CHALLENGES IN METHOD DEVELOPMENT FOR ANALYSIS OF SELECTED PHENOTHIAZINE DRUGS IN BIOLOGICAL SAMPLES: OXIDATION OF DRUGS AND METABOLITES DURING SAMPLE PREPARATION

By

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Abstract

The main goal of this research project was the detection and semi-quantitation of the parent drugs promethazine (PMZ), and chlorpromazine(CPZ) along with selected metabolites promethazine sulfoxide (PMZSO), desmethylpromethazine (DPMZ), chlorpromazine sulfoxide (CPZSO), and desmethylchlorpromazine (DCPZ) in the skeletal remains of rats. We sought to evaluate the relative distribution of structural analogues (promethazine (PMZ) and chlorpromazine (CPZ)), and the relationship of multiple metabolites to the parent drug. The first phase of the project was to consist of method development and validation and the second phase would be examining a large number of rat bone samples exposed to different drug exposure patterns. For the analytical method, drug extraction is completed using microwave assisted extraction (MAE), followed by sample clean-up by microplate solid-phase extraction (MPSPE) and instrumental analysis by ultra-high performance liquid chromatography coupled to photodiode array detection (UHPLC-PDA). During method development and validation, extraneous compounds appeared in the chromatograms. The production of unknown compounds hindered the ability to meet the validation criteria. Thus, the new objective of the research was characterization of the unknown compounds by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-qTOF-MS), comparing the extent of degradation for different extraction conditions, and validation of a new method that does not promote degradation.

Keywords: Forensic toxicology, Promethazine, Chlorpromazine, Oxidation, UHPLC-PDA, UHPLC-QTOF-MS

Co-Authorship Statement

Chapter 1 of this thesis is an overview of the background information relevant to this study. It discusses the theoretical concepts and includes a review of the literature. I am the sole author of this chapter however, James Watterson provided guidance with the outline of this chapter.

Chapter 2 is a manuscript that has been submitted to the Journal of Analytical Toxicology. Also, sections have been presented at the American Academy of Forensic Science Conference (2015) and the Canadian Society of Forensic Science Conference (2016). I am the first author and James Watterson is a co-author.

Chapter 3 includes the conclusion and a brief review of future research that can be conducted as an extension to the research presented in this thesis. I am the sole author with contributions from James Watterson.

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Lastly, I would like to thank my parents, sisters and boyfriend for the unwavering encouragement and moral support. I have the utmost appreciation for your patience and understanding throughout the last two years. Your belief in me gave me the confidence to continue my education and accomplish goals along the way. Thank you everyone!

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Definition
ACN	Acetonitrile
APCI	Atmospheric Pressure Chemical Ionization
BTE	Bone Tissue Extract
EA	Ethyl Acetate
CE	Capillary Electrophoresis
CE-ECL	Capillary Electrophoresis-Electrochemiluminescence
CI	Chemical Ionization
CNS	Central Nervous System
CPZ	Chlorpromazine
CPZ-D3	Chloropromazine-deuterated
CPZSO	Chlorpromazine Sulfoxide
CPZNO	Chlorpromazine N-Oxide
CV	Coefficient of Variation
DCPZ	Desmetyhlchlorpromazine
DCM	Dichloromethane
DPMZ	Desmethylpromethazine
EI	Electron Impact
ESI	Electrospray Ionization
FTPE	Filtration/Pass Through Extraction
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionization Detection
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet Detection
IS	Internal Standard

iPrOH	Isopropanol
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC/MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLE	Liquid-liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave Assisted Extraction
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MPSPE	Microplate Solid-Phase Extraction
MS	Mass Spectrometry
PMZ	Promethazine
PMZ-D3	Promethainze-deuterated
PMSO	Promethazine Sulfoxide
R^2	Linearity
RPM	Revolutions Per Minute
SPE	Solid-Phase Extraction
TIC	Total Ion Chromatogram
TOF	Time-of-Flight
SWGTOX	Scientific Working Group for Forensic Toxicology
UHPLC	Ultra High Performance Liquid Chromatography
UHPLC-PDA	Ultra High Performance Liquid Chromatography-Photodiode Array Detection
UHPLC-q-TOF-MS	Ultra High Performance Liquid Chromatography-Quadrupole-Time-of-Flight-Mass Spectrometry
UV	Ultraviolet
VIS	Visible

Chapter 1

1.1 Forensic Toxicology

Forensic toxicology is the application of toxicology where the adverse effects of drugs and chemicals on the human body have legal repercussions (1). Forensic toxicologists work with pathologists, coroners, and police officers to help establish the role of alcohol, drugs, or poisons in the cause of death, or in the contribution of the crime. It is a multidisciplinary field with four main disciplines: postmortem forensic toxicology, human performance toxicology, and workplace drug testing (1,2). Therefore, toxicological investigations commonly involve confirming a lethal dose of drugs as the cause of death, incidents of driving under the influence, and detecting the use of drugs in the workplace. It includes testing for both therapeutic drugs and drugs of abuse (1). The most commonly analyzed biological specimen is blood because you can relate drug concentrations to pharmacological effects and databases of drug levels in blood exist to assist with interpretation (1). Depending on the nature of the case and specimens available, other biological samples may be analyzed such as vitreous humour, urine, liver, stomach contents, hair or saliva. Toxicological analysis of bone is not very common because interpretation of drug concentrations is complicated given that a reference database does not exist, and there is a lack of published research and casework containing bone as the biological sample. However, in cases of extreme decomposition, bone may be considered for analysis because the conventional specimens such as blood, urine, or other tissues are not present. The sample analysis in a toxicological examination consists of four main steps: detection of any drugs or chemicals using a screening method, identification of any parent drugs, metabolites or

chemicals using a confirmatory method, quantification of the analytes that were identified and lastly interpretation of the analytical results (2).

1.2 Phenothiazines: Chlorpromazine and Promethazine

Promethazine and chlorpromazine belong to the drug classification known as phenothiazine derivatives due to their structure. The chemical structure of phenothiazines consists of a tricyclic structure in which two benzene rings are joined by a sulfur and a nitrogen atom. The nature and position of the substituents determines the pharmacological activity. The chlorine substituent at position 2 for chlorpromazine is said to increase the antipsychotic efficacy (3). The aliphatic side chain at position 10 is also responsible for differing pharmacological properties between promethazine and chlorpromazine (3). Promethazine only has two carbon atoms separating the amino group from position 10 whereas chlorpromazine has three carbon atoms.

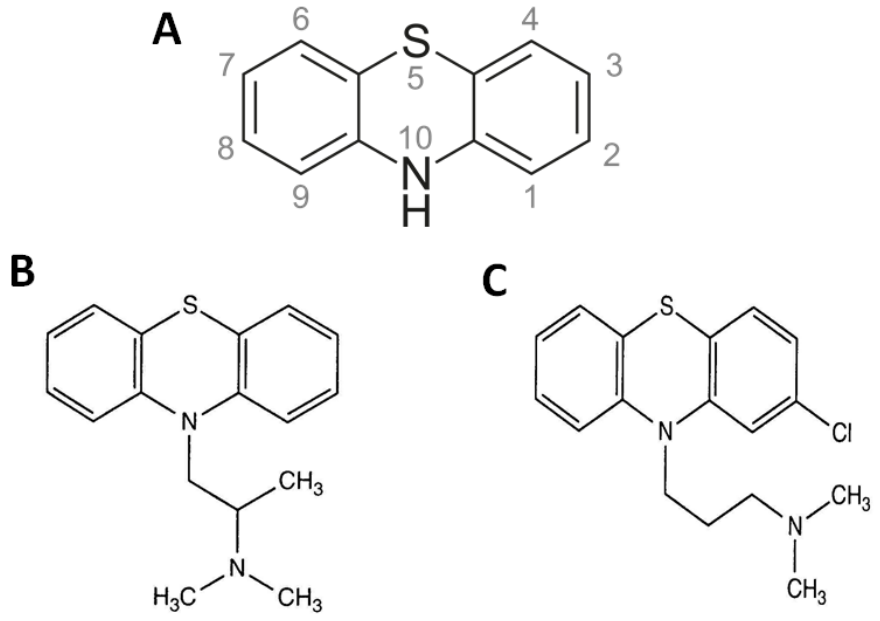


Figure 1:General phenothiazine structure (A), Promethazine (B), and Chlorpromazine (C).

1.2.1 Chlorpromazine

Chlorpromazine(CPZ) is marketed under the trade names Thorazine and Largactil and is an older antipsychotic drug. It was first available as a prescription drug in 1953 and is prescribed for the treatment of psychotic disorders, mainly schizophrenia (4). It is distributed in the form of tablets and a syrup for oral administration in a hydrochloride salt composition (4). A single dose of chlorpromazine for adults is typically 25-100mg (4). Although knowledge of the exact actions underlying antipsychotic drugs remains incomplete and is drug-specific, studies have shown that the principal mechanism of action of antipsychotics is the affinity of these drugs to bind to dopamine D_2 receptors (3,5). By binding to the dopamine D_2 receptors they block the dopamine receptors by acting as receptor antagonists. This reduces dopamine levels in the brain which are abnormally elevated in a psychotic state (5). CPZ is absorbed readily from the gastrointestinal tract after oral administration but undergoes considerable first-pass metabolism in the liver (1). Thus, depending on the extent of first-pass metabolism, its bioavailability and effectiveness varies. CPZ has a large volume of distribution of 20 L/kg which demonstrates it is extensively distributed throughout the body (1). It is highly bound to plasma proteins which results in localization in tissues with vast blood supply such as the brain, lungs, and liver (1). It can also cross the blood-brain barrier and transfer across the placental barrier (3,6). CPZ is primarily metabolized in the liver by cytochrome P450 to form mostly polar metabolites which are excreted in the urine (3,6). Biotransformation of chlorpromazine commonly occurs by sulfoxidation, demethylation, hydroxylation and glucuronidation (3,6). Therefore, it is metabolized to a great extent, with at least 12 different metabolites identified (1). However, the predominant metabolites are the sulfoxide, demethylated, and hydroxy products. The elimination phase of chlorpromazine is slow and is characterized by a half-life of 18-30 hours (3,6). As with

most prescribed drugs, side effects can occur with chlorpromazine depending on the dose consumed. There may be some initial drowsiness, slowness in response, confusion, and blurred vision however; some tolerance to the adverse effects usually develops after a few days (3). At high dose levels, more serious side effects can develop such as orthostatic hypotension, acute dystonia, akathisia, parkinsonism, and neuroleptic malignant syndrome (3). Unfortunately, the photochemical properties of CPZ can also induce undesirable side effects (7-11). The interaction of light with CPZ present in the skin and eyes often results in photosensitization (7-11). This may take the form of phototoxic or photoallergic side effects which include exaggerated sunburns, hyperpigmentation of the skin, and ocular opacity (7-11). Therefore, individuals who are taking CPZ should refrain from exposure to sunlight and artificial light. Based on laboratory studies, single oral doses of chlorpromazine as low as 25 mg given to normal healthy adult volunteers can negatively impact psychomotor performance (4). CPZ can also influence the actions of other drugs, most notably other CNS depressants (3). It can prolong or intensify the effect of sedatives, analgesics, alcohol, and cold remedies. A therapeutic range for serum drug concentrations of chlorpromazine has been established to be 50-500 ng/ml with concentrations greater than 750 ng/ml considered toxic (1). However, serum concentrations ranging from 800 to 1,500 ng/ml have been observed in patients that survived (6). This significant overlap that can exist between therapeutic and toxic drug concentration is due to tolerance and inter-individual variation and is a problem in the interpretation of chlorpromazine drug concentrations. Another factor that confounds interpretation is the fact that blood levels do not correlate well with clinical effects because many metabolites of chlorpromazine are active and may contribute to biological activity (3,6). Although new generation antipsychotics are replacing conventional older ones such as chlorpromazine, new experimental uses of CPZ have been proposed for the treatment of

anorexia nervosa (12), and anti-migraine therapies (13,14). Currently, reports of fatalities from CPZ overdose are rare because it not prescribed as frequently, but it is possible it will be detected in overdoses with ingestion of other drugs. The analytical methods which have been developed to detect and identify CPZ in biological samples are shown in Table 1.

