. In this study, it was evaluated by running a double blank, blank matrix without internal standard, immediately after the highest calibrator of every calibration curve. Any signal higher than 10% of the lowest calibrator response observed in the double blank at the correct retention time was treated as carryover and the sample was re-analyzed.

5.3 Detection Limits

There are two types of detection limits: limit of detection (LOD) and limit of quantitation (LOQ). LOD indicates the lowest possible concentration at which an analyte can be detected, while LOQ expresses the lowest possible concentration at which an analyte can be detected and quantified. LOD can be evaluated using background noise by preparing at least three different blank matrices (double blanks) and fortified samples at decreasing concentrations in duplicate over three separate runs. A concentration level is regarded as the LOD if the samples fortified at that level 1) has an average response signal

greater than or equal to the average background noise of blank matrix ($X_{Blank\ Matrix}$) plus 3.3 times of its standard deviation ($S_{Blank\ Matrix}$). expressed by the following formula:

$$LOD = X_{Blank\ Matrix} + 3.3(S_{Blank\ Matrix})$$

and 2) is acceptable for other predefined criteria, such as ion ratio within ±20% of average, peak shape, ±5% retention time, etc.

Limit of quantitation is usually evaluated concurrently with LOD by preparing at least three different blank matrices fortified at decreasing concentrations in duplicate over three separate runs. This approach requires concentrations to be within the previously established calibration model. Therefore, when attempting to quantify samples at a concentration lower than the lowest calibrator, control samples along those levels are needed. Similar to LOD, fortified samples need to meet the acceptable predefined criteria, including bias and precision (to be discussed in section 5.4), in order to be deemed as LOQ.

In this study, three sets of blank matrices and their fortified samples at 5, 10, 15, and 20 ng/mL were analyzed in duplicate with controls at 10, 15, and 20 ng/mL over three separate runs for the evaluation of LOD and LOQ.

5.4 Bias and Precision

Bias (or accuracy) indicates how close the concentrations measured by a method are to their expected values, while precision is an indicator of how close the results are to each other. Bias and precision are usually evaluated together. This is done by analyzing three different samples per concentration at three concentration levels (low, medium, and high) over five separate runs. The low concentration should be at least 3 times the lowest calibrator; the high concentration should be at least 80% of the highest calibrator; and the medium concentration should be near the midpoint of the low and high controls. Bias for each concentration can be calculated by the following formula:

$$Bias\ (\%)\ at\ a\ Concentration$$
$$= \left(\frac{Average\ of\ Calculated\ Concentration - Expected\ Concentration}{Expected\ Concentration}\right) \times 100\%$$

For precision, within-run and between-run precision is of interest for method validation. Within-run precision is the precision at each control replicate with each of the five runs. Between-run precision is the precision at each concentration over five runs. Precision is expressed by coefficient of variation (%CV) and calculated by the formula below:

$$Precision\ (\%CV)\ at\ a\ Concentration$$
$$= \frac{Standard\ Deviation\ \ of\ Calculated\ Concentration}{Average\ of\ Calculated\ Concentration} \times 100\%$$

Within-run precision is calculated by:

$$Within\text{-}Run\ Precision\ (\%CV)\ at\ a\ Concentration$$
$$= \frac{Standard\ Deviation\ \ of\ Calculated\ Concentration\ over\ a\ Single\ Run}{Average\ of\ Calculated\ Concentration\ over\ a\ Single\ Run} \times 100\%$$

And between-run precision is calculated by:

$$Between\text{-}Run\ Precision\ (\%CV)\ at\ a\ Concentration$$

$$= \frac{Standard\ Deviation\ \ of\ Calculated\ Concentration\ over\ Five\ Runs}{Average\ of\ Calculated\ Concentration\ over\ Five\ Runs} \times 100\%$$

For a method to be acceptable, its bias, within-run and between-run precision at each concentration level have to be within ±20% or 20%CV, respectively. The ion ratio for each calculated concentration against the calibration curve needs to be within ±20% of the average ratio as well.

In this study, bias and precision were evaluated concurrently by running matrix-matched controls in triplicate per concentration over the three concentration levels in five separate runs. The concentrations for low, medium, and high controls were 60, 950 and 1800 ng/mL, respectively.

5.5 Stability

There are circumstances in which samples may need to be re-analyzed a few days later after it was first extracted. Unforeseen situations include poor injection, carryover, instrumental error, etc. The length of time an extracted sample can stay unchanged at room temperature (i.e. in autosampler) before re-injection needs to be studied in order to evaluate method robustness. It is commonly assessed by preparing matrix-matched samples fortified at low and high concentrations in triplicate, followed by comparing the average response ratio (area of IS over area of analyte) of those analyzed immediately after sample preparation (time zero, T0) to that of those re-analyzed at regular intervals later. A sample is stable for re-analysis at certain times later only if the bias of average response ratio at that time point is within ±20%, which is calculated by the following formula:

$$Bias\ (\%)\ of\ Average\ Response\ Ratio\ at\ a\ Concentration$$
$$= \left(\frac{Average\ Response\ Ratio\ at\ a\ Time\ Point - Average\ Response\ Ratio\ at\ Time\ Zero}{Average\ Response\ Ratio\ at\ Time\ Zero}\right) \times 100\%$$

The stability in this study was evaluated by also having matrix-matched controls in triplicate per concentration over three concentration levels, similar to the approach mentioned in section 5.4, yet re-injected at different times. The concentrations of controls were at 60, 950 and 1800 ng/mL. The time points for re-analysis were 24 hours (T24), 48 hours (T48) and 72 hours (T72) after initial analysis (T0).

5.6 Dilution Integrity

Drugs detected in samples received by a laboratory can be at concentrations above the established limit of quantitation or with low sample volume which may require dilution. Dilution integrity is therefore an important factor to be assessed during method validation. This parameter is usually evaluated by monitoring the bias and precision of samples diluted at common dilution ratios, such as 1:2, 1:10, 1:50, etc., depending on the laboratory's needs. Sample dilution at a particular ratio is only allowed if the bias, precision, and ion ratio are within the acceptable range, i.e., ±20%.

In this study, blank matrix was first fortified at 4000 ng/mL to mimic a high concentration sample. Ten times (400 ng/mL) and fifty times (80 ng/mL) dilutions of the fortified sample were then prepared in triplicate over five separate runs for the assessment of dilution integrity.

5.7 Interference Studies

Common substances, such as components in matrix or other drugs, may generate signals interfering with those of the target analyte, hence affecting the specificity of a method. Signals from common sources can be deemed insignificant to signals from target analyte(s) but this needs to be demonstrated before method implementation. Three types of interferences are commonly assessed during method validation: matrix interference, IS interference, and interference from common analytes. Matrix interference is examined by looking at the signal responses of a minimum of 10 different blank matrices for each matrix type without the addition of IS. IS interference is evaluated by analyzing blank matrix individually fortified with IS at high concentration only and then blank matrix spiked with only target analyte at the upper end of calibration range. This is for the purpose of examining if the purchased IS contains target analyte or interfering ions, and vice versa. Interference from common analytes are assessed by looking at the signal responses of blank matrix fortified with other commonly encountered analytes (e.g., drugs of abuse, metabolites) at high concentrations without IS.

In this study, 10 different lots of blank matrix without IS were used to assess matrix interference. For IS interference, matrix-matched samples fortified with IS only and the highest calibrator only were used. Interferences from common analytes were evaluated by having matrix-matched samples fortified separately at 2000 ng/mL with a total of 39 different drugs classified into three groups (Table 7). For all types of interference, any signal greater than the LOD (IS and common analyte interferences) or LOQ (matrix interference) signals would be regarded as an interference.

Table 7. Thirty-Nine Common Drugs of Abuse Tested for Interference at 2000 ng/mL

Intermix 1 (2000 ng/mL)				
Benzoylecogonine	Clonazepam	Cocaine	Codeine	Diazepam
Etizolam	Fentanyl	Hydrocodone	Lidocaine	Methadone
Morphine	Norcocaine	Oxycodone	α-hydroxyalprazolam	6-acetylmorphine
7-aminoclonazepam				
Intermix 3 (2000 ng/mL)*				
AB-FUBINACA	AB-FUBINACA metabolite 2a	AB-FUBINACA metabolite 3	AMB-FUBINACA	AB-PINACA-blood
AB-PINACA pentatonic acid metabolite	Tetrahydrocannabinol	11-Hydroxy-Δ^9-Tetrahydrocannabinol	11-nor-9-Carboxy-Δ^9-Tetrahydrocannabinol	
Intermix 4 (2000 ng/mL)				
Amitriptyline	Amphetamine	Citalopram	Ethylone	Fluoxetine
Lysergic acid diethylamide	Methamphetamine	Phencyclidine	Trazadone	α-Pyrrolidinopentiophenone
3,4-Methylenedioxyamphetamine	3,4-Methylenedioxy methamphetamine	3,4-Methylenedioxy-N-ethylamphetamine	25I-NBOMe	

* Intermix 2 contained barbiturates and was not used for the interference study

5.8 Ionization Suppression/Enhancement

Analyte signals may be subject to ionization suppression or enhancement by co-eluting substances, such as biological matrices, when being analyzed by LC-MS with ESI. This parameter is therefore also called matrix effect. It is important to evaluate the matrix effect of a method to ensure such signal suppression or enhancement is within an acceptable range. Ionization suppression or enhancement evaluated by the post-extraction addition approach is a comparison of signal responses between the neat standard (target analyte alone neither matrix nor extraction) and the blank matrices fortified with target analyte after extraction. Since the target analyte in both types of samples does not undergo extraction, any change of the analyte signal would be solely the consequence of the presence or absence of matrix. This signal comparison will be conducted at two different concentrations (low and high). At one of the concentrations, the average signal response from injecting neat standard a minimum of six times will be compared to the average signal response from injecting at least 10 different blank matrices fortified with target analyte after extraction in duplicate. The ionization suppression or enhancement at a concentration can then be calculated using the formula below:

$$Ionization\ Suppression\ or\ Enhancement\ (\%)\ at\ a\ Concentration = \left(\frac{Average\ Signal\ Response\ of\ Blank\ Matrices\ (at\ least\ ten)}{Average\ Signal\ Response\ of\ Neat\ Standard} - 1\right) \times 100\%$$

For a method to be valid, the average signal suppression or enhancement has to be within ±25%, or its coefficient of variation must be less than 15%CV. If not, no or minimal impact

of the suppression or enhancement to other validation parameters (e.g., bias, LOD, LOQ) has to be demonstrated, i.e. other parameters have to be within their own acceptable ranges.

In this study, the two concentrations examined were at 200 and 1500 ng/mL. At each concentration, neat standard was injected six times and a total of 10 different matrix-matched samples fortified with target analyte and IS after extraction were made in duplicate for assessing the ionization suppression or enhancement.

5.9 Recovery

Recovery indicates the ability of a method to recover target analyte after extraction. It is generally evaluated concurrently with ionization suppression or enhancement by comparing the signal responses between blank matrices fortified with target analyte before extraction and after extraction. At one of the two concentrations mentioned in Section 5.8, blank matrices (at least ten) fortified before extraction are made along with those fortified post-extraction. Their average signal response was compared with that of the post-extraction samples for assessing recovery. The formula for calculating recovery is as follow:

$$Recovery\ (\%)\ at\ a\ Concentration$$
$$= \left(\frac{Average\ Signal\ Response\ of\ Blank\ Matrices\ fortified\ BEFORE\ Extraction}{Average\ Signal\ Response\ of\ Blank\ Matrices\ fortified\ AFTER\ Extraction}\right) \times 100\%$$

In this study, at each of the two concentrations (200 and 1500 ng/mL) mentioned in Section 5.8, 10 different matrix-matched samples fortified with target analyte and IS before and after extraction (in duplicate) were made. A minimum of 50% recovery of target analyte was desired.

6. RESULTS AND DISCUSSION

6.1 Liquid-Liquid Extraction (LLE)

6.1.1 Calibration Model, Carryover, and Detection Limits

For amobarbital, butalbital, and phenobarbital extracted using LLE, the inversely weighted ($\frac{1}{x}$) calibration model with six calibrators (20, 50, 150, 500, 1000, and 2000 ng/mL) was reproducible in both blood and urine with individual and average r^2 greater than or equal to 0.997 over five separate runs (Table 8). The concentrations for calibrators and controls (60, 950 and 1800 ng/mL) calculated against the calibration curve were all within ±20% of their expected values. Their ion ratios in each run were also within ±20% of the average ion ratio. No carryover was observed for each double blank run immediately after the 2000 ng/mL calibrator of the calibration model. Standardized residual plots for barbiturates in blood and urine (Figure 16) all showed random distribution of residuals around the zero line, which indicate the use of the appropriate linear model. However, for butalbital and phenobarbital in blood and urine, some residuals at the high end of the calibration model (500, 1000 and 2000 ng/mL) did lie outside ±3 standard deviation of the expected value. This suggests re-analysis or adjusting the concentrations of high-end calibrators may be needed. LOD evaluated for amobarbital, butalbital, and phenobarbital in blood and urine ranged from 5 to 15 ng/mL, while the LOQ was either at 15 or 20 ng/mL (Table 9). Overall, the LOD and LOQ for barbiturates in blood was lower than that in urine when using LLE.

Table 8. Individual and Average r^2 for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Run 1	0.99956	0.99991	0.99853	0.99919	0.99927	0.99885
Run 2	0.99982	0.99994	0.99877	0.99941	0.99814	0.99827
Run 3	0.99981	0.99972	0.99949	0.99943	0.99889	0.99818
Run 4	0.99981	0.99972	0.99949	0.99943	0.99889	0.99818
Run 5	0.99983	0.99953	0.99917	0.99903	0.99958	0.99797
Average	0.99977	0.99976	0.99909	0.99930	0.99895	0.99869