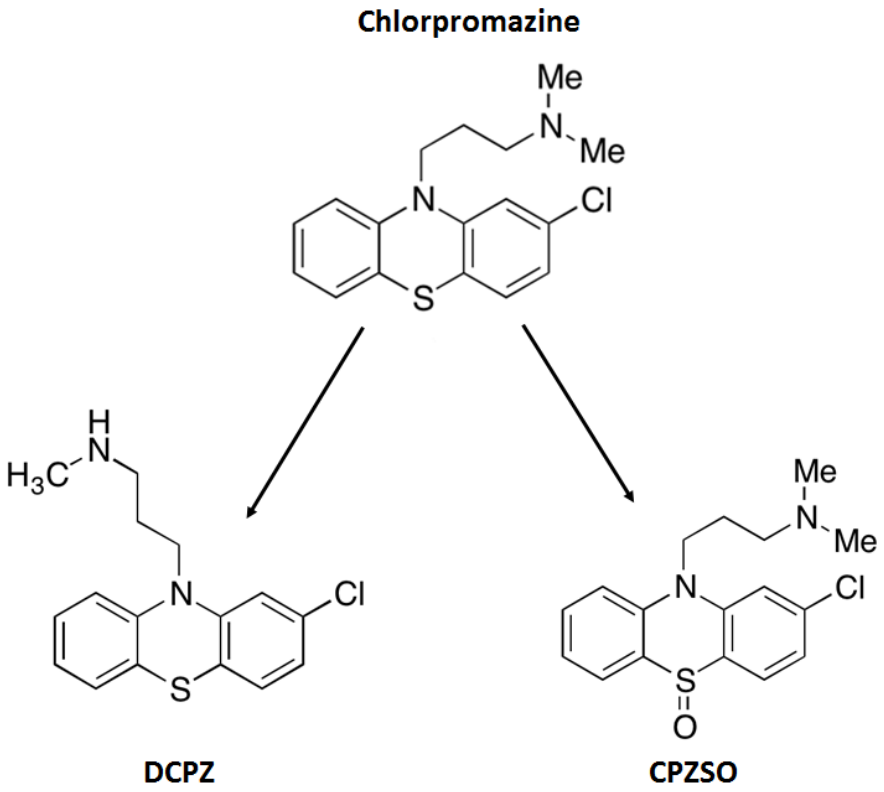


Figure 2:Chlorpromazine and metabolites: desmethylchlorpromazine (DCPZ) and chlorpromazine sulfoxide (CPZSO)

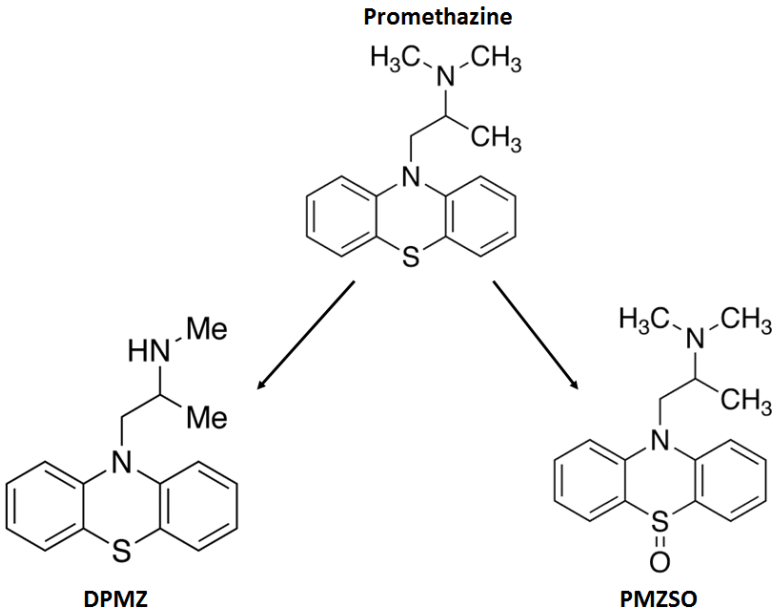


Figure 3: Promethazine and metabolites: desmethylpromethazine (DPMZ) and promethazine sulfoxide (PMZSO)

1.2.2 Promethazine

Promethazine(PMZ) is available as an over-the-counter drug known as Phenergan. It is an antihistamine used to treat allergic or anaphylactic reactions, motion sickness, nausea, and vertigo (4). It also exhibits sedative effects and therefore can be found as an ingredient in sleeping pills. Similar to CPZ, PMZ is supplied as a hydrochloride salt in tablets or syrup for oral administration (4). Other routes of administration include rectal, intravenous and intramuscular (5). However, complications appear most often when administering PMZ intravenously (15,16). It is a frequently used drug in emergency departments to treat headaches and nausea but needs to be given slowly because it is highly corrosive to blood vessels and surrounding tissue (15,16). There have been cases of promethazine related-tissue toxicity, some requiring amputation (15,16). The mechanism of action of PMZ is poorly understood, however it is known that it is an H1 antagonist. The structure of PMZ is comparable enough to histamine

that is penetrates the blood-brain barrier and binds to central H1 receptors, blocking the actions of histamine on these receptors (5). Following oral administration, PMZ is well absorbed and peak concentrations are achieved within two hours with effects that usually last four to six hours (3). The major site of metabolism is the liver by the cytochrome P450 enzyme system and most of the metabolites are excreted in the urine (1,3). Both N-demethylation and sulfoxidation biotransformation are common, producing the predominant metabolites which are promethazine sulfoxide and desmethylpromethazine (1,3). The elimination half-life of PMZ is approximately 9-16 hours (3). Consumption of PMZ may be associated with side effects, though the incidence and severity of side effects will vary between individuals. The most common side effect is sedation, others include drowsiness, blurred vision, tinnitus, and disorientation (3,5). Like chlorpromazine, Promethazine is known to cause both phototoxic and photoallergic side effects in individuals due to its photochemical properties (7-11). Individuals taking PMZ should be warned not to drive or drink alcohol because of its sedation potential; simultaneous ingestion with other CNS depressants will produce additive effects that impair motor skills (3,4). Laboratory studies have demonstrated that a single oral dose of promethazine given to healthy volunteers can impair task performance and cause sedation for up to 12 hours (4). Although, antihistamines are deemed relatively safe, cases of promethazine poisoning have been reported. In instances where poisoning occurred the dose ingested ranged from 350 mg-1250 mg, where a daily oral dose in adults ranges from 25 mg to 150 mg (4,18). According to Parker S.D *et al.* (18), it is possible the abuse and dependence of promethazine is under-recognized and needs to be more acknowledged (19). Promethazine has been encountered in post-mortem examinations of multiple drug toxicity and more recently is being found in combination with codeine in a mixture referred to as “purple drank” (20). Hence, it is a possibility PMZ will be encountered in

forensic toxicological analysis. Presently, PMZ and some of its metabolites in biological samples have been analyzed by multiple methods. These are listed in Table 1.

Table 1: Analytical Methods Used to Identify Promethazine and Chlorpromazine in Biological Samples

Analyte	**Sample Matrix**	**Sample Preparation**	**Analytical Method**	**Limit of Detection (ng/ml)**	**Reference**
PMZ	Plasma	Pipette Tip SPE C_{18}	GC-MS	20n	Hasegawa *et al.*, 2006 (20)
PMZ and metabolites	Urine	Online solid-phase extraction and column switching	HPLC-UV	PMZ:3.7 PMZSO:2.5 DPMZSO:2.5 DPMZ:2.5	Song, Q., and Putcha, L., 2001 (21)
PMZ	Blood	Hollow-fiber drop-to-drop solvent microextraction	GC-MS	18	Tapadia, K., *et al*., 2011 (22)
PMZ	Urine and plasma	Liquid-liquid extraction	LC-MS	1.0	Campanero, M. A., *et al*., 1998 (23)
PMZ and metabolites	Plasma	Liquid-Liquid Extraction	HPLC-UV	PMZ:1.0 PMZSO:2.0 DPMZSO:1.5 DPMZ: 1.5	Vanapalli, S. R., *et* al. ,2001 (24)
PMZ	Serum	Liquid-Liquid Extraction	HPLC-UV	1.0	Wallace, J. E., *et al*., 1981 (25)
CPZ	Urine	Liquid-Liquid Extraction	CE-ECL	1.5	Li, *et al*., 2006 (26)
CPZ	Blood	Solid-Phase Extraction	GC-NPD	46	de la Torre, C. S.,*et al*.,2005 (27)

CPZ	Urine and serum	Hollow fiber liquid phase microextraction	HPLC-UV	0.5	Sobhi, H. R., *et al*. 2007 (28)
CPZ	Plasma	Solid-phase extraction	HPLC-Coulometric Detection	0.17	Saracino, M. A.,*et al*. 2008 (29)
CPZ	Serum	Stir-bar sorptive extraction	HPLC-UV	0.7	Bazhdanzadeh, S., *et al*. ,2011 (30)
CPZ	Urine	Molecularly imprinted solid-phase extraction	HPLC-UV	80	Song, S., *et al*.,2008 (31)
PMZ and CPZ	Plasma	Dilution	HPLC-UV	100	Pistos, C., Stewart, J. T., 2003 (32)
PMZ and CPZ	Urine	Hollow-fiber liquid phase microextraction	GC-FID	PMZ:1.4 CPZ:9.9	Xiao, Q., Hu, B, 2010(33)
PMZ and CPZ	Plasma	Micropipette tip solid-phase extraction	GC-MS	PMZ: 6 CPZ: 2	Kumazawa, T., *et al*. 2011 (34)
PMZ and CPZ	Urine	Dynamic liquid-phase microextraction	HPLC-UV	PMZ:48 CPZ:31	Cruz-Vera, M., *et al*.,2009 (35)

1.3 Stability of Phenothiazines

In forensic toxicology, knowledge of the stability of drugs in biological samples during storage, handling and analysis is important for the interpretation of findings. Drug concentrations can change from the time of death to the sample acquisition. This may lead to a discrepancy between the initial concentration and the concentration result from the toxicological analysis, which can result in unreliable interpretations. In reference to the literature, stability is defined as the

"influence of time on the concentration of the analyte in a given matrix under specific conditions" (36,37). Evaluation of the stability of drugs is often completed as part of the method development and validation, but unless the stability experiments are stated in the title, information about a particular drug is very difficult to retrieve. This is the case for promethazine and chlorpromazine; information concerning long-term stability or stability in a variety of matrices under different conditions is hard to locate. The different types of stability experiments that should be performed include: long-term stability in the sample matrix, freeze/thaw stability, stability of the analyte during sample preparation, and stability of the analyte in extracts under conditions of analysis (36,37). The literature publications regarding the stability of chlorpromazine and promethazine mainly involve the effect of sample storage at different temperatures. None of the articles describe promethazine as unstable during storage, yet chlorpromazine is described as having stability issues. The stability results for CPZ are contradictory; one article mentioned CPZ showed losses when stored at -20^0C in blood after 20 weeks (36), on the other hand, another study found CPZ to be stable in blood over 84 days when stored at -20^0C (38). Other articles investigated the stability of CPZ in serum and plasma where the results indicated it was stable, however one study did report an increase in CPZ concentration in a patients' blood serum sample stored below 0^0C due to reconversion of the metabolite to the parent drug (37).

1.3.1 Oxidation of Chlorpromazine and Promethazine

The phenothiazines exhibit many chemical properties which may account for their instability or reactivity. Their most well-known property is their susceptibility to oxidation by a number of different oxidizing agents, ex.,$K_2Cr_2O_7$, NH_4VO_3, $Ce(SO_4)_2$, $KBrO_3$, KIO_3, KIO_4,$NaNO_2$, H_2O_2

or by exposure to light (39,40). This property enables their application as redox indicators and use in catalytic methods (39). They also react with some metals and organic substances to form ion-association complexes (39). The general reaction mechanism for the oxidation of phenothiazines describes the first step as reversible and is the loss of an electron to produce a coloured radical cation (Fig 4, 39, 40-42) In the second step the free radical is oxidized further to generate a colourless sulfoxide (Fig 4,40-42). The susceptibility of phenothiazines to oxidation and the type of oxidation products that form depend on the following factors: acidity of the solution, concentration of reagents, temperature and substituents at position 2 and 10 on the ring (40-42). Therefore, although the sulfoxide commonly forms, depending on the reaction conditions the nitrogen atoms may also be oxidized to form the N-oxide (43). Other oxidation products result from cleavage of the lateral side chain and electrophilic substitutions (44). Literature studies have demonstrated that chlorpromazine and promethazine are easily oxidized (45-49). They will degrade in the presence of acid or base and degradation is increased if external factors such as light or temperature are involved (45-49). Furthermore, promethazine sulfoxide and chlorpromazine sulfoxide are the most commonly identified degradation products (45-49). According to the toxicology literature, depending on the extraction conditions CPZ metabolites in plasma samples were converted back into CPZ (50). The reduction of CPZNO to CPZ leads to deceptively high levels of CPZ (51). The same authors observed the oxidation of CPZ to CPZSO in whole blood due to the set-up of the analytical procedure (51,52). They also noted that whole blood presents a challenge compared to plasma because the hemoglobin catalyzes the sulfoxidation (51,52). In summary, the oxidation of phenothiazines by chemical, photochemical, electrochemical, and enzymatic methods has been extensively studied, however it is a complex subject that is very dependent on the experimental conditions. Thus, the

mechanisms and products formed vary considerably. Also, there are very few articles that highlight the measures that should be taken to avoid degradation. Even more concerning is the rare mention of the problem of instability of the phenothiazines in the toxicology literature.

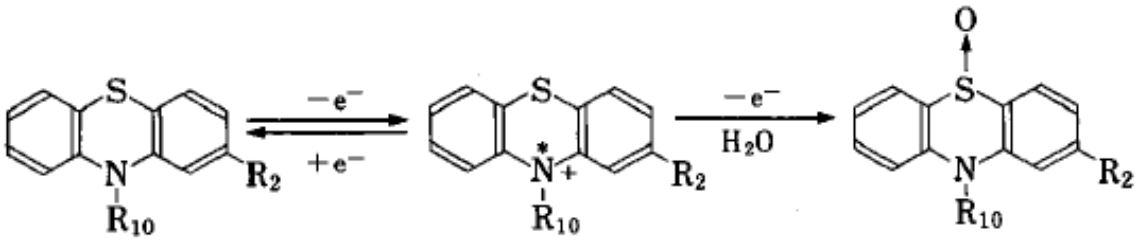


Figure 4: Mechanism of oxidation for phenothiazine derivatives

1.4 Sample Preparation

In most cases, prior to separation and detection by analytical instrumentation the sample will require some form of treatment and preparation. The sample preparation can be the most time-consuming, tedious and expensive step of the analytical procedure. Furthermore, it has an influence on the selectivity, sensitivity, accuracy and precision of the results obtained. Therefore, optimization of the sample preparation procedure is very important. Although, sample preparation can be employed using a wide range of techniques, all methods share the same primary goal: to remove as many interfering compounds as possible. Many factors are taken into consideration when choosing a particular sample preparation procedure, such as the nature of the sample matrix, the properties of the target analyte, the limit of detection required and the type of separation and detection utilized. The different types of sample preparation methods generally include: dilution, precipitation, centrifugation, filtration, and extraction. Solid-phase extraction and filtration are the two sample preparation methods discussed below based on their inclusion in this research project.