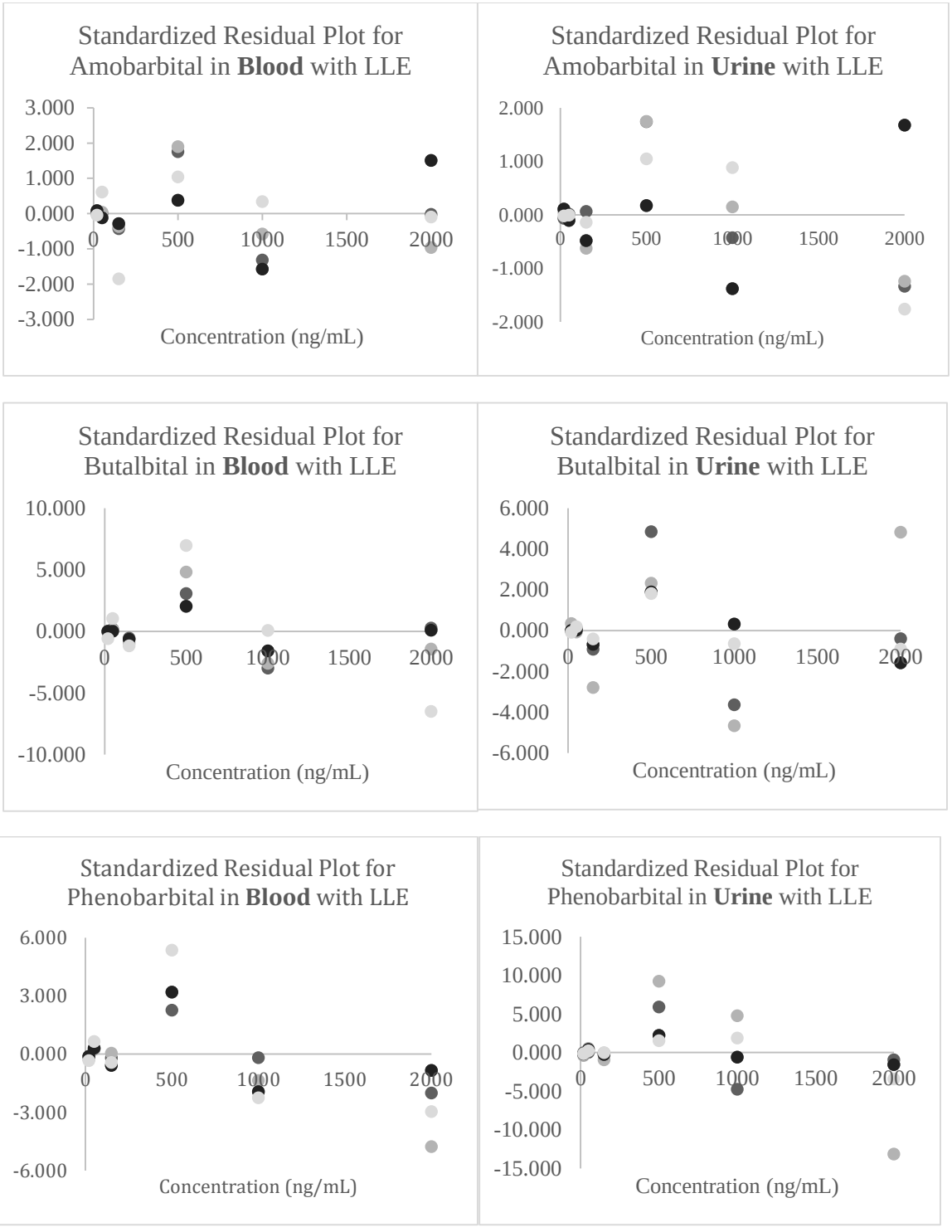


Figure 16. Standardized Residual Plots for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

Table 9. LOD and LOQ for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD (ng/mL)	5	15	10	15	10	15
LOQ (ng/mL)	15	20	20	15	20	20

6.1.2 Bias and Precision, Stability, and Dilution Integrity

With the ion ratio within ±20% of the average ion ratio for each control in each run, biases for amobarbital, butalbital, and phenobarbital in both matrices were all within ±15%; within-run precisions were all smaller than 15%CV; and between-run precisions were all less than 10%CV (Table 10-12). The three barbiturates extracted with LLE were also found to be stable up to 72 hours at room temperature after initial analysis (T0) based on their biases of average response ratio (Table 13). Bias and between-run precision of the diluted samples diluted 1:10 (400 ng/mL) and 1:50 (80 ng/mL) in both matrices were within ±15% and less than 10%CV, respectively (Table 14 and 15).

Table 10. Bias (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	-6.18	-4.74	-11.69	-10.38	-7.94	-9.16
Medium (950 ng/mL)	-0.17	-0.88	7.44	4.41	6.43	6.66
High (1800 ng/mL)	0.27	3.71	1.12	3.06	-1.89	0.77

Table 11. Within-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

		Amobarbital		Butalbital		Phenobarbital	
		Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	**Run 1**	2.25	1.47	2.29	2.57	2.17	3.63
	Run 2	4.00	2.33	2.28	1.67	5.74	3.62
	Run 3	2.22	2.87	1.63	0.86	3.31	0.36
	Run 4	3.79	3.61	3.06	2.18	5.37	7.10
	Run 5	2.17	2.35	1.13	5.79	3.14	4.08
Medium (950 ng/mL)	**Run 1**	2.69	0.69	1.61	1.20	2.30	0.39
	Run 2	0.83	1.29	1.98	2.83	1.76	1.02
	Run 3	0.21	1.15	1.49	0.21	1.46	5.82
	Run 4	2.66	0.64	0.67	1.29	2.06	1.83
	Run 5	0.44	3.35	0.35	3.61	0.70	3.02
High (1800 ng/mL)	**Run 1**	1.80	2.75	1.34	2.03	2.12	3.51
	Run 2	1.66	1.25	0.98	0.75	3.53	6.86
	Run 3	0.79	1.01	0.76	1.12	0.90	2.59
	Run 4	0.45	0.63	1.14	1.02	1.28	0.76
	Run 5	0.74	11.16	0.99	9.51	1.60	9.31

*The highest within-run precision in each run are highlighted

Table 12. Between-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	3.58	3.79	2.85	3.95	4.70	5.40
Medium (950 ng/mL)	2.51	2.05	1.80	2.69	3.35	4.51
High (1800 ng/mL)	1.76	9.04	1.56	8.06	2.27	7.05

Table 13. Bias (%) for Average Response Ratio for Amobarbital, Butalbital, and Phenobarbital Stability in Blood and Urine Using LLE

		Amobarbital		Butalbital		Phenobarbital	
		Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	**T24**	2.93	6.85	-5.46	-2.22	-2.59	-0.26
	T48	-5.32	-3.90	-0.07	1.69	-1.36	-2.97
	T72	3.95	4.51	3.21	-2.04	-0.87	1.84
Medium (950 ng/mL)	**T24**	-0.64	1.12	0.18	1.28	-6.06	-2.30
	T48	-2.69	-1.10	1.50	1.66	-4.61	-0.87
	T72	2.00	7.71	-1.37	-1.56	-0.40	1.04
High (1800 ng/mL)	**T24**	-1.19	1.40	0.68	0.21	-2.17	2.42
	T48	-3.06	1.31	2.37	2.35	-2.27	1.62
	T72	2.21	5.08	-3.80	-1.06	1.24	4.66

Table 14. Bias (%) for Amobarbital, Butalbital, and Phenobarbital Diluted in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
1:10 (400 ng/mL)	2.31	4.73	9.18	8.91	9.76	12.92
1:50 (80 ng/mL)	-7.10	-3.35	-5.18	-0.63	-4.78	1.01

Table 15. Between-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital Diluted in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
1:10 (400 ng/mL)	3.32	2.56	2.31	2.27	3.47	3.98
1:50 (80 ng/mL)	6.02	4.91	7.17	5.64	7.50	5.57

6.1.3 Interference Studies

Amobarbital, butalbital, and phenobarbital extracted from blood and urine using LLE showed no interferences from matrices, IS, and common analytes tested. None of the

tested signal response was higher than the LOD or LOQ responses of the three barbiturates (Table 16-18).

Table 16. Matrix Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOQ*	16714.78 15 ng/mL	19733.33 20 ng/mL	20853.33 20 ng/mL	11831.17 15 ng/mL	17143.33 20 ng/mL	17812.22 20 ng/mL
Blank Matrix 1	53.61	10.95	273.10	580.20	40.54	7.67
Blank Matrix 2	110.50	62.60	170.20	86.15	37.29	63.31
Blank Matrix 3	38.93	19.27	439.00	97.52	10.96	107.70
Blank Matrix 4	13.07	171.50	246.10	62.58	79.32	13.11
Blank Matrix 5	25.84	36.50	63.99	750.60	121.70	131.30
Blank Matrix 6	67.09	9.57	252.90	159.80	107.60	10.99
Blank Matrix 7	40.53	20.74	164.00	249.50	13.49	27.05
Blank Matrix 8	66.77	9.70	163.70	266.50	13.53	13.07
Blank Matrix 9	26.71	10.99	231.60	33.66	13.51	66.50
Blank Matrix 10	53.43	40.49	141.00	144.50	40.29	27.02
Matrix Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified

Table 17. Internal Standard Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD*	5682.50 5 ng/mL	13527.78 15 ng/mL	10257.17 10 ng/mL	11831.17 15 ng/mL	8136.61 10 ng/mL	12274.78 15 ng/mL
IS^{+}	187.40	197.50	44.53	164.70	133.70	13.52
Highest Calibrator^	34.32	53.82	51.78	107.70	26.50	12.91
IS Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified
$^{+}$Signal area of the highest calibrator in matrix-matched sample fortified with IS only
^Signal area of IS in matrix-match sample fortified with the highest calibrator only

Table 18. Common Analytes Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD*	5682.50 5 ng/mL	13527.78 15 ng/mL	10257.17 10 ng/mL	11831.17 15 ng/mL	8136.61 10 ng/mL	12274.78 15 ng/mL
Intermix 1	92.15	19.39	159.70	184.20	13.53	269.60
Intermix 3	6.63	73.76	42.49	53.34	78.92	108.20
Intermix 4	39.21	100.90	13.23	406.10	13.10	27.02
Common Analytes Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified

6.1.4 Ionization Suppression/Enhancement and Recovery

Comparing the responses from neat sample (un-extracted barbiturates, including IS) to sample fortified with barbiturates after extraction, amobarbital, butalbital, and phenobarbital in blood extracted with LLE showed an ionization enhancement effect in both concentration levels (200 and 1500 ng/mL). The enhancement effect by blood was between 10 to 20% at low concentration, while it was within 5% at high concentration. On the contrary, suppression effect was observed for the three barbiturates extracted in urine. That matrix effect was between -20% to -30% at low concentration and was within -20% at high concentration (Table 19). Although the ionization suppression effect in urine at low concentration exceeded the ±25% criteria, it had minimal impact to the analysis since other validation parameters for urine mentioned in previous sections all fell under the acceptable ranges and each analyte has its own deuterated internal standard.

Recovery was evaluated as a comparison between the responses of samples fortified with barbiturates before and after extraction. Extraction of amobarbital, butalbital, and

phenobarbital using LLE demonstrated recoveries 53-68% (Table 20). There was no significant difference in recovery between barbiturates in blood or in urine.

Table 19. Ionization Suppression/Enhancement (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (200 ng/mL)	11.53	-25.97	12.52	-25.89	15.60	-20.00
High (1500 ng/mL)	2.39	-17.64	3.06	-15.81	1.07	-15.77

Table 20. Recovery (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using LLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (200 ng/mL)	61.34	57.41	61.63	60.75	64.13	67.89
High (1500 ng/mL)	59.72	62.03	55.23	55.17	57.11	53.46

6.2 Supported-Liquid Extraction (SLE)

6.2.1 Calibration Model, Carryover, and Detection Limits

The six-point calibration model for amobarbital, butalbital, and phenobarbital extracted from blood and urine using SLE was reproducible with individual and average r^2 greater than or equal to 0.994 over five separate runs (Table 21). Concentrations and ion ratios of calibrators and controls were all within the ±20% criteria. Carryover was not observed throughout the entire SLE validation. Standardized residual plots of barbiturates revealed random distribution of residuals around the zero line for both blood and urine (Figure 17), indicating the use of a linear model was appropriate. All the residuals were within the ±3 standard deviation of the expected value. This shows none of the calibration points used were outliers in this model. LOD for barbiturates in blood and urine was found to be either 10 ng/mL or 15 ng/mL, while the LOQ ranged from 10 ng/mL to 20 ng/mL (Table 22).

Table 21. Individual and Average r^2 for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Run 1	0.99821	0.99892	0.99859	0.99980	0.99891	0.99864
Run 2	0.99846	0.99445	0.99969	0.99869	0.99807	0.99884
Run 3	0.99920	0.99581	0.99821	0.99974	0.99740	0.99902
Run 4	0.99642	0.99736	0.99928	0.99555	0.99671	0.99769
Run 5	0.99933	0.99920	0.99891	0.99891	0.99970	0.99970
Average	0.99832	0.99715	0.99894	0.99854	0.99816	0.99878

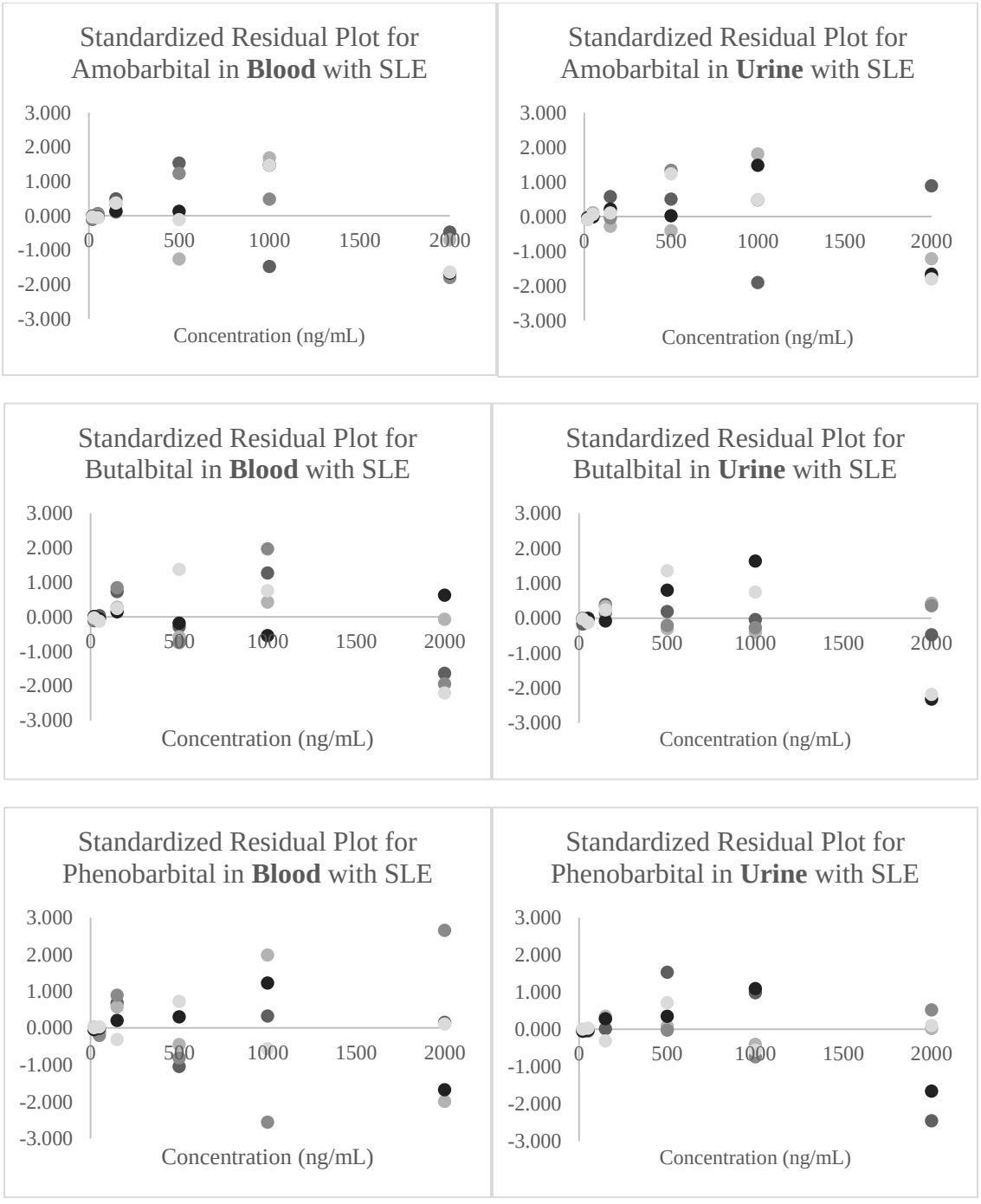


Figure 17. Standardized Residual Plots for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

Table 22. LOD and LOQ for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD (ng/mL)	15	10	10	10	10	10
LOQ (ng/mL)	15	20	10	15	20	15

6.2.2 Bias and Precision, Stability, and Dilution Integrity

Biases for all three barbiturates in blood and urine were all within ±20%, yet the accuracy at low concentration (60 ng/mL) was significantly less satisfactory than at medium (950 ng/mL) and high (1800 ng/mL) concentrations (Table 23). Within-run and between-run precision all exhibited %CV less than 20% (Table 23-25). For stability, barbiturates in blood and urine extracted with SLE were stable up to 72 hours at room temperature after initial analysis (T0) (Table 26). Dilution integrity also revealed that barbiturates in blood and urine were able to be diluted 1:50 (80 ng/mL). Bias and between-run precision of the diluted samples were within ±8% and less than 12%CV, respectively (Table 27 and 28).