1.4.1 Solid-Phase Extraction

For many years, liquid-liquid extraction (LLE) was the most common and widespread sample preparation method, however currently, solid-phase extraction (SPE) is gaining popularity at the expense of LLE procedures. The principle of SPE is similar to that of LLE, which involves the partitioning of the analyte between two phases, in LLE it is two immiscible liquid phases (53). In SPE, the analytes to be extracted are distributed between a solid phase (sorbent) and a liquid (sample matrix) (53,54). The separation is based on the analytes having a greater affinity for the solid phase than the sample matrix (53,54). The advantages of SPE over LLE which have contributed to its growth and widespread use include: decreased solvent consumption, versatility (as many sorbent formats and protocols are available), ease of automation, increased extraction efficiency (stronger retention and selectivity available), and reduction in time and labour (simultaneous analysis of multiple samples) (53-55). There are various types of SPE sorbent formats available

such as cartridges, disks, syringes, pipette tip, and 96-well plate (55,56). The 96-well plate configuration is a newer format that has become common because you can process 96 individual samples simultaneously in parallel (55,56). The principle SPE objectives consist of 1. Removal of interferences: to identify and quantitate the analyte of interest, and reduce ion suppression or enhancement 2. Increase the concentration of the analyte: to achieve low limits of detection depending on the application 3. Ability to fractionate the sample matrix: to separate different classes of compounds. (53,54).

Compounds are retained by the SPE sorbent based on the mechanism of interaction between the sorbent and the analyte (53,54). The different mechanisms of retention are based on van der

Waals forces, hydrogen bonding, dipole-dipole interactions, pi-pi interactions, hydrophobic interactions, hydrophilic interactions and electrostatic interactions (53,54). In reversed phase SPE, the separation involves a non-polar stationary phase and a polar matrix, with the analyte being mostly non-polar (53,54). In normal phase SPE, the analyte has polar functional groups to interact with the polar stationary phase and the sample matrix is non-polar (54,57). For ion-exchange SPE, the compounds must be charged in solution which is achieved by manipulating the pH (54,57). The analyte and the sorbent must be oppositely charged for retention to occur. A cation exchange stationary phase is used to retain positively charged compounds and an anion exchange stationary phase is used to retain negatively charged compounds (54,57). If a mixed-mode sorbent is utilized, then two modes of retention are included in the stationary phase (54,55). For example, the sorbent can incorporate reversed-phase and ion-exchange to enable greater retention and selectivity (54,55). In forensic toxicology mixed-mode sorbents are commonly employed because many drugs contain aromatic and alkyl functionalities with a nitrogen that can be readily protonated, allowing them to participate in hydrophobic, aromatic, and electrostatic interactions and separate from acidic compounds.

The experimental procedure for SPE consists of five major steps: conditioning, equilibration, loading, washing, and elution (53,54). Each step requires optimization because the solvent selection is important and depends on the analyte and stationary phase. For the first step, a strong organic solvent conditions the sorbent by activating the stationary phase so the compounds can properly interact with the sorbent pores (53,54). The purpose of the equilibration step is to replace the organic solvent by a liquid that is similar to the composition of the sample matrix (53,54). Next, the sample which contains the analytes of interest is applied to the cartridge or

well and allowed to flow by gravity (53,54). The wash step provides for the removal of interferences while retaining the analyte (53,54). A vacuum manifold can be utilized to dry the sorbent if changing between aqueous and organic solvents and to control the flow rate (53,54). The last step is the elution of the analyte from the sorbent with a solvent that has the required eluent strength, with the goal of recovering as much of the analyte as possible (53,54). Finally, a dry down and reconstitution step is often required to evaporate the strong elution solvent and add mobile phase to ensure the sample is compatible with the chromatography system (54).

SPE can be used for numerous applications and as a result there are many publications describing SPE methods (55,56). It is an active area of research with recent developments focused on new formats, new selective sorbents, and automation (55,56).

1.4.2 Filtration

Filtration is an easy and straightforward method of sample preparation. Most filtration methods consist of a porous membrane that involves a size-exclusion mechanism which allows small molecules to pass through freely but stops the passage of larger molecules (58). Its purpose is to remove particles from solutions or dissolved substances. It is important to remove these particles because they can affect instrument hardware such as columns, flow lines, valves and frits (53). The efficiency of the filtration is determined by the porosity of the filter (58). Therefore, a lower porosity provides a cleaner extract but a longer filtering time. The membrane of fibers can be made of paper, glass, cellulose, or other plastic substances (58). The most common filtration systems operate under pressure differences and simply flow by gravity but there are more advanced micro-and ultra-filtration devices that require vacuum (53,58). A popular filtration format is the 96-well flow through plate with an array of small membrane filters built as a sorbent bed (53). It resembles the 96-well plates for solid-phase extraction and offers the same

benefit of simultaneous analysis of multiple samples at once (53). With the 96-well filtration plates manufacturers offer various membranes and porosities to choose from (53). Depending on the application, filtration can be utilized as the only sample preparation method, or prior to an extraction method such as SPE. An important consideration is to ensure the solvent is compatible with the filter and whether or not rinsings must be applied before and after applying the sample. Furthermore, the analyst must be aware that adsorption of the analytes onto the filter or within the pores is possible.

1.5 Instrumental Analysis

1.5.1 Liquid Chromatography

Chromatographic separations are widely used in analytical toxicology prior to a detection mechanism. The goal of a separation technique is to obtain sharp, symmetrical peaks because then the sensitivity and selectivity are optimized. Liquid chromatography (LC) is the broad term used to describe any chromatographic procedure in which the mobile phase is a liquid. It is a widely used analytical separation technique because it can be applied to the separation of any compound that can be dissolved, and thus is amenable to a greater variety of compounds than GC (59). Furthermore, the complications of volatility, stability at high temperatures and derivatization are avoided by utilizing liquid chromatography compared to gas chromatography (59). Another added benefit is the increased control over separation efficiency because in LC you can manipulate both stationary and mobiles phases (59). The separation process in liquid chromatography is based on the differential distribution of sample components between a liquid mobile phase and a stationary phase which is fixed in place in a column (59-61). The separation between components is achieved as a result of differing attraction to the stationary phase. If the

component is strongly attracted to the stationary phase it will be retained which will delay its movement through the chromatographic system. In contrast, the components that are less attracted will be weakly retained and move more rapidly. Therefore, the migration rates of components will differ resulting in distinct, zones, bands, or peaks. There are many factors that influence the chromatographic separation, the most important being column efficiency (60). Column efficiency is used to compare column performance and is quantitatively measured by plate height and number of theoretical plates (60). The plate theory refers to a column made up of numerous discrete layers termed plates and at each plate equilibration of the component between the mobile and stationary phase takes place (60). As the number of plates increases, and the plate height decreases, the efficiency of the column becomes greater producing more narrow peaks (59-61). On the other hand, band broadening indicates a loss of column efficiency due to a slow rate of mass-transfer between the two phases (61). The parameters which affect column efficiency include diameter, length, and particle size of the column, flow rate and viscosity of the mobile phase, and type of stationary phase (59). Resolution describes the power of the column to separate the peaks of interest and is defined as the difference in their retention times relative to their widths (60). Consequently, the higher the resolution, the easier it is to obtain baseline separation between peaks, which is often achieved by lengthening the column (60). Columns for LC are available in many dimensions with regards to length, diameter, and particle size to suit the needs of the application. They range from short (50 mm) for high throughput to long (150 mm) for greater resolution. Silica is commonly utilized for the particles because it can withstand high pressures and has a large surface area (59). The surface of the silica particles can subsequently be modified by chemical or physical means to produce stationary phases with different mechanisms of interaction. A decrease in particle size (<3μm) is effective because it

improves speed and resolution but a greater backpressure is produced. Ultra-High Performance Chromatography (UHPLC) refers to instruments that can operate at higher pressures and accommodate smaller particles sizes (60,61). The injection volume is another important factor to consider because it can impact peak shape and detection limits (60). If the injection volume is too large it can clog the column leading to peak broadening. Conversely, if the injection volume is too small it may be hard to detect the analytes but this problem can be overcome by incorporating an evaporation step to concentrate the analytes. LC can be divided into different modes or mechanisms based on the stationary phase used in the column. The most popular modes include reversed-phased, normal phase, and ion-exchange (60). The principles are the same as described in the previous section on solid-phase extraction. Reversed-phase chromatography is most commonly utilized, and consists of a non-polar stationary phase and polar mobile phase (60). As a result, the most polar components elute first, followed by mid-polar and then non-polar. Reversed-phase chromatography is typically associated with a C18, or C8, which indicates the number of carbons on the alkyl chains, or a phenyl structure. The more components in the sample the more likely it is that a gradient elution method will be used. Gradient elution refers to changing the mobile phase composition over time by increasing solvent strength (59-61). The benefit is sharper peaks, compared to when an isocratic method is used (59). Two to four mobile phase solvents are used and in reversed-phase A is the weaker solvent (water) and B is the stronger organic solvent (often ACN). It is important to use high-quality-grade solvents that are compatible with the materials on the instrument. Unlike in GC, temperature does not have much of an effect on resolution in LC. However, an increase in temperature does reduce the viscosity of the mobile phase which increases the rate of mass transfer (61). Also, if the temperature is kept constant the chromatograms obtained are more

reproducible (61). A chromatogram depicts the results of the separation and provides the retention time of each analyte which is defined as the time required for the analyte to reach the detector after injection (61). To conclude, the overall goal in LC method development is to optimize the separation of the components. It is important to choose the correct experimental conditions such as: the mode for the intended application, a column with high efficiency, the stationary phase chemistry, the length of the column, the composition of the mobile phase solvents, and the type of elution.

1.5.2 Photodiode Array Detection (PDA)

In most instances, a chromatographic method is combined with a detector to identify and quantify the eluted components. A common combination is the use of LC with a UV/VIS detector which is based on the absorption of light in these regions. Therefore, a requirement is that substances should absorb strongly in the UV-visible region at different wavelengths or a chemical modification must be used to transform the substance into a derivative that can absorb (62). This is because the energy absorption properties of the substance are what is used to quantify the concentration in solution. The relationship between the absorbance and the concentration of the absorbing analyte is known as Beer's law (61-63). The equation for Beer's law is shown below

$$A=\varepsilon c l \quad (61)$$

where A is the absorbance, ε is the molar absorptivity which is characteristic of the substance and wavelength, c is the concentration of the solution and l is the path length of light through the solution (61). Based on this equation, concentration is linearly related to absorbance (62). However, it is a limiting law because at high concentration deviations from the linear

relationship can occur (61). As the concentration increases, the extent of solute-solvent or solute-solute interactions can affect the analyte environment and may alter its absorptivity (61). There are many variations for UV-Vis instrumentation, some are designed for operation in only the UV range while others operate in both the UV and VIS range (63). A UV/VIS spectrometer consists of five essential components: a light source, a monochromator, sample container, detector, and a readout device (61,62). For UV, the light source is commonly a deuterium lamp, the monochromator provides wavelength selection, and the sample container or sample format must be transparent so the light can pass through (61). The light that is transmitted is quantified by the detector which produces an electrical signal when it is struck by the photons (62). There are different types and designs of detectors which consist of a photon transducer which can be in the form of photovoltaic cells, phototubes, photomultiplier tubes, or photodiodes (61). The signal from the detector is then amplified and converted to readable form that is displayed on a computer. There are three types of multichannel detectors used in spectroscopic instruments: photodiode arrays, charge-injection devices, and charge-coupled devices (61).

With regards to photodiode array detection, after the light passes into the monochromator it is dispersed onto a photodiode-array transducer, which consists of a linear array of several hundred photodiodes mounted on a silicon chip (62). The slit of the monochromator is identical to the width of one of the diodes, as a result, each diode receives a different wavelength (62). The outputs from the diodes are scanned rapidly and sequentially which allows for the simultaneous measurement of multiple wavelengths and collection of the entire absorbance spectrum (62). The resulting data is a plot of absorbance as a function of wavelength. Identification of a component is based on the combination of both retention time and UV spectrum. For quantification, it is important to select the wavelength where the analyte demonstrates maximum absorption because

this will increase sensitivity (62,63). Once an appropriate wavelength is selected, peak areas are integrated which are proportional to the amount of the analyte in the sample. Interferences will arise only if compounds co-elute with the analyte and absorb radiation in the same spectral region as the analyte. There are many advantages to using PDA detection which include: robustness, reproducibility, lower operating costs than MS instrumentation, simple design which needs minimal maintenance and no recalibration (61-63). It allows monitoring a sample at more than one wavelength and peak purity analysis (61). Lastly, when coupled to LC it provides useful qualitative and quantitative information for multiple compounds in a single run.

1.5.3 Quadrupole-Time-of-Flight-Mass Spectrometry (Q-TOF-MS)

Another detection system often coupled with UHPLC is mass spectrometry (MS) which offers increased sensitivity and selectivity for both qualitative and quantitative analyses. In recent years, technological advances have enabled the development of various ionization mechanisms and mass analyzers, making MS a very flexible detection system with various applications. MS detection can provide elemental and structural information by means of molecular weight determination, fragmentation patterns, and isotope ratios (64). A basic mass spectrometer has five components: a sample inlet; to introduce the sample into the MS, an ionization source; where the sample molecules are ionized, next is the mass analyzer; which separates the ions based on their mass-to-charge-ratio (m/z), then a detector records and counts the ions and finally a data processing system plots and manipulates the data to produce a mass spectrum (65). A mass spectrum provides the results as a plot of ion intensity versus m/z. The most intense peak called the base peak is assigned an intensity of 100 and all other peaks are assigned an abundance relative to the base peak (65). The type of ionization method utilized depends on the

separation step (GC, LC or CE), the nature of analyte, the information required, and the capabilities of the instrumentation. The various ionization methods include: electron impact ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and desorption ionization techniques. EI and CI are commonly used with GC, and ESI and APCI are commonly used with LC, whereas desorption ionization techniques are often used for larger biomolecules and involve *in-situ* analysis with no prior separation (64). The most well-known and simple method to ionize the sample is EI. In EI, the sample which is in vapour phase is bombarded by a beam of electrons emitted from a filament (64,65). The high-energy bombardment causes the molecules to become ionized by the loss an electron, excited and then relax which often occurs by fragmentation to produce ions of lower masses (61,64,65). The ionized molecule that loses an electron but does not undergo fragmentation forms a positively charged molecular ion (M+) which has the same molecular mass as the analyte of interest and appears at the highest value of m/z on the spectrum (61,64,65). The ionized molecules are directed to the mass analyzer based on a potential difference between two plates where they are sorted according to their mass-to-charge ratios which are then displayed in the form of a mass spectrum (61,64,65). EI is a "hard" ionization method meaning it leads to significant fragmentation (61). However, the fragmentation pattern is characteristic of the analyte and reproducible so it is useful in identification (61,64).