Table 23. Bias (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	17.97	10.14	10.41	14.44	2.74	12.29
Medium (950 ng/mL)	-2.03	-4.36	-3.45	-4.13	-3.96	-5.20
High (1800 ng/mL)	-7.62	-10.97	-6.31	-8.80	-10.24	-9.95

Table 24. Within-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

		Amobarbital		Butalbital		Phenobarbital	
		Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	**Run 1**	8.35	6.83	8.68	3.13	13.79	12.50
	Run 2	11.13	4.23	12.49	2.05	13.18	4.13
	Run 3	0.42	6.91	0.70	8.14	10.57	12.59
	Run 4	19.17	7.10	4.98	4.04	5.26	2.33
	Run 5	11.29	0.42	4.77	5.45	6.62	6.62
Medium (950 ng/mL)	**Run 1**	1.17	5.89	4.51	1.82	3.32	3.22
	Run 2	3.90	6.71	7.08	1.12	10.10	2.17
	Run 3	2.51	3.83	11.58	11.57	3.68	13.70
	Run 4	8.77	4.67	5.98	6.52	5.30	3.03
	Run 5	10.54	2.51	0.48	0.48	1.16	1.16
High (1800 ng/mL)	**Run 1**	4.48	5.06	2.77	5.31	4.89	7.13
	Run 2	7.05	3.74	8.21	4.49	4.04	3.77
	Run 3	2.75	3.75	7.93	2.15	5.27	2.70
	Run 4	6.49	6.05	3.62	3.79	3.19	9.39
	Run 5	1.07	2.75	0.93	0.93	3.04	3.04

*The highest within-run precision in each run is highlighted

Table 25. Between-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	16.62	8.46	14.84	11.64	12.97	12.77
Medium (950 ng/mL)	9.37	6.68	14.92	10.89	9.28	9.55
High (1800 ng/mL)	6.09	5.37	8.01	13.39	6.71	9.71

Table 26. Bias (%) for Average Response Ratio for Amobarbital, Butalbital, and Phenobarbital Stability in Blood and Urine Using SLE

		Amobarbital		Butalbital		Phenobarbital	
		Blood	Urine	Blood	Urine	Blood	Urine
Low (60 ng/mL)	T24	3.80	7.13	4.19	6.80	-4.73	9.88
	T48	1.32	4.51	1.76	-0.14	1.56	4.44
	T72	5.01	2.87	3.02	-1.43	1.50	4.91
Medium (950 ng/mL)	T24	0.96	-0.05	0.08	-0.67	1.27	4.98
	T48	-1.05	-2.74	-2.89	-1.33	0.59	4.32
	T72	-2.67	-3.91	-4.51	-4.88	4.11	2.00
High (1800 ng/mL)	T24	5.16	1.32	-0.58	-1.61	3.05	6.99
	T48	-2.36	-3.73	-1.54	-1.11	-2.94	5.85
	T72	1.92	-2.00	-6.22	-4.59	-1.65	3.38

Table 27. Bias (%) for Amobarbital, Butalbital, and Phenobarbital Diluted in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	Urine	Blood	Urine	Blood	Urine
1:10 (400 ng/mL)	4.06	7.55	-2.48	4.60	4.69	5.96
1:50 (80 ng/mL)	-4.29	-2.23	-5.03	-1.24	-3.22	5.52

Table 28. Between-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital Diluted in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	Urine	Blood	Urine	Blood	Urine
1:10 (400 ng/mL)	9.32	4.49	7.19	8.01	7.32	6.56
1:50 (80 ng/mL)	10.67	6.01	11.89	4.82	10.59	6.81

6.2.3 Interference Studies

No signal response from matrices, IS, or common analytes tested was found higher than the LOD or LOQ responses of the three barbiturates (Table 29-31). Therefore,

interference was not observed when extracting barbiturates from blood and urine using SLE.

Table 29. Matrix Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOQ*	12126.61 15 ng/mL	16870.00 20 ng/mL	7315.89 10 ng/mL	10868.00 15 ng/mL	13864.83 20 ng/mL	7707.00 15 ng/mL
Blank Matrix 1	13.02	65.03	14.34	84.53	22.80	147.60
Blank Matrix 2	42.72	13.53	19.35	15.36	49.81	26.67
Blank Matrix 3	13.23	269.20	27.90	11.01	26.36	13.50
Blank Matrix 4	38.73	47.98	20.43	64.41	54.05	81.05
Blank Matrix 5	72.14	85.72	27.16	21.72	12.93	13.50
Blank Matrix 6	10.95	47.98	23.01	26.03	13.52	40.52
Blank Matrix 7	26.50	80.09	13.08	13.49	13.25	80.73
Blank Matrix 8	88.86	46.13	9.57	22.30	67.60	13.49
Blank Matrix 9	10.95	67.03	67.64	7.43	54.02	40.50
Blank Matrix 10	66.42	68.81	10.85	13.53	13.52	40.52
Matrix Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified

Table 30. Internal Standard Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD*	12126.61 15 ng/mL	9692.78 10 ng/mL	7315.89 10 ng/mL	7863.44 10 ng/mL	6787.28 10 ng/mL	6604.56 10 ng/mL
IS^{+}	68.20	23.80	42.96	29.21	53.23	37.81
Highest Calibrator^	114.40	74.13	177.70	268.00	13.52	27.03
IS Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified
$^{+}$Signal area of the highest calibrator in matrix-matched sample fortified with IS only
^Signal area of IS in matrix-match sample fortified with the highest calibrator only

Table 31. Common Analytes Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD*	12126.61 15 ng/mL	9692.78 10 ng/mL	7315.89 10 ng/mL	7863.44 10 ng/mL	6787.28 10 ng/mL	6604.56 10 ng/mL
Intermix 1	93.18	119.90	27.54	41.38	10.95	20.53
Intermix 3	10.85	11.24	35.26	166.60	12.54	36.72
Intermix 4	208.70	60.58	60.15	35.03	10.95	9.58
Common Analytes Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified

6.2.4 Ionization Suppression/Enhancement and Recovery

Ionization enhancement was observed for barbiturates in blood and urine at both low and high concentrations. The enhancement by matrices were up to 10%, yet urine yielded a slightly greater signal enhancement than blood at both concentration levels (Table 32). Barbiturates extracted using SLE demonstrated recoveries up to 86%. The three barbiturates in urine were consistently found to have higher recoveries than in blood at both concentrations (Table 33). However, recoveries of barbiturates from blood were below the desired 50% at high concentration.