Electrospray ionization (ESI) takes place under atmospheric pressure and temperature and is tailored to ionize molecules from liquid streams, thus, samples are directly introduced from UHPLC (1,64). In ESI, a solution of sample is passed through a stainless steel capillary needle held at high voltage potential, creating charged droplets that are expelled into the ionization chamber (1,64). The charged droplets come in contact with a nebulizing inert gas that disperses

the liquid into an aerosol (1). As the solvent evaporates from the droplets, their charge density becomes greater until the surface tension can no longer support the charge and they break apart into smaller droplets (64). This process continues until all the solvent evaporates and the charge is transferred to the analyte (64). The ions are then drawn towards the mass analyzer by an electric field (64). Ions formed can be positive or negative and modern ESI sources can be programmed to analyze in positive or negative mode depending on the application (64). Furthermore, the fragmentation parameters can be optimized by running a standard solution at different voltage, pressure, and temperature settings. A concern in LC/MS techniques is the possibility of ion suppression. Ion suppression can occur when excessive ions are produced from the sample and the signal from the analyte of interest is suppressed in the background noise (1). This can arise from concentrated analytes, the matrix, solvents, or glassware and can negatively affect the limit of detection, precision and accuracy of the results. Therefore, it is important to examine the method for the presence of ion suppression and try to minimize it. Also, the formation of adducts is possible but can be controlled by using highly pure mobile phase solvents (1).

As mentioned previously, several different mass analyzers are available for separating ions. With regards to the quadrupole-time-of-flight mass spectrometer (Q-TOF-MS), there are two different mass analyzers combined; the quadrupole and time-of-flight analyzer. The performance of the mass analyzer is measured according to its mass range limit, its analysis speed, the ion transmission rate, its mass accuracy and its resolution (65). The term quadrupole refers to a linear quadrupole designed with rods arranged in parallel and opposite (64). It operates by varying two electrostatic fields, one direct current and one radio frequency which creates a resonance frequency for each m/z value (64). Therefore, only ions of a specified m/z pass

through the quadrupole and reach the detector. The full mass range can be scanned so that ions of sequential m/z pass through the analyzer or it is possible to operate the quadrupole so that only ions with a certain m/z pass through, this is called selected ion monitoring (64,65).

A time-of-flight mass(TOF) analyzer functions on the relationship between kinetic energy, velocity and mass. Ions produced are accelerated into a flight tube by an electric field pulse ensuring all the ions entering acquire the same kinetic energy (61,64). Since the ions have the same energy, they will travel through the tube at different speeds due to differences in mass (64,65). Separation by mass occurs, with the lighter particles arriving at the detector before the heavier ones (64). The mass-to-charge ratios are determined by measuring the time it takes for the ions to arrive at the detector (64). A TOF analyzer can measure an analytes mass to four decimal places providing accurate mass measurement. Thus, many different drugs or metabolites with close molecular masses can be effectively separated due to mass determination with high accuracy. The added advantage of the quadrupole as a scanning device allows for accurate mass measurement of precursor and fragment ions for increasing specificity and sensitivity.

1.6 Method Validation

The accuracy and reliability of analytical data is very important in forensic toxicology for the correct interpretation of findings. If unreliable scientific data is entered as evidence, serious legal implications could result. Therefore, it is crucial that method validation is performed to ensure the analytical measurements obtained are reliable, precise, and consistent so that the method's performance is acceptable for its intended use. It is also important for recognizing the method's limitations. An analytical method needs to be validated or revalidated in the following examples: a new method is developed, an existing method does not meet current validation requirements, the method is transferred to another laboratory, modifications of an established

method such as adding new compounds or to demonstrate equivalence when switching to a new instrument (66-68). The requirements for method validation in forensic toxicology have been defined in different working groups and committees such as The American Academy of Forensic Sciences (AAFS), The Scientific Working Group for Forensic Toxicology (SWGTOX), The American Board of Forensic Toxicology (ABFT), and the Society of Forensic Toxicologists (SOFT). These organizations provide documents outlining the standards for method validation and information pertinent to understanding the parameters and definitions. Laboratories are responsible for choosing which standard of practice to follow and implementing a validation plan. Analytical method validation is the process of performing a series of validation experiments that statistically evaluates the efficacy, reliability, and applicability of a method (66-68). The type and extent of validation experiments depends of the scope of the method for example, whether it is used for screening, qualitative, or quantitative purposes (66-68). For a proper validation plan, it is important to outline the validation parameters that should be evaluated and their corresponding acceptance criteria. In forensic toxicology, there is general agreement that the following parameters should be examined: precision, accuracy, linearity, stability, limit of detection, and each are described below (66-68).

Precision

Precision refers to the degree of closeness between a series of measurements when a procedure is applied to multiple replicate samples (66-68). Precision should be evaluated for each concentration using at least three different samples over five different runs (66-68). Therefore, intraday precision and interday precision should be assessed and under the same operating conditions each time. It is calculated as the coefficient of variation (%) and should be within 20% (66-68). However, acceptance criteria can vary depending on the matrix, certain matrices may require better reliability and thus a lower CV (66-68).

Accuracy

It is the closeness of agreement between the measured experimental value and the theoretical value (66-68). Accuracy is determined by blinded analysis of standard samples with known concentrations and is described as bias studies (66-68). It should be measured using triplicate samples at a minimum of two concentration levels (low and high) (66-68). It is calculated as a percent deviation from the theoretical value using the equation: mean measured concentration-theoretical concentration/theoretical concentration) x 100% (66-68). The maximum recommended acceptable bias is within 20% of the true value (66-68).

Limit of Detection

This is the lowest concentration that is detectable and differentiated from the background noise but not subject to precision criteria (66-68). It needs to yield a peak with a height greater than or equal to three times the baseline noise level from the negative control (66-68).

Limit of Quantification (LOQ)

The lowest concentration that can be quantitatively determined with acceptable precision (66-68). The selected concentration should have a precision that does not exceed 20% (66-68). A signal-to-noise approach can also be applied where the signal-to-noise ratio is required to be equal to or greater than 10 (66-68).

Linearity (Calibration Curve Model)

Linearity is evaluated as the relationship between detector response and concentration. A calibration curve is plotted to demonstrate that response is directly proportional to the concentration (66-68). The concentration range chosen should reflect the concentrations you would expect in the matrix of interest (66-68). It is ideal to have at least six non-zero concentrations covering the expected range (66-68). The resulting curve is usually graphically

displayed and evaluated for linearity by calculating the regression line (66-68). The most common indicator utilized is the correlation coefficient (>0.99) (66-68). In some cases, it is possible that the relationship is modelled more effectively with a quadratic regression rather than a linear regression.

Stability

Method validation must include stability experiments for the various stages of analysis and the conditions used should reflect situations likely to be encountered during sample handling, storage and analysis. Stability experiments should evaluate long-term storage (frozen), short-term storage (room-temperature), freeze-thaw stability, and under conditions of analysis (sample preparation stability, autosampler stability) (66-68). The experiment should be designed to analyze samples at both low and high concentrations for preselected time intervals that cover the expected maximum time (66-68). The response at time zero is compared to the response from each time interval to see if there was any change in response.

Additional validation parameters that may be investigated depending on the application and instrumentation used are: carryover, dilution integrity, recovery, and matrix effects.

Internal standards are also a very important factor in obtaining accurate and reliable quantitative results. It improves precision and accuracy of the method because the effect from sources of error are minimized. The internal standard should have chemical and physical properties that are very close to the analyte, and as a result behave similarily, but are well-resolved and produce a signal that can be distinguished from all analytes (2). Therefore, any factors that affects the internal standards will also affect the analytes to the same degree. This is important because the internal standards serve the purpose of correcting for any differences in extraction efficiencies, or

variations that might result from small errors during handling or preparation of the samples (2). Quantification is based on the ratio of response between the analyte and IS (2). Since both compounds will undergo the same losses their ratio will remain unchanged during the procedure. For GC/MS or LC/MS procedures it is very common to use deuterated internal standards (1).

1.7 Drug Detection in Bone

Although blood is typically the matrix of choice in post-mortem forensic toxicology, the use of other biological matrices may be necessary in cases where blood is unavailable. For example, in cases of extreme decomposition, where only skeletonized remains exist, bone may be the only suitable matrix for analysis. For many years, researchers have been trying to determine whether drugs can be detected in bone and if they are detected whether the levels can be accurately and quantitatively interpreted. Therefore, the main question is: do drug levels in bone samples correlate to the blood concentrations at time of death (69-71)? So far, a general agreement on the answer to this question has not been reached. There are instances where a correlation between bone and blood concentrations was demonstrated and other instances where correlations prove non-significant (70,71). The issues that may cause the discrepancies are: drug deposition and distribution in bone, differences in sampling location, and the use of animal models (69-71) There are several factors that can influence the deposition and distribution of drugs in bone and bone marrow, such as bone type, physicochemical characteristic of the drug and metabolites, drug exposure (acute vs chronic) and distribution at the time of death (69) The mechanisms involving drug deposition in bone are not fully understood, therefore, studies should be conducted using different drugs, exposure types, and environmental conditions. A variety of bone types have been analyzed in the literature and in our laboratory and the results demonstrate that depending on the specimen chosen (femur, vertebrae, tibia etc.) the drug levels can vary (70-

82) In the literature, several articles describe the analysis of bone or marrow and generally involve the same steps of dissection to remove tissues, washing and drying, extraction of the drugs from the bone using an organic solvent followed by analysis of the supernatant (70,71) Furthermore, multiple drug classes have been detected in bone such as benzodiazepines, stimulants, antipsychotics, opioids, and tricyclic antidepressants (70-82). Thus, common drugs may be detected in bone using current methods which proves bone may be a useful alternative specimen to blood. However, caution must be exercised because most studies have been conducted on animal models and application of the data obtained to human autopsy cases requires careful consideration. In our laboratory, we conduct studies using controlled drug exposure in experimental animals with the hopes of establishing standardized methods and a reference database for bone drug measurements. Thus far, the work of Watterson and colleagues has demonstrated interesting findings with regards to detection of drugs in bone such as 1) there is substantial variation in drug and metabolite levels between bone types, 2) the vertebral, pelvic and femoral bone exhibit the largest drug levels, 3) the measurement of the ratio of the metabolite-to-parent drug levels show less variability than individual drug concentrations across different bone types 4) drug exposure may be distinguished based on the ratio of levels of parent to metabolite and 5) body position and microclimate influence drug levels (72-81). The findings illustrate there are many factors to consider when interpreting toxicological analysis of bone making it a challenge. In the future, as the research in this field continues, validated standardized methods will be implemented, and eventually quantitative interpretation will be achievable.

Chapter 2

Characterization of degradation products of selected phenothiazine drugs formed during a standard SPE approach is described. This project was intended to develop an analytical method for promethazine (PMZ), chlorpromazine (CPZ) and their respective N-desmethyl and sulfoxide metabolites in biological samples (bone tissue extract and blood) by UPLC-PDA, using mixed-mode SPE for basic drugs, using ethyl acetate:isopropanol:ammonium hydroxide (80:18:2) as the elution solvent. During the validation process, extraneous peaks were observed that were absent in the negative control. Analysis of extracts of PMZ and CPZ individually yielded extraneous peaks, including peaks with retention time and UV spectra suggesting the formation of the sulfoxide metabolites, amongst others. With the hypothesis that the analytes were being oxidized during the sample preparation process, each analyte was extracted individually and analyzed by UPLC-qTOF-MS using the same chromatographic column and mobile phase program. The resulting data supported that hypothesis, as we confirmed through analysis of reference standards that PMZ was being oxidized to its corresponding sulfoxide and N-oxide while CPZ was oxidized to its corresponding sulfoxide. Therefore, the oxidation products included naturally occurring metabolites of the drugs being assayed. Oxidation was also observed in analysis of whole blood, and thus not specific to bone tissue extract. To minimize oxidation, SPE using a different elution solvent was evaluated, as was filtration/pass through extraction (FPTE) with and without evaporation. The results demonstrated that the sample preparation method highly influence the extent of oxidation. FPTE without an evaporation step was the only method that did not measurably induce analyte oxidation. The method was validated for the analysis of PMZ, CPZ and their corresponding metabolites in bone tissue extract by UPLC-qTOF-MS according to SWGTOX standards.

2.1 Introduction

Methods for extraction of basic drugs from postmortem tissue samples are often based on liquid-liquid extraction (LLE) (82,83) or solid-phase extraction (SPE) (83,84), in order to provide sufficiently clean extracts so that they may be analyzed by GC/MS or HPLC with optical detection, or to reduce matrix effects in methods based on LC/MS/MS. Work in our laboratory is focused on analysis of drugs and metabolites in skeletal remains. Following a solvent extraction procedure to extract analytes from the bone matrix, SPE-based methods generically designed for basic drugs, with minor variations to improve cleanliness or recovery, are used to assay a range of analytes (85,86,87). At the onset of this work, we sought to examine the relative distribution of two basic drugs from the same class (phenothiazines) in skeletal tissues, with the intent of investigating the effect of slight structural differences on the relative distribution of each drug and its metabolites across the skeleton.

Herein, we describe our initial efforts to validate a method for the analysis of promethazine, chlorpromazine and their respective N-desmethyl and sulfoxide metabolites in skeletal remains using UPLC-PDA. Throughout the validation efforts, evidence of analyte degradation occurring during the sample preparation process was observed. Here, we describe our characterization of the extent of the degradation, data collected to identify the degradation products by UPLC-PDA and UPLC-qTOF-MS, and the influence of modifications to the extraction process on the extent of oxidation. Lastly, we developed and validated a simple sample preparation method for the analysis of promethazine, chlorpromazine and corresponding metabolites that does not induce any measurable oxidation of the analytes.

2.2 Materials and Methods

2.2.1 Chemicals

Promethazine (PMZ), chlorpromazine (CPZ), desmethylpromethazine (DPMZ), desmethylchlorpromazine (DCPZ), promethazine sulfoxide (PMZSO), chlorpromazine sulfoxide (CPZSO), promethazine N-oxide (PMZNO) and internal standard Promazine (PZ) were purchased from Toronto Research Chemicals (Toronto, ON). The internal standards promethazine-d3 and chlorpromazine d-3 were purchased from Cerilliant (Round Rock, TX, USA). Methanol (MeOH) and acetonitrile (ACN) used in sample preparation and extraction were both HPLC grade, and obtained from EMD chemicals (Gibbstown, NJ). and J.T Baker (Center Valley, PA), respectively. For UHPLC-PDA mobile phase, high-performance liquid chromatography (HPLC)-grade water was obtained through a Milli-Q water purification system. ACN and formic acid used for UPLC-PDA mobile phase was LC/MS grade and obtained from Fisher Scientific. For UHPLC-QTOF-MS mobile phase and washes, Acetonitrile (ACN), methanol (MeOH), and water were all LC/MS grade and purchased from OmniSolv (EMD, Millipore). Leucine enkephalin is used as a reference material and is obtained from Waters Corporation (Milford, MA).

2.2.2 Sample Preparation

2.2.2.1 Solid Phase-Extraction (SPE)

The analytical matrix used in method validation was bone tissue extract (BTE), prepared by subjecting samples (1 g) of drug-free decomposed bone to microwave-assisted extraction in 5 ml of methanol and then reconstituting in 1ml of phosphate buffer (PBS 0.1M, pH 6). Each sample

was prepared in 1 mL of BTE, using 500 ng of promazine as internal standard. Lipids and proteins were precipitated by addition of 3 mL of ACN:MeOH (1:1) followed by storage at -20 ^{o}C overnight. Samples were centrifuged for 10 min (4000 rpm) and the supernatant was collected and evaporated to 1 mL using a Centrivap vacuum concentrator (Labconco, Kansas City, MO, USA). Following evaporation, samples were diluted with 2 mL of PBS and then acidified with 100 μL of glacial acetic acid prior to solid phase extraction (SPE).

Samples underwent SPE using Clean Screen XCEL I (130 mg) 48-well plates (United Chemical Technologies, Bristol, PA). Wells were conditioned using sequential additions of 3 mL MeOH, 3 mL water, and 3 mL of PBS. After loading samples by gravity, wells were washed with 3 mL of PBS, followed by 3 mL of 0.1 M acetic acid. Wells were then dried under vacuum (~350 mmHg) for 5 min. After drying, wells were washed with 3 mL of MeOH and then sorbents were dried for a second time under vacuum for 10 min (~350 mmHg). Two different elution solvent systems were used: a solvent mixture based on dichloromethane (DCM), consisting of DCM:isopropanol:ammonium hydroxide (80:17:3) or one based on ethyl acetate (EA) consisting of EA:iPrOH:NH_4OH (80:17:3). Extracts were then evaporated to dryness at 40 ^{o}C by vacuum centrifugation and reconstituted in 500 μL of mobile phase A (0.1 % formic acid in 90:10 water:ACN). Samples were centrifuged for 10 min at 13,000×*g* and then transferred to autosampler vials, and 15μL of sample was injected into the UPLC.

2.2.2.2Filtration/Pass-Through Extraction (FPTE)

Samples were prepared in 500 μL of BTE and underwent protein precipitation by addition of 1 mL of (ACN:MeOH, 1:1). Following incubation at -20 ^{o}C overnight, the samples were centrifuged at 4000 RPM and then poured directly into wells of the FASt 96-well plate (United

Chemical Technologies, Bristol, PA). After filtration, the samples were collected, evaporated to dryness by vacuum centrifugation, reconstituted in 500 μL of mobile phase and centrifuged for 10 min at 13,000 RPM. Samples (2 μL) were injected for instrumental analysis following the same parameters mentioned above.

2.2.2.3 FPTE without Evaporation

The stock solutions were prepared in 1ml of BTE. A 200 μL volume was removed from the stock solutions and transferred to clean test tubes where 800 μL of ACN:H_2O (1:1) was added for a final volume of 1mL. The internal standards were added, the samples were vortexed and then poured directly into the wells of a FASt 96-well plate. Once the samples were filtered, they were collected and centrifuged for 10 min at 13,000 ×*g*. After centrifugation, the samples were transferred from microcentrifuge tubes to autosampler vials, and 2 μL of sample was injected into the UPLC.

2.2.3 Characterization of Analytical Performance – UPLC-PDA of SPE Extracts

The criteria followed in initial efforts to validate the method were based on the guidelines set forth by the Scientific Working Group For Forensic Toxicology (SWGTOX). Standard analyte samples were prepared in 1 mL of BTE at concentrations ranging from 0 to 10,000 ng/mL in triplicate. Standard curves were prepared on five different days from extracted standards to assess precision, linearity, accuracy, and limits of detection and quantification (LOD and LOQ).

2.2.4 Method Validation – FTPE without Evaporation and UPLC-qTOF-MS

The criteria followed to validate the final method were consistent with the guidelines set forth by the Scientific Working Group For Forensic Toxicology (SWGTOX). Standard analyte

samples were prepared in 1 mL of BTE at concentrations ranging from 0 to 2,000 ng/mL in triplicate. Standard curves were prepared from extracted standards to assess precision, concentration dependence, accuracy, matrix effect, recovery and limits of detection and quantification (LOD and LOQ). The concentration dependence of six non-zero concentrations over the concentration range 10-2000 ng/mL was assessed by plotting the best-fit ($R^2 \geq 0.99$) of peak area ratios versus concentration. The intraday and interday precision was measured as the coefficient of variation (CV), of triplicate analyses over the assayed concentration range on five different days. Accuracy was determined through blinded analysis of triplicate samples at two different concentrations of each analyte, the measured concentration must be within 20% of the theoretical concentration. The limit of detection for a given analyte was defined as the lowest concentration assayed with S/N ≥ 3, but not subject to precision criteria, while the limit of quantification (LOQ) for a given analyte was defined as the lowest concentration assayed where precision (CV) was $\leq 20\%$. The matrix effect (ion enhancement or suppression experienced by analytes in an extract) was determined by the post-extraction spike method and was evaluated at three concentrations levels: low (25 ng/ml), mid-range (100 ng/ml), and high (1000 ng/ml). The response of the analyte in standard solution was compared to the response of the analyte spiked into a blank matrix sample that underwent the sample preparation process. It was calculated as the response of the post-extracted spiked sample divided by the response of the non-extracted neat sample, minus one * 100%. Analyte recovery was defined as the ratio of analyte peak area in an extracted sample to the peak area of that analyte, spiked at the same nominal concentration, into a drug-free, matrix-matched extract.

2.2.5 Ultra-Performance Liquid Chromatography (UPLC) Conditions

An Acquity^{TM} UPLC equipped with a photodiode array detector (UPLC-PDA; Waters Corp., Milford, MA) or with a quadrupole time-of-flight mass spectrometer ((UPLC-QTOF-MS; Waters Corp., Milford, MA) was used for the analysis of extracts. The column used was a Raptor biphenyl column (150 mm × 2.1 mm, 2.7 μm particle diameter; Restek, Bellefonte, PA) with column temperature set to 50 ^{o}C. A binary gradient elution (A: 0.1 % formic acid in 90:10 water:ACN and B: 0.1% formic acid in 90:10 ACN:water) was used. The gradient was as follows: 95:5 A:B held for 1 min, linear increase to 70:30 A:B over 4 min and held for 1 min; linear increase to 20:80 A:B over 3 min; reversion back to 95:5 A:B, for 1 min. The total run time was 10 min with a constant flow rate of 0.300 mL/min and the injection volume was 15 μL. The wavelength range was set from 210 to 400 nm, and for quantitative comparisons, the sulfoxide metabolites were monitored at 240 nm, while the remaining analytes were monitored at 250 nm. Data acquisition was performed using Waters MassLynx software version 4.1.

2.2.6 UPLC-TOF MS Settings and Conditions

Mass spectrometry was performed on a Waters Acquity UPLC equipped with a Waters Xevo G2-XS-qTOF (Waters Corp., Milford, MA). Data was acquired in sensitivity mode under positive electrospray ionization with resolution > 20,000 at full width half maximum. The acquisition range was from *m/z* 50 to 601, using a scan time of 0.1 sec. Capillary voltage and cone voltage were set to 0.5 kV and 25 V, respectively. The source temperature was 150 ^{o}C, the desolvation gas flow was set to 1000 L/hr at a temperature of 500 ^{o}C and the cone gas was set to 50 L/hr. Data acquisition was achieved using MS^{e} mode, with low collision energy set to 6.0 eV, and the high-energy ramp ranged from 10-40 eV. Nitrogen was used as both the drying and nebulizing gas and the collision gas was argon. Verification of calibration of the mass axis from *m/z* 50 to 601 was conducted daily with 5 mM sodium formate. Leucine enkephalin was used as

the lockmass reference compound in positive mode at *m/z* 278.2641 and infused at a flow rate of 10 μL/min. MassLynx® Software (version 4.1) was used for data acquisition and processing.

2.2.7 Autosampler Stability

The stability of analytes while on the instrument autosampler (maintained at 25 ^{o}C) was evaluated on the UHPLC-PDA by repeated injection of the extracted samples at two different concentration levels (100 ng/mL and 2000 ng/mL, n=3) for 0, 6, 12, 18, 24, 30, 36 hr. Analytes were considered stable if there was no deviation in analyte response in excess of 20% from the response of the corresponding sample at t=0 hr. Autosampler stability was also assessed on the UHPLC-Q-TOF-MS by repeated injection of extracted analytes analyzed individually at two different concentration levels (100 ng/mL and 1000 ng/mL per analyte, n=3) after 0, 12, 24 and 36 hr incubation. Analytes were considered to be stable if there was no deviation in analyte response more than 20% from the response of the corresponding analyte at t=0 hr.

2.3 Results

2.3.1 Characterization of Analytical Performance – UPLC-PDA

Analytical performance data for UPLC-PDA analyses are summarized in Table 2. The response ratio was linear (R^2>0.99) from 25 ng/mL to 10,000 ng/mL for all analytes. Analytical precision (CV) ranged from 0.9%-30% for PMZ and its metabolites (PMZSO, DPMZ), and from 0.8%-29% for CPZ and its metabolites (CPZSO, DCPZ). While precision criteria were not met in all cases, the data in Table 2 indicate that CV values in excess of 20% were observed in 5 or

fewer cases of a total of 90 different sets of triplicate extractions for PMZ and its metabolites, and in 6 or fewer cases for CPZ and its metabolites.

Accuracy (bias) was assessed through blind analysis of standard samples prepared in BTE at concentrations ranging from 150 to 2000 ng/mL. Bias was acceptable when the measured concentration deviated from the target concentration by no more than 20%. For PMZ and metabolites, the deviation range was -101% to 48% and for CPZ and metabolites was -36% to 23%. While accuracy criteria were not strictly met, the data in Table 2 indicate that absolute bias values in excess of 20% were observed in 8/90 cases or less for PMZ and its metabolites, and in no more than 6/90 cases for CPZ and its metabolites.

The stability of the analytes while resident on the autosampler tray was assessed at two different concentrations over a 36 hour time period. For all analytes, there was no change in response ratio in excess of 20% of the initial response (t=0 hr), indicating that they remained stable while on the instrument waiting to be run.

Table 2: Summary of validation parameter results (LOD, LOQ, precision, linearity, bias). Solid-phase extraction with ethyl acetate elution solution was used as the sample preparation method and a UPLC-PDA as the analytical instrument. Data were collected over 9 different sets of extractions of analyte standard mixtures ranging from 25-10,000 ng/mL, where each standard concentration was analyzed in triplicate.