Table 32. Ionization Suppression/Enhancement (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (200 ng/mL)	1.25	4.73	3.40	9.63	7.68	10.00
High (1500 ng/mL)	3.07	4.06	4.05	6.70	4.79	7.74

Table 33. Recovery (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SLE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (200 ng/mL)	52.83	77.03	55.59	74.55	53.34	82.37
High (1500 ng/mL)	46.32	86.07	48.56	80.09	47.38	81.93

6.3 Solid-Phase Extraction (SPE)

6.3.1 Calibration Model, Carryover, and Detection Limits

Six-point calibration model was also reproducible for the extraction of amobarbital, butalbital, and phenobarbital in blood and urine using SPE. The individual and average r^2 were greater than or equal to 0.996 over all five runs (Table 34). Concentrations and ion ratios calculated all met the ±20% criteria. Carryover was not detected throughout the entire validation. Residuals in standardized residual plots were all randomly distributed around the zero line (Figure 18), thus indicating the use of a linear model. However, a few of the butalbital and phenobarbital residuals at the high end of the calibration model (500, 1000, and 2000 ng/mL) were found outside the ±3 standard deviation limit. Re-analysis or method adjustment might be needed to improve the reliability of the calibration model. LOD and LOQ for barbiturates in blood and urine were either 15 or 20 ng/mL (Table 35). The limits of detection for blood were found to be equal to or lower than that of the urine.

Table 34. Individual and Average r^2 for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Run 1	0.99961	0.99980	0.99944	0.99913	0.99974	0.99889
Run 2	0.99979	0.99914	0.99980	0.99947	0.99993	0.99945
Run 3	0.99926	0.99621	0.99993	0.99796	0.99961	0.99757
Run 4	0.99952	0.99936	0.99982	0.99919	0.99970	0.99932
Run 5	0.99961	0.99992	0.99980	0.99924	0.99981	0.99986
Average	0.99956	0.99889	0.99976	0.99900	0.99976	0.99902

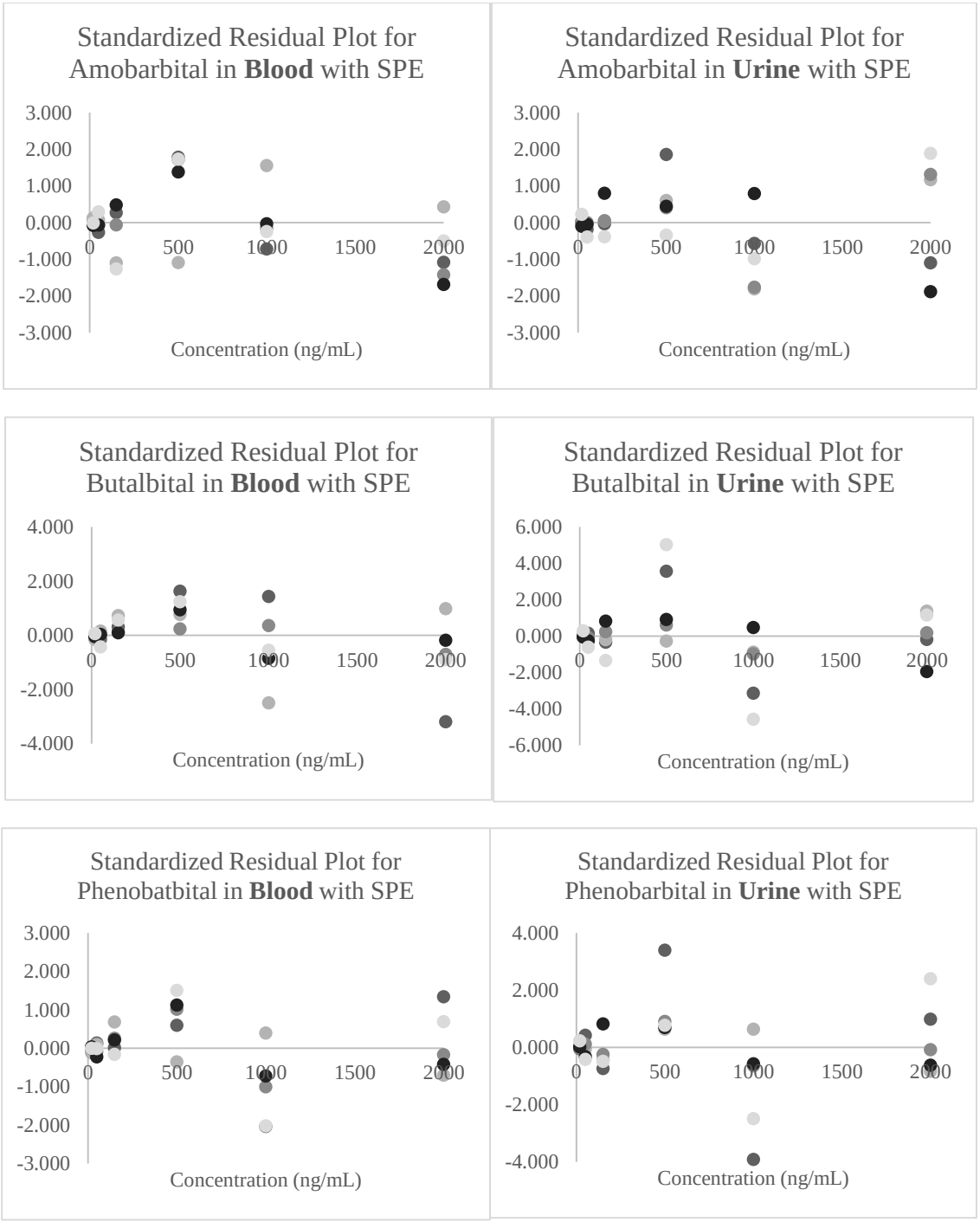


Figure 18. Standardized Residual Plots for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

Table 35. LOD and LOQ for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD (ng/mL)	15	15	15	20	15	20
LOQ (ng/mL)	15	20	15	20	20	20

6.3.2 Bias and Precision, Stability, and Dilution Integrity

Biases and within-run precisions for barbiturates in blood and urine were within ±15% and less than 15%CV, respectively. Between-run precisions were less than 20%CV (Table 36-38), yet barbiturates in blood were found to have lower between-run precisions than in urine. Similar to LLE and SLE, barbiturates extracted using SPE were stable up to 72 hours at room temperature after initial analysis (T0) (Table 39). Barbiturates in blood and urine were also able to be diluted 1:50 (80 ng/mL) since bias and between-run precision of the diluted samples were within ±20% and less than 15%CV, respectively (Table 40 and 41).

Table 36. Bias (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	2.03	3.81	7.44	-2.26	5.72	0.26
Medium (950 ng/mL)	-4.42	-9.67	-4.41	-2.69	-5.09	-2.57
High (1800 ng/mL)	-0.66	-11.52	0.54	-2.79	0.55	-8.59

Table 37. Within-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

		Amobarbital		Butalbital		Phenobarbital	
		Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	**Run 1**	3.87	2.91	3.64	2.36	4.63	8.40
	Run 2	5.48	11.18	1.92	4.01	5.71	6.69
	Run 3	6.00	6.96	5.19	4.01	3.87	6.63
	Run 4	3.54	0.79	3.07	1.44	2.09	8.33
	Run 5	7.11	3.76	1.23	1.20	3.33	3.40
Medium (950 ng/mL)	**Run 1**	0.87	9.69	1.27	12.27	2.36	1.83
	Run 2	2.66	6.12	2.16	3.20	2.85	6.69
	Run 3	3.33	0.92	2.17	1.29	2.37	1.34
	Run 4	0.54	4.35	2.04	3.60	1.71	7.63
	Run 5	1.83	4.02	2.43	1.13	2.55	4.62
High (1800 ng/mL)	**Run 1**	1.06	13.31	3.09	8.30	1.49	1.72
	Run 2	2.39	4.42	4.76	4.69	3.52	1.47
	Run 3	3.77	1.66	4.37	4.91	2.06	5.91
	Run 4	1.32	5.30	3.52	2.73	2.60	2.47
	Run 5	2.61	1.63	3.27	2.24	2.74	2.66

*The highest within-run precision in each run is highlighted

Table 38. Between-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	7.20	8.56	4.35	8.35	5.78	17.29
Medium (950 ng/mL)	3.02	7.39	2.22	9.06	2.78	6.68
High (1800 ng/mL)	4.25	10.16	4.39	11.44	2.70	5.86