Analyte	Retention Time (min, ±0.05)	Limit of Detection (LOD, ng/mL)	Limit of Quantitation (LOQ, ng/mL)	Precision (CV, %) (Acceptance Criteria: ≤ 20%) [# failed]	Linearity (Acceptance Criteria: $R^2 \geq 0.99$)	Bias (%) (Acceptance Criteria: ≤ 20%) [# failed]
PMZ	7.13	10	25	0.9-22.6 [5/90]	0.9990	-0.3-(-101.4) [8/90]

PMZSO	4.32	10	25	0.4-30.0 [5/90]	0.9986	-0.9-(-44.0) [6/90]
DPMZ	6.76	10	25	0.9-18.8 [2/90]	0.9987	0.8-(-18.1) [5/90]
CPZ	8.09	10	25	1.8-22.2 [2/90]	0.9994	0.1-(-23.6) [1/90]
CPZSO	5.32	10	25	0.8-29.2 [6/90]	0.9932	0.5-(-36.5) [6/90]
DCPZ	7.95	10	25	1.7-21.7% [3/90]	0.9988	-0.3-(30.5) [2/90]

2.3.2 Appearance and Putative Identification of Extraneous Peaks in Chromatograms of Extracted Standards

A closer examination of the chromatograms from extracted standards revealed extraneous peaks that were not present in the negative control or unextracted standard mixture. Four minor chromatographic peaks (labeled 1-4) and drug standard peaks were detected in the chromatograms as shown in Figure 5. We first considered whether the extra peaks were the result of impurities in the solvents used in extraction or mobile phases. In analysis of multiple unextracted standard mixtures, analyte-free samples of mobile phase A and samples of extraction reagents that had been evaporated and reconstituted in mobile phase A, no extraneous peaks were observed. We then hypothesized that analytes were undergoing chemical degradation under the sample preparation conditions used. To determine which analyte was generating each extraneous peak, analyte standards were extracted from BTE individually (n_i=3). Chromatograms of extracted standards of PMZ and CPZ produced multiple extra peaks that were not present in the drug-free negative control, as shown in Figures 2 and 3. The peaks labeled 1, 2 and 3 from Figure 6 had retention times of 4.62 min, 4.70 min, and 7.59 min, respectively. The retention time of peak 2 from Figure 6 and peak 1 from Figure 3 (4.70 min and 5.32 min, respectively), from each extracted sample corresponded to the same retention time as PMZSO and CPZSO, respectively, and the UV spectrum of each sulfoxide standard was indistinguishable from the UV spectrum of the corresponding extraneous peak (Figs. 6-7). These results support

the putative identification of the compounds corresponding to peak 1 (Fig. 7) and peak 2 (Fig. 6) as chlorpromazine sulfoxide and promethazine sulfoxide, respectively.

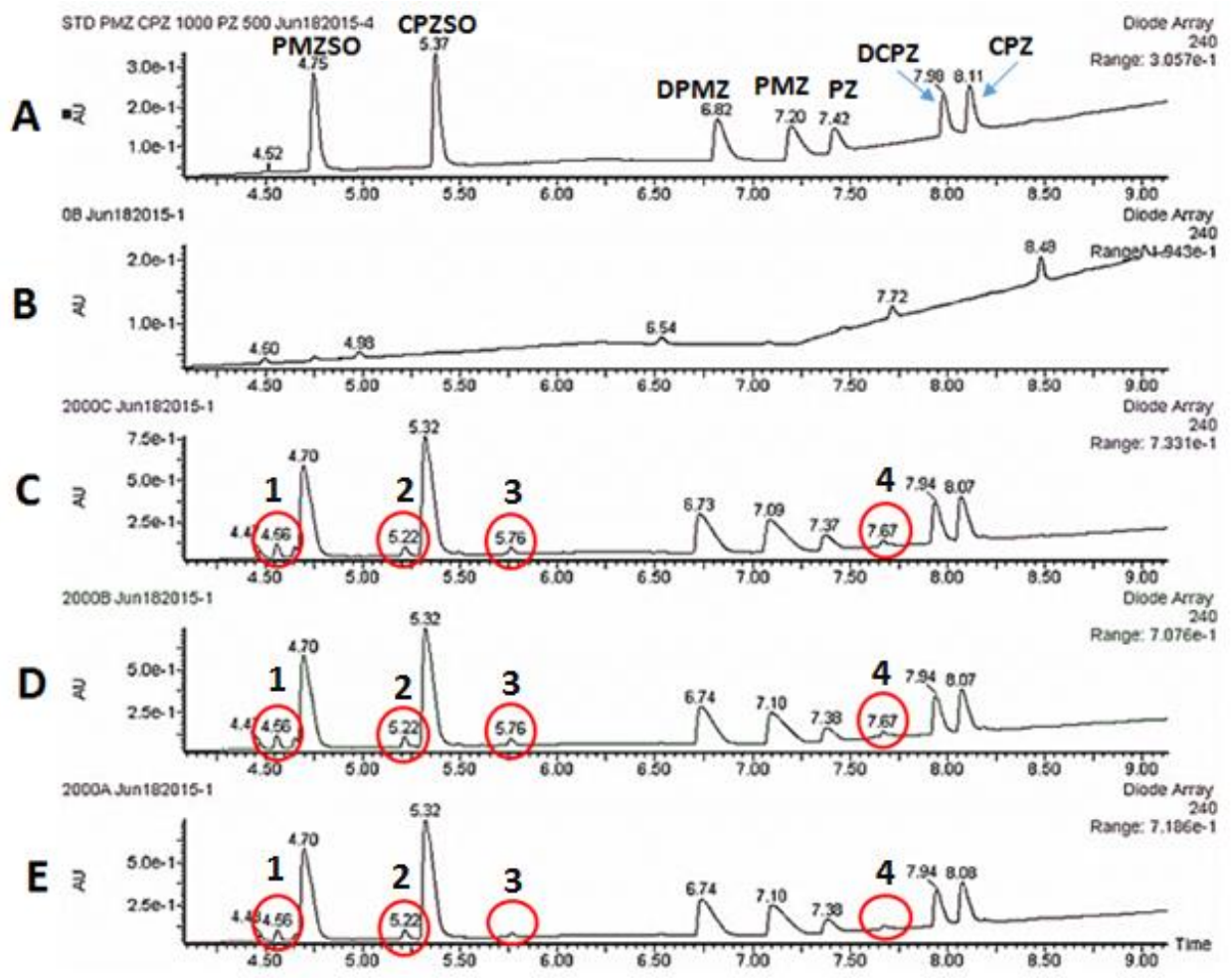


Figure 5: (C-E) UHPLC-PDA chromatograms (240nm, 4.50-9.00 min window) of extracted standards (n=3) at a concentration of 2000 ng/ml. Extraneous peaks are labeled 1-4. (A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control.

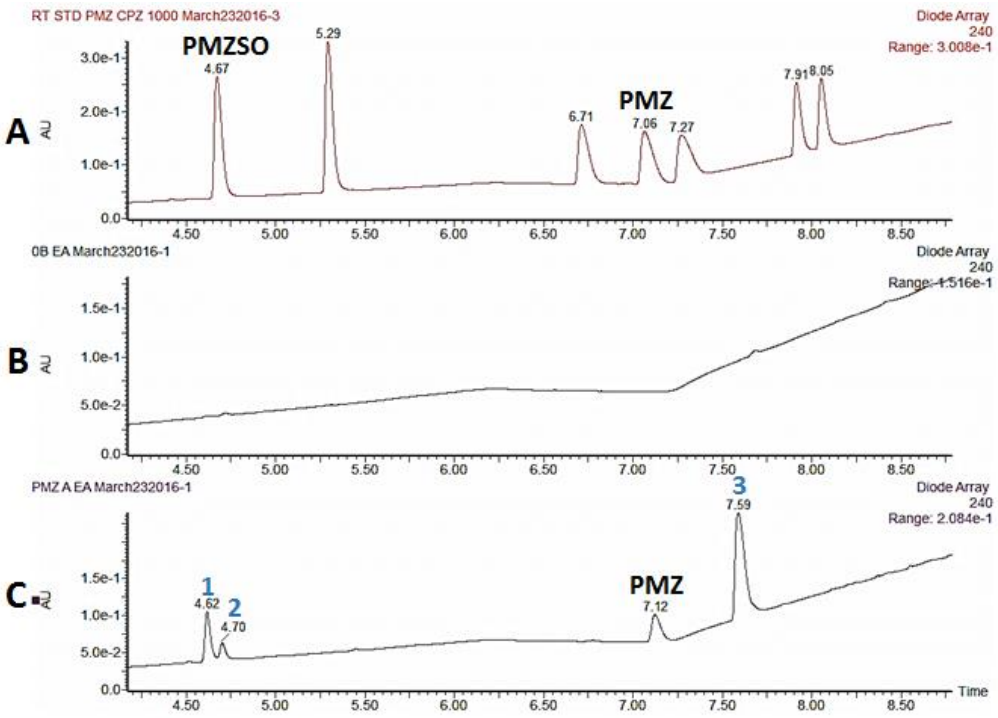
2.3.3 Confirmation of Extraneous Products by UPLC-TOF-MS: Phenothiazene Oxidation

In order to confirm that PMZ and CPZ were being oxidized to their corresponding sulfoxides, high resolution mass spectral data was acquired. The same set of individually extracted standards was analyzed by UPLC-qTOF-MS using the same column and chromatographic method to obtain accurate mass measurements for the compounds corresponding to the extraneous peaks. These data served as additional parameters for characterization and identification. The extraneous peaks that were observed in the chromatograms from the UPLC-PDA (Figs. 6 and 7) were also observed in the total ion chromatograms (TICs) from the UPLC-q-TOF-MS (Fig. 8). The TIC for the extracted PMZ sample indicated the presence of compounds eluting at 4.71 min, 4.81 min, and 7.7 min with measured masses of 317.1356, 301.1355, and 301.1426 Da, respectively (Fig. 8C, 8MS-C). The TIC for the extracted CPZ sample indicated the presence of compounds eluting at 5.45 min, 5.82 min, 6.18 min and 8.23 min with measured masses of 335.1017, 351.1020, 376.1273, and 335.1054 Da respectively (Fig. 8D, 8MS-D). The accurate mass data is summarized in Table 3. For both extracted PMZ and CPZ samples, two of the extraneous peaks had the same accurate mass but differed in retention time. If we consider the accurate mass (M) measured for the parent drug molecules (285.1419 Da for PMZ and 319.1090 Da for CPZ), a pattern of M+16 or M+32 was observed for the mass of the extraneous

compounds, suggesting the occurrence of oxidation (Fig. 7, Table 3). As expected, comparison of the results with reference material indicated that PMZ was oxidized to its corresponding sulfoxide (Peak 2) and N-oxide (Peak 3) while CPZ was oxidized to its corresponding sulfoxide (Peak 1). The PMZNO standard was acquired after the samples had been analyzed to confirm the identity of peak 3 in Figure 8C and as a result, is not included in the chromatograms for unextracted standard mixtures shown in any of the figures. Where reference standards were available, the parameters used for compound identification were retention time, accurate mass and fragmentation pattern. Possible candidates for the remaining oxidation products based on accurate mass data include the sulfone, sulfoxide-N-oxide or hydroxylated form of the parent drug.

Table 3: Summary of accurate mass data for labeled peaks in Figs. 4 and 5.

Extracted Promethazine Standard (Fig. 4)		*Extracted Chlorpromazine Standard (Fig. 5)*	
Compound	**Accurate Mass (±0.005)**	**Compound**	**Accurate Mass (±0.005)**
Peak 1	317.136	Peak 1	335.102
Peak 2	301.136	Peak 2	351.102
Peak 3	301.143	Peak 3	376.127
Promethazine	285.142	Peak 4	335.105
		Chlorpromazine	319.109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Diode Array
240
Range: 3.008e-1
PMZSO
4.67
5.29
PMZ
6.71
7.06
7.27
7.91
8.05
A
AU
3.0e-1
2.0e-1
1.0e-1
0.0
4.50
5.00
5.50
6.00
6.50
7.00
7.50
8.00
8.50
0B EA March232016-1
Diode Array
240
Range: 1.516e-1
B
AU
1.5e-1
1.0e-1
5.0e-2
0.0
PMZ A EA March232016-1
Diode Array
240
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1
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2
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3
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PMZ
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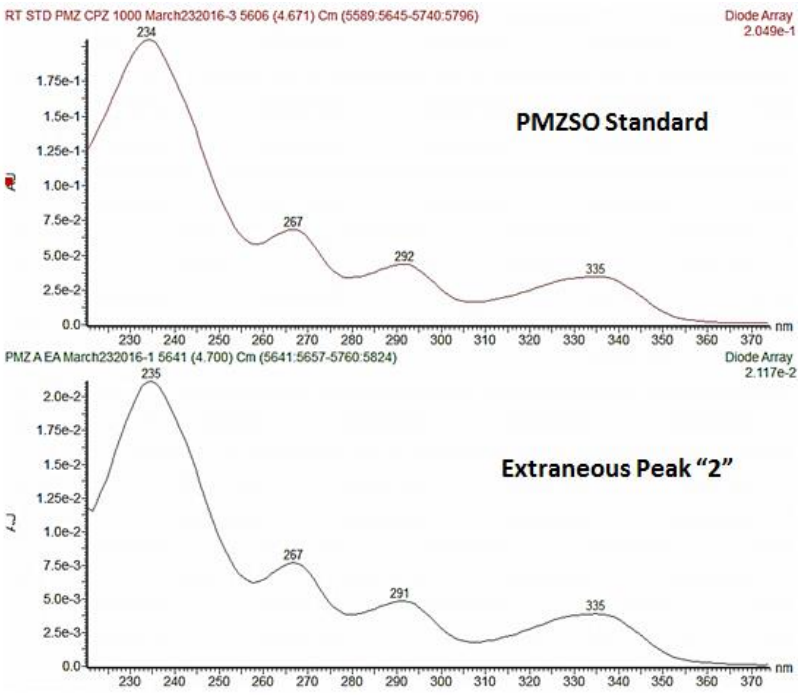

Figure 6:(A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. (C) UHPLC-PDA chromatogram (240 nm, 4.5-9.0 min window) of promethazine standard prepared at a concentration of 1000 ng/ml in BTE, and extracted by SPE. Extraneous peaks are labeled 1-3. UV absorbance spectrum associated with peak 2, retention time of 4.70 min is compared to the spectrum of a PMZSO standard with retention time of 4.67 min.

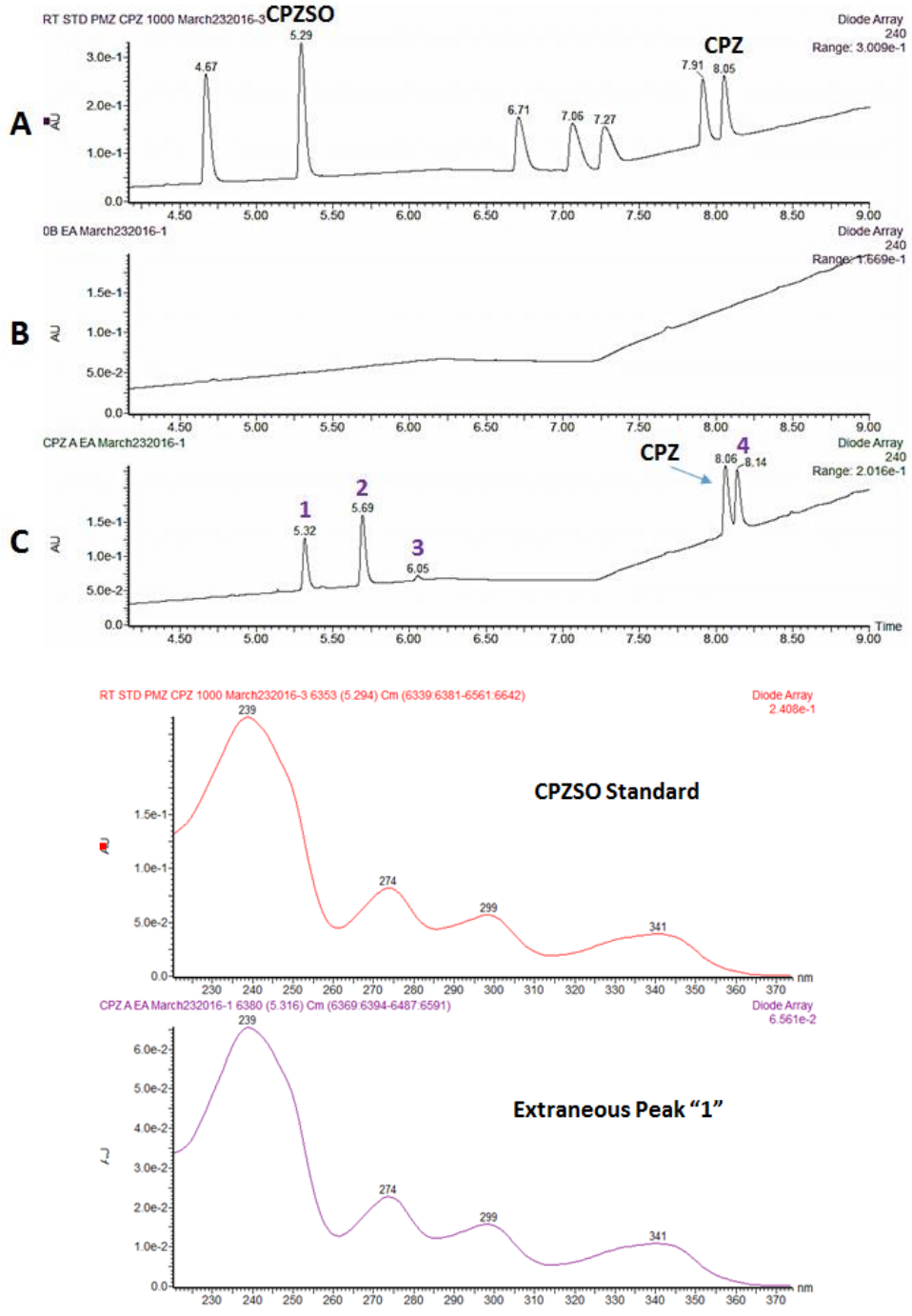

Figure 7: (A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. C) UHPLC-PDA chromatogram (240 nm, 4.5-9.0 min window) of chlorpromazine standard prepared at a concentration of 1000 ng/ml in BTE, and extracted by SPE. Extraneous peaks are labeled 1-3. UV absorbance spectrum associated with peak 1, retention time of 5.32 min, is compared to the spectrum of CPZSO standard, retention time of 5.29.

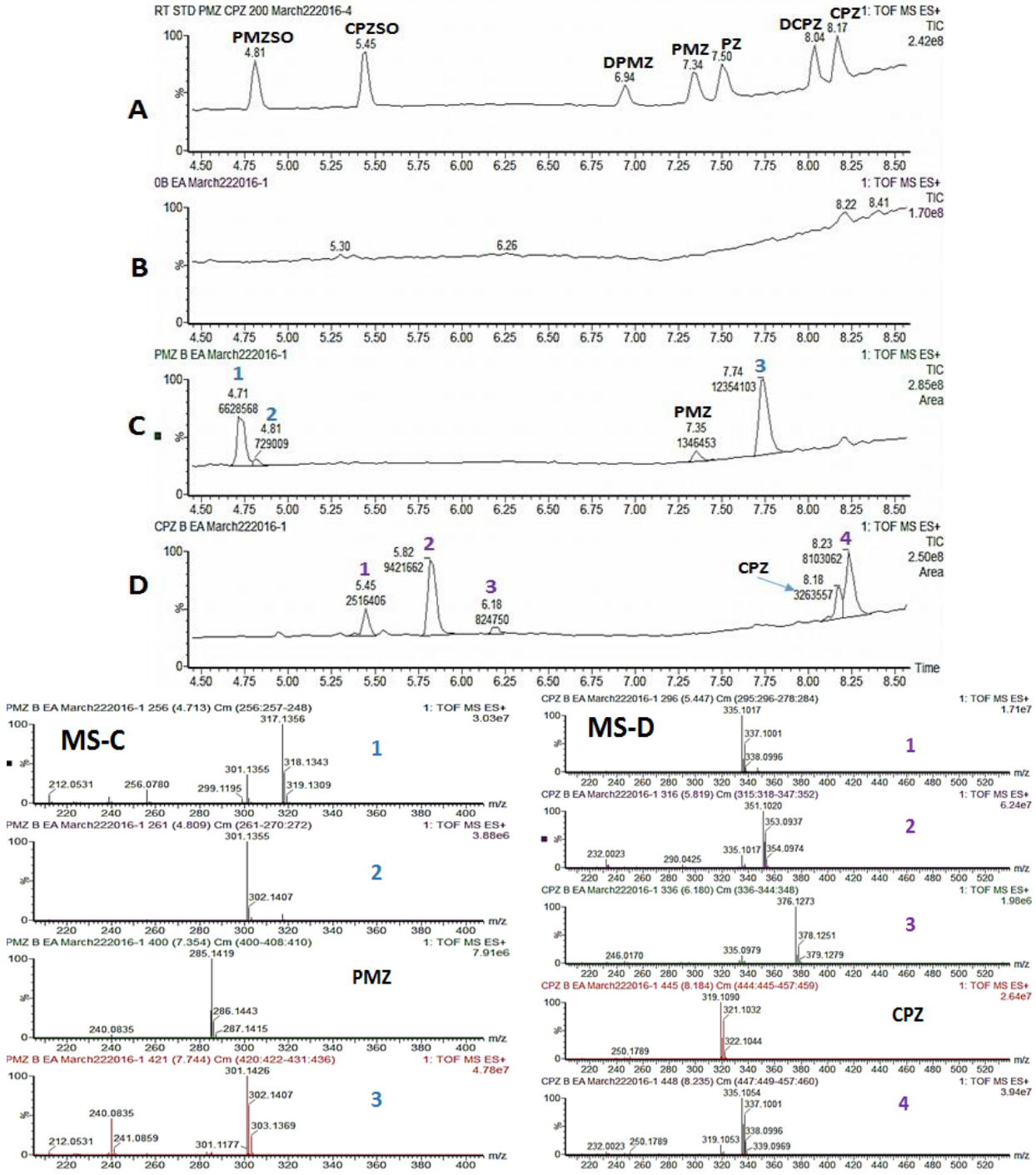

Figure 8: (A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. (C,D) UHPLC-q-TOF-MS total ion chromatograms of extracted promethazine and chlorpromazine standards at a concentration of 1000 ng/ml in BTE. Extraneous peaks are labeled 1-4. Below the TICs are the corresponding accurate mass spectra (MS) for each labeled peak in chromatograms C and D (MS-C, MS-D).

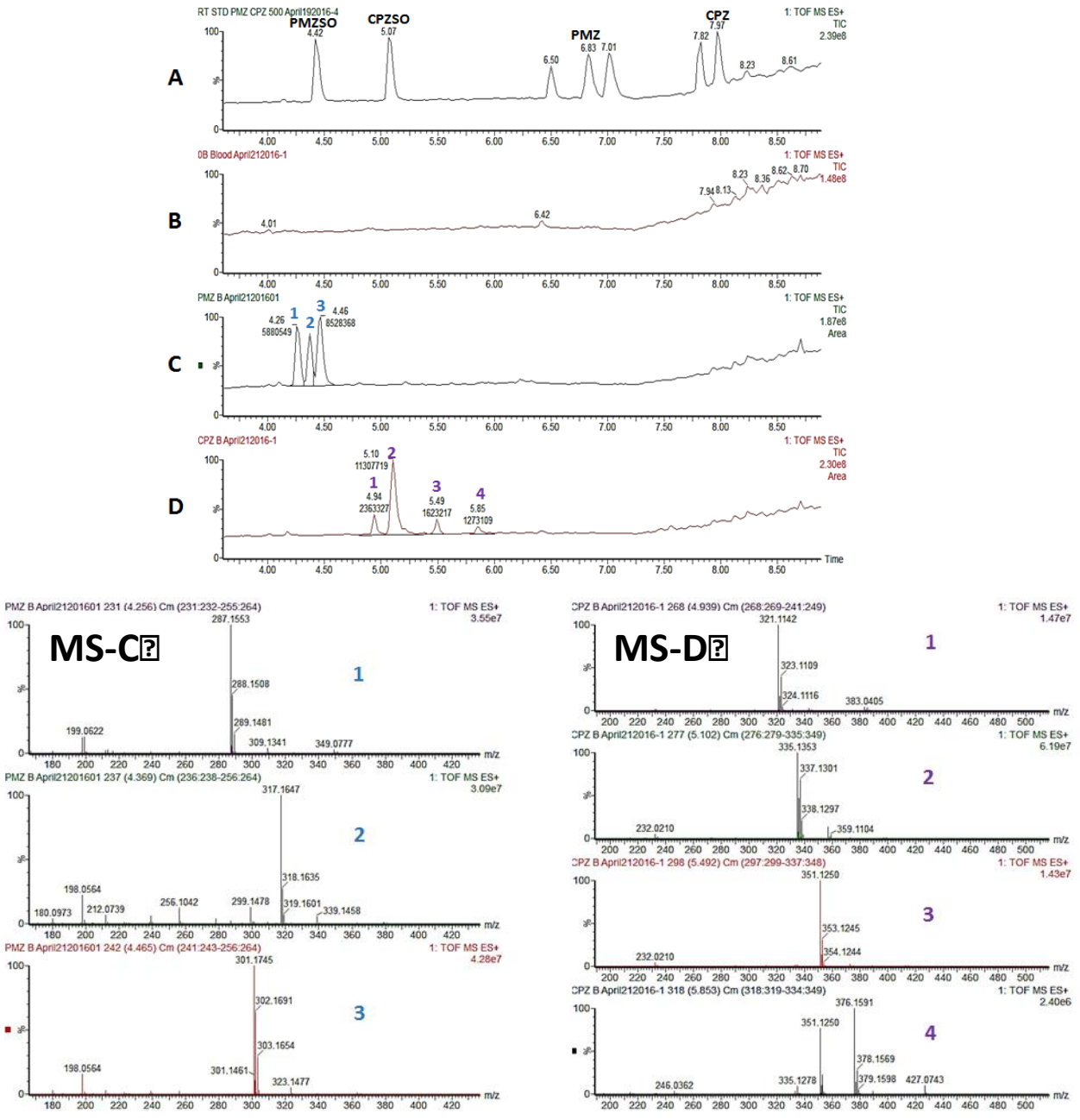

Figure 9:(A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. (C,D) UHPLC-q-TOF-MS total ion chromatograms of extracted promethazine and chlorpromazine standards at a concentration of 1000 ng/ml in blood. Extraneous peaks are labeled 1-4. Below the TICs are the corresponding high-resolution mass spectra (MS) for each labeled peak in chromatograms C and D (MS-C, MS-D, respectively).

As with bone tissue extract, extraneous peaks were observed in the TICs for extracted PMZ and CPZ standard samples when blood was utilized as the sample matrix (Fig. 9) that were not present in the analyte-free control. However, there was the presence of a new peak with retention time of 4.26 min and predominant ion with m/z 287.1553 in chromatogram C, and a new peak with retention time 4.94 min and predominant ion with m/z of 321.1142 in chromatogram D. Additionally, the peaks corresponding to PMZ and PMZ-N-oxide were not detected.

2.3.3 Semi-Quantitative Comparison of Oxidation Products - Bone Tissue Extract vs Blood

Table 4 summarizes the relative extent oxidation of the parent compounds (PMZ and CPZ), indicated by increases in the peak area ratio ($A_{sulfoxide}/A_{parent\ analyte}$). A baseline level of oxidation products were present in the analytical drug standards, however the extent of oxidation, indicated by increases in the peak area ratio (extra peak/parent analyte) increased drastically once the drug standards have been subjected to these sample preparation and extraction conditions. Data in Table 4 showed strong differences in the relative extent of oxidation between extracts from different sample matrices, with the greatest extent of oxidation observed when blood was used as the sample matrix.

Table 4: A semi-quantitative comparison of the relative extent of formation of PMZSO and CPZSO from the corresponding parent compound, based on the sample matrix: The mobile phase matrix an unextracted drug standard prepared in mobile phase A and analyzed. The peak area ratio is the ratio of the area of PMZSO or CPZSO relative to that of the parent analyte originally added (i.e., PMZ or CPZ). The peak area ratio is represented as a mean percentage (n=3).