Table 39. Bias (%) for Average Response Ratio for Amobarbital, Butalbital, and Phenobarbital Stability in Blood and Urine Using SPE

		Amobarbital		Butalbital		Phenobarbital	
		Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (60 ng/mL)	**T24**	-9.37	8.74	-1.63	2.65	-7.46	-10.07
	T48	9.83	-5.87	5.07	3.05	-13.94	-13.60
	T72	-7.59	-6.28	-6.05	1.43	-18.23	-8.28
Medium (950 ng/mL)	**T24**	-2.89	-11.25	4.04	2.44	-3.60	-9.83
	T48	11.59	-6.41	6.02	1.52	-5.81	-6.92
	T72	-0.64	-7.03	2.46	-1.39	-4.78	-6.68
High (1800 ng/mL)	**T24**	-2.74	-8.67	4.03	0.40	3.13	-9.09
	T48	4.96	-5.58	4.44	-0.88	2.58	-8.04
	T72	-0.48	-2.07	6.89	-0.14	3.78	-6.18

Table 40. Bias (%) for Amobarbital, Butalbital, and Phenobarbital Diluted in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
1:10 (400 ng/mL)	-5.99	-6.06	4.10	3.16	-7.56	3.17
1:50 (80 ng/mL)	6.65	-4.02	16.22	5.57	11.32	-4.60

Table 41. Between-Run Precision (%CV) for Amobarbital, Butalbital, and Phenobarbital Diluted in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
1:10 (400 ng/mL)	5.78	6.28	6.19	11.23	6.75	5.21
1:50 (80 ng/mL)	6.36	8.23	4.95	9.15	5.58	8.75

6.3.3 Interference Studies

Signal responses from matrices, IS, or common analytes tested were all below that of the barbiturates LOD or LOQ (Table 42-44). Therefore, no significant interference was observed during the extraction of amobarbital, butalbital, and phenobarbital using SPE.

Table 42. Matrix Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOQ*	10296.39 15 ng/mL	4977.33 20 ng/mL	8092.50 15 ng/mL	3341.61 20 ng/mL	4249.83 20 ng/mL	2308.56 20 ng/mL
Blank Matrix 1	135.40	54.11	80.17	26.55	10.89	62.47
Blank Matrix 2	10.83	13.26	53.79	416.90	39.27	66.33
Blank Matrix 3	77.17	13.55	74.16	80.93	27.00	26.79
Blank Matrix 4	334.40	27.06	88.33	54.45	39.43	13.53
Blank Matrix 5	84.78	13.50	67.48	27.00	16.80	40.56
Blank Matrix 6	52.11	13.52	10.97	13.53	40.09	13.53
Blank Matrix 7	134.50	13.50	136.70	40.55	26.42	40.58
Blank Matrix 8	539.20	27.05	220.70	599.90	50.25	13.51
Blank Matrix 9	582.70	13.51	116.10	67.56	167.90	13.53
Blank Matrix 10	48.89	20.68	47.75	53.19	21.66	94.90
Matrix Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified

Table 43. Internal Standard Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD*	10296.39 15 ng/mL	3269.56 15 ng/mL	8092.50 15 ng/mL	3341.61 20 ng/mL	3396.56 15 ng/mL	2308.56 20 ng/mL
IS^{+}	121.10	71.42	12.94	51.73	138.10	15.29
Highest Calibrator^	105.40	44.95	36.62	45.27	125.20	23.68
IS Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified
$^{+}$Signal area of the highest calibrator in matrix-matched sample fortified with IS only
^Signal area of IS in matrix-match sample fortified with the highest calibrator only

Table 44. Common Analytes Interference for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LOD*	10296.39 15 ng/mL	3269.56 15 ng/mL	8092.50 15 ng/mL	3341.61 20 ng/mL	3396.56 15 ng/mL	2308.56 20 ng/mL
Intermix 1	54.11	14.36	37.50	143.40	27.05	111.80
Intermix 3	27.04	1.52	81.48	59.77	27.05	39.56
Intermix 4	13.50	9.58	108.20	83.40	175.80	66.15
Common Analytes Interference?	No	No	No	No	No	No

*All values are signal responses unless otherwise specified

6.3.4 Ionization Suppression/Enhancement and Recovery

Barbiturates extracted from blood and urine using SPE exhibited the most ionization suppression. For most analytes, values were within 25%, yet signal responses of barbiturates extracted from urine were suppressed by over 70% (Table 45). Although the matrix effect was substantial, analysis using SPE was only subjected to minimal impact as each analyte had its own deuterated internal standard and other validation parameters were all acceptable. For recovery, SPE was able to recover over 50% in blood and; up to 70% of barbiturates from urine. However, recovery of barbiturates from blood was closer to 50% (Table 46).

Table 45. Ionization Suppression/Enhancement (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (200 ng/mL)	-23.28	-71.24	-24.68	-79.92	-27.87	-83.88
High (1500 ng/mL)	-14.28	-68.45	-14.59	-76.77	-24.37	-80.82

Table 46. Recovery (%) for Amobarbital, Butalbital, and Phenobarbital in Blood and Urine Using SPE

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Low (200 ng/mL)	46.31	70.30	47.73	72.61	51.42	69.07
High (1500 ng/mL)	45.42	63.93	48.95	56.96	48.22	54.15

7. COMPARISONS

Apart from validating different sample preparation methods, the purpose of this study is to also compare the efficiency and effectiveness of LLE, SLE, and SPE, thus to recommend the most suitable method to recover barbiturates from blood and urine under the laboratory setting at BUMC. Efficiency evaluates the time and ease of a method to extract target analyte, while effectiveness is an assessment of one's ability to selectively recover target analyte at a reasonably low concentration.

7.1 Efficiency

Among the three extraction methods investigated, LLE and SPE required the longest time to prepare samples for instrumental analysis, which was approximately 4 hours. The time for SLE to finish the overall pretreatment and extraction for roughly 36-48 samples was only 2-3 hours. The major reason LLE was more time-consuming was the involvement of the manual transfer step. Physical separation of target analyte was still needed even after the 3-step LLE in one single container. Training is therefore required as it takes time for the analyst to master the hand-eye coordination for separating the immiscible layers (Table 47).[8] When the number of samples go up and no automation is available, LLE can take a long time to complete. This is the reason why LLE is commonly known as labor intensive.[7]

Solid-phase extraction in this study was also more time intensive than SLE. It was not the procedure that took the time but rather the evaporation step. Since DI water was

used for conditioning, almost an hour waiting was needed to evaporate the eluate to dryness and to proceed to reconstitution. Although it took time for evaporation, the entire process only involved 2-3 steps and is actually amenable to automation. Therefore, only minimal training is needed for analyst (Table 47).

Among the three sample preparation methods, SLE took the least amount of time, which was only 2-3 hours. It did not require manual transfer and required much less drying time due to the use of organic solvents. There was also no solvent waste at all when compared to LLE and SPE. The 3-step procedure can be automated as well so only minimal training is required (Table 47). Based on the comparison, SLE was the most efficient sample preparation method among the three for the extraction of barbiturates in blood and urine under the laboratory setting at BUMC, however, a method's effectiveness still needs to be considered in order to determine the most efficient and effective method for quantifying a specific barbiturate in a particular matrix.

Table 47. Comparison of the Efficiency of LLE, SLE, and SPE

	LLE	**SLE**	**SPE**
Time	~ 4 hours	2-3 hours	~ 4 hours
Number of Extraction Steps	4*	3^	3[+]
Number of Solvents	3	2	3
Automatable?	No	Yes	Yes
Training Required?	Yes	Minimal	Minimal

*1) Addition of Extraction Solvent 2) Rock 3) Centrifuge 4) Transfer of Organic Layer
^1) Sample Load 2) Wait 3) Elute
[+] 1) Condition with Methanol 2) Condition with DI Water 3) Elute

7.2 Effectiveness

For quantitation of amobarbital in blood, LLE offered the highest drug recovery, while the recoveries for SLE and SPE did not meet the minimum expectation of 50%. LLE also provided a more accurate and precise detection with LOD down to 5 ng/mL, the lowest among the three methods. SLE, in contrast, provided the best results for the extraction of amobarbital in urine. Not only was it able to recover up to 86% of amobarbital, it also yielded the cleanest extract and demonstrated the least amount of matrix effects. SLE had the lowest LOD among the three methods as well (Table 48).

Quantifying butalbital in blood was the best using either LLE or SLE. LLE offered a recovery over 60%, while SLE provided a recovery around 50% but with a cleaner extract and lower LOQ. If recovery is highly desired over the quantification limit, SLE is recommended. Quantitation of butalbital in urine was the most effective using SLE because recovery was up to 80% with low matrix effect, unlike LLE and SPE which suffered from ionization suppression (Table 49). LOD was able be go as low as 10 ng/mL as well when using SLE.

Similar to amobarbital, LLE and SLE were the most effective methods for quantifying phenobarbital in blood and urine, respectively. LLE offered the highest recovery among the three methods with decent extract cleanliness for the blood extraction. Samples extracted via SLE, in this case, had recovery values barely reaching 50%, while samples extracted via SPE partly suffered from matrix effect. For the quantification in urine, SLE performed well in most criteria, including providing a recovery up to 80%; offering

great extract cleanliness; and reaching the lowest level of LOD and LOQ among the tested methods (Table 50).

Although SLE was the most efficient sample preparation method for extracting barbiturates in this study, LLE was the most effective at quantifying barbiturate in blood, while SLE was the most suitable for barbiturates from urine under the setting in the laboratory. Table 51 illustrates the sample preparation method recommended for extracting a particular barbiturate in a certain type of biological matrix.

Table 48. Comparison of Effectiveness of LLE, SLE, and SPE in Extracting Amobarbital from Blood and Urine

	LLE		SLE		SPE	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Recovery	59-61%	57-62%	46-52%	77-86%	~45%	63-70%
Extract Cleanliness*	+12%	-26%	+3%	+5%	-23%	-71%
LOD	5 ng/mL	15 ng/mL	15 ng/mL	10 ng/mL	15 ng/mL	15 ng/mL
LOQ	15 ng/mL	20 ng/mL	15 ng/mL	20 ng/mL	15 ng/mL	20 ng/mL
Reproducibility^	< ±7%	< ±12%	< ±20%	< ±11%	< ±8%	< ±14%

*Evaluated by the largest Matrix Effect
^Evaluated by Overall Bias and Precision

Table 49. Comparison of Effectiveness of LLE, SLE, and SPE in Extracting Butalbital from Blood and Urine

	LLE		SLE		SPE	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Recovery	55-61%	55-61%	48-55%	74-80%	~48%	56-72%
Extract Cleanliness*	+13%	-26%	+4%	+10%	-25%	-80%
LOD	10 ng/mL	15 ng/mL	10 ng/mL	10 ng/mL	15 ng/mL	20 ng/mL
LOQ	20 ng/mL	15 ng/mL	10 ng/mL	15 ng/mL	15 ng/mL	20 ng/mL
Reproducibility^	< ±12%	< ±11%	< ±15%	< ±15%	< ±8%	< ±13%

*Evaluated by the largest Matrix Effect
^Evaluated by Overall Bias and Precision

Table 50. Comparison of Effectiveness of LLE, SLE, and SPE in Extracting Phenobarbital from Blood and Urine

	LLE		SLE		SPE	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
Recovery	57-64%	53-68%	47-53%	~82%	48-51%	54-69%
Extract Cleanliness*	+16%	-20%	+8%	+10%	-28%	-84%
LOD	10 ng/mL	15 ng/mL	10 ng/mL	10 ng/mL	15 ng/mL	20 ng/mL
LOQ	20 ng/mL	20 ng/mL	20 ng/mL	15 ng/mL	20 ng/mL	20 ng/mL
Reproducibility	< ±8%	< ±10%	< ±14%	< ±14%	< ±6%	< ±18%

*Evaluated by the largest Matrix Effect
^Evaluated by Overall Bias and Precision

Table 51. Recommendations of Sample Preparation Method for the Extraction of Barbiturates from Blood and Urine

	Amobarbital		Butalbital		Phenobarbital	
	Blood	**Urine**	**Blood**	**Urine**	**Blood**	**Urine**
LLE	✓		✓		✓	
SLE		✓	✓	✓		✓
SPE						

8. CONCLUSIONS

8.1 Summary and Significance of Findings

The first objective of this study was to validate LLE, SLE, and SPE for the quantitation of amobarbital, butalbital, and phenobarbital in blood and urine using LC-MS/MS. As demonstrated in Section 6, the six-point linear calibration model (20, 50, 150, 500, 1000 and 2000 ng/mL) with inversely weighted concentration ($\frac{1}{x}$) was reproducible in all three sample preparation methods for both blood and urine with r^2 greater than or equal to 0.994. Bias and precision evaluated from low, medium, and high controls (60, 950 and 1800 ng/mL) were all within ±20% and ±20% CV, respectively. Neither carryover nor interference from matrix, IS or common analytes were observed throughout the entire validation and the detection limits (LOD and LOQ) were evaluated down to 5 ng/mL. Samples can be diluted 1:50 prior to instrumental analysis. Previously injected samples were stable at room temperature for up to 72 hours before re-analysis. Recovery of barbiturates from blood and urine using LLE, SLE, and SPE all ranged from 45% to 86%, whilst the entire study was only subject to minimal impact by the effect of ionization suppression or enhancement. Having met the ASB validation guidelines, this study has successfully validated three sample preparation methods (LLE, SLE, and SPE) for the quantitation of three barbiturates (amobarbital, butalbital, and phenobarbital) in blood and urine using LC-MS/MS.

With three extraction methods available, the second objective of this study was to choose the most suitable method to recover a specific barbiturate by comparing a method's

efficiency and effectiveness. Efficiency is an evaluation of time required and ease of a method to extract a target analyte. As shown in Section 7.1, SLE was the most efficient method for extracting barbiturates under the setting at BUMC laboratory as it required the least amount of time to perform due to short drying time, least number of solvents, and the absence of a manual transfer step. Its procedure can be easily automated as well so only minimal training was required. Effectiveness is another crucial factor for determining the most suitable sample preparation method for a particular barbiturate. Based on the consideration of each methods' recovery, extract cleanliness, detection limits, and reproducibility, LLE was over overall the most successful at quantitating barbiturates in blood and SLE was the most suitable for barbiturates in urine under the laboratory setting at BUMC.

Since this study constitutes part of the BUMC collaborative project with RTI, all the analytical data collected have been already reported to RTI, which completed the third objective of this study. These findings can be used for interlaboratory comparison and for examining the overall reliability and reproducibility of methods. Results obtained can then be used to explore the possibility of streamlining sample preparation in the forensic laboratory, and hence reducing the case backlog.

8.2 Future Directions

To provide a more comprehensive comparison, future studies can consider increasing the diversity of drugs analyzed. This can be done by including more common barbiturates for analysis, such as pentobarbital, secobarbital, and butabarbital, etc. More sample preparation methods can also be included for comparison. For example, traditional SPE using a UCT cartridge can be included and compared to the data obtained from Waters SPE cartridge in this study. A novel extraction method, such as disposable pipette extraction, can also be studied to offer a more complete comparison. Increasing the number of different biological matrices is also an option. Oral fluid is a good third matrix to be included as the demand for toxicological analysis on oral fluid has grown in recent years due to its ease of collection and the prevalence of roadside screening.[8] Finally, to increase the reliability of this collaborative study, data from authentic case samples using those validated sample preparation methods should also be collected.

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