	Promethazine	**Chlorpromazine**
Matrix	**Mean Peak Area Ratio** $(A_{PMZSO}/A_{PMZ} \times 100\%)$	**Mean Peak Area Ratio** $(A_{CPZSO}/A_{CPZ} \times 100\%)$
Mobile Phase A	3.9	3.9
Bone Tissue Extract	70.8	71.3
Blood	10,013	9,947

2.3.4 Relative Extent of Phenothiazine Oxidation: Influence of Extraction Conditions

As described above, PMZ and CPZ oxidize during sample preparation producing the corresponding sulfoxides as well as other oxidation products. When the analyte standards were extracted from BTE individually using the sample preparation method of solid-phase extraction using an elution solution based on ethyl acetate, extraneous peaks were present in the total ion chromatogram (Figure 10). The TIC for the extracted PMZ sample indicated the presence of compounds eluting at 4.71min (*m/z* 317.1356, Peak 1), 4.81 (*m/z* 301.1355, Peak 2), and 7.74 (*m/z* 301.1426, Peak 3) (Figure 10, C). Peak 2 has been identified as PMZSO and Peak 3 has been identified as PMZNO and is the most abundant peak and oxidation product formed. The TIC for the extracted CPZ sample indicated the presence of compounds eluting at 5.45min (335.1017, Peak 1), 5.82 (351.1020, Peak 2), and 6.18 (335.1054, Peak 3) and 8.23 (335.1054,

Peak 4). (Figure 10, D). CPZSO has been identified as Peak 1 and Peak 4 is the most abundant oxidation product formed and is presumed to be chlorpromazine N-oxide.

In an effort to minimize analyte oxidation, various sample preparation methods were evaluated. The next sample preparation method involved a simple switch to a different elution solvent while keeping all other variables constant. The elution solution used was 80:17:3 ,dichloromethane (DCM):iPrOH:NH_4OH, as it is a commonly used solvent system for the elution of basic drugs. However, once again, precision and bias exceeded the 20% threshold. (Table 5). The chromatograms of extracts using a DCM-based elution solution also bore extraneous peaks, though, as shown in Figure 10 and Figure 11, it is apparent that the distribution and quantity of the extraneous peaks differs from experiments using an elution solution based on ethyl acetate. The TIC for the extracted PMZ sample indicated the presence of compounds eluting at 4.83 min (*m/z* 317.1665, Peak 1), 4.90 (*m/z* 301.1355, Peak 2), and 7.82 (*m/z* 301.1497, Peak 3) (Figure 11, C). PMZSO (Peak 2) and PMZNO are present (Peak 3) in the sample, however the parent drug is the most abundant peak and the sulfoxide is the most abundant oxidation product. The TIC for the extracted CPZ sample indicated the presence of compounds eluting at 5.54min (*m/z* 335.1166, Peak 1), 7.61 (*m/z* 285.1557, Peak 2). (Figure 11, D). The peak corresponding to CPZ is the most abundant peak, and CPZSO is the most abundant oxidation product (Peak 1). When the elution solvent was based on DCM, PMZ was formed as a degradation product (Peak 2) but this was not the case when ethyl acetate was utilized.

The third sample preparation method involved FPTE instead of SPE. Substituting FPTE as the extraction method removed an elution step, which was presumed to be a factor contributing to the oxidation because of the strong organic nature of the elution solvent. The TIC for the extracted PMZ sample only contains one extra peak at a retention time of 4.89 (*m/z* 301.1264)

which corresponds to PMZSO (Figure 12, C). The TIC for extracted CPZ contains three extra peaks, Peak 1(5.49min) is CPZSO, Peak 2 (7.50min) corresponds to the formation of PZ, and Peak 3 was a new extraneous peak at 7.73 min with an accurate mass of *m/z* 315.1455 (Figure 12, D). Although this method also caused oxidation products to form, the peak areas of the oxidation products have been reduced.

The final sample preparation method that was developed was FPTE with no evaporation steps. As a result, it was a very simple preparation that included minimal steps that would influence or change the sample. Figure 13 demonstrates that this method did not cause any extra peaks to appear in the total ion chromatogram. Therefore, it is reasonable to conclude this method did not induce any measurable oxidation.

Table 5: Summary of validation parameter results (LOD, LOQ, precision, linearity, bias). Solid-phase extraction with dichloromethane elution solution was used as the sample preparation method and a UPLC-PDA as the analytical instrument. Data were collected over 5 different sets of extractions of analyte standard mixtures ranging from 25-10,000 ng/mL, where each standard concentration was analyzed in triplicate.

Analyte	**Limit of Detection (LOD, ng/mL)**	**Limit of Quantitation (LOQ, ng/mL)**	**Precision (CV, %) (Acceptance Criteria: ≤ 20%) [# failed]**	**Linearity (Acceptance Criteria: $R^2 \geq 0.99$)**	**Bias (%) (Acceptance Criteria: ≤ 20%) [# failed]**
PMZ	10	25	0.9-18.7 [0/50]	0.9974	0.4-(197.6) [5/20]
PMZSO	10	25	0.8-24.1 [3/50]	0.9917	0.3-(180.3) [7/20]
DPMZ	10	25	0.7-21.9 [2/50]	0.9918	0.4-(55.6) [5/20]
CPZ	10	25	0.9-38.3[1/50]	0.9960	-0.25-(61.8) [11/20]
CPZSO	10	25	0.9-22.9[2/50]	0.9923	-1.9-(-306.9) [6/20]
DCPZ	10	25	0.3-20.8 [1/50]	0.9938	-2.6-(-85.4) [12/20]

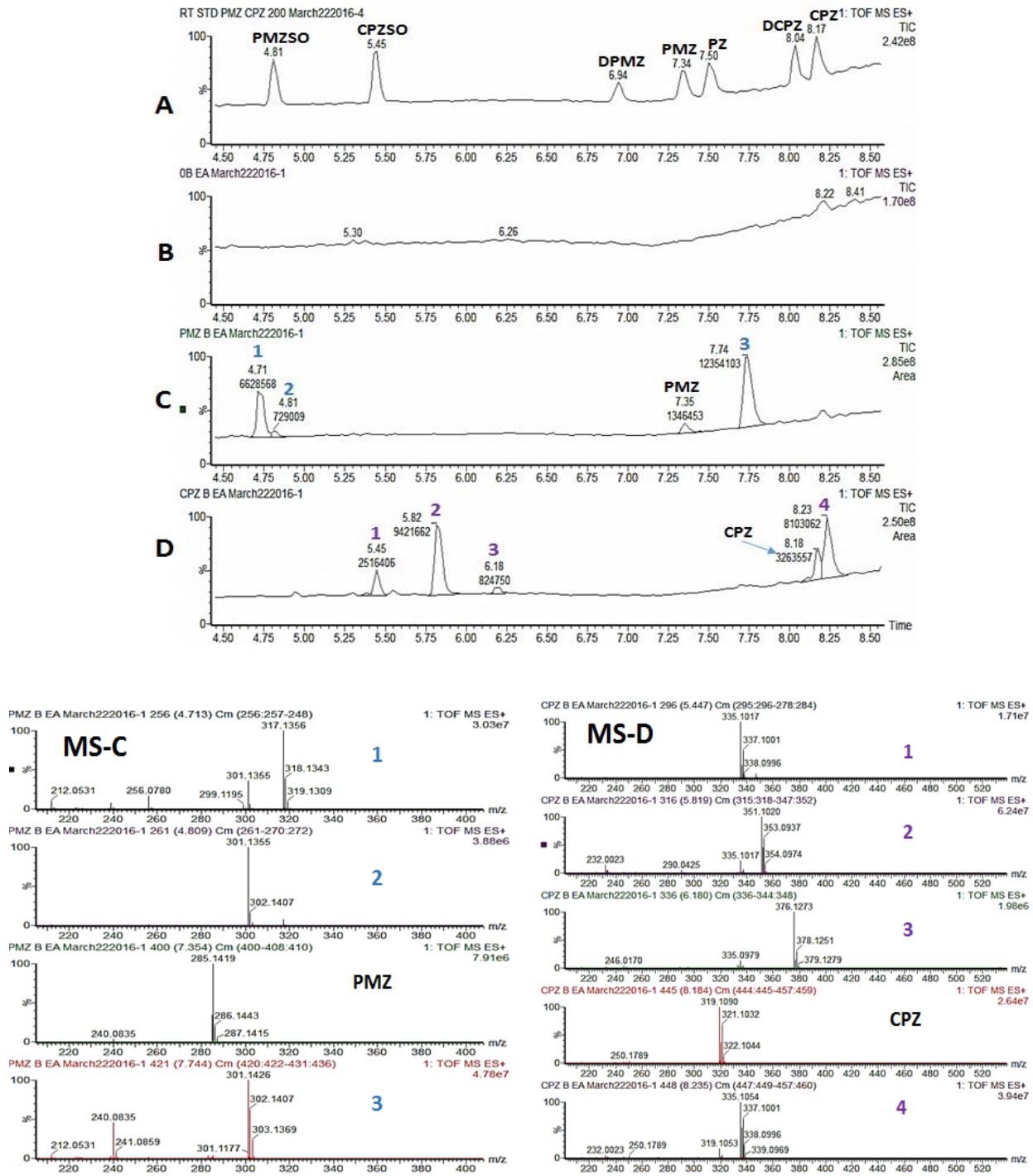

Figure 10: (A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. (C,D) UHPLC-q-TOF-MS total ion chromatograms of extracted promethazine and chlorpromazine standards at a concentration of 1000 ng/ml in BTE using solid-phase extraction with ethyl acetate as the elution solution. . Extraneous peaks are labeled 1-4. Below the TICs are the corresponding accurate mass spectra (MS) for each labeled peak in chromatograms C and D (MS-C, MS-D).

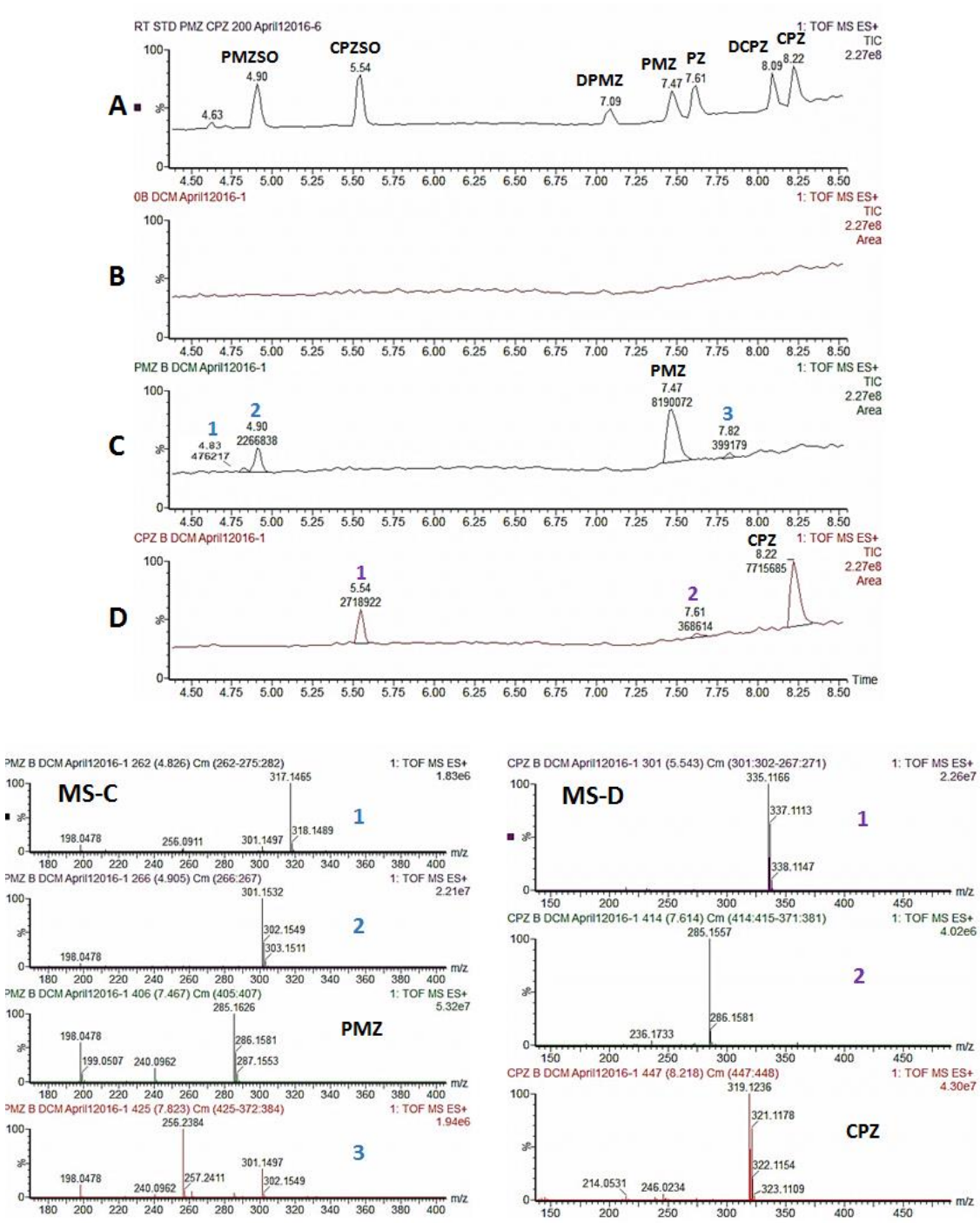

Figure 11: (A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. (C,D) UHPLC-q-TOF-MS total ion chromatograms of extracted promethazine and chlorpromazine standards at a concentration of 1000 ng/ml in BTE using solid-phase extraction with dichloromethane as the elution solution. Extraneous peaks are labeled 1-3. Below the TICs are the corresponding accurate mass spectra (MS) for each labeled peak in chromatograms C and D (MS-C, MS-D).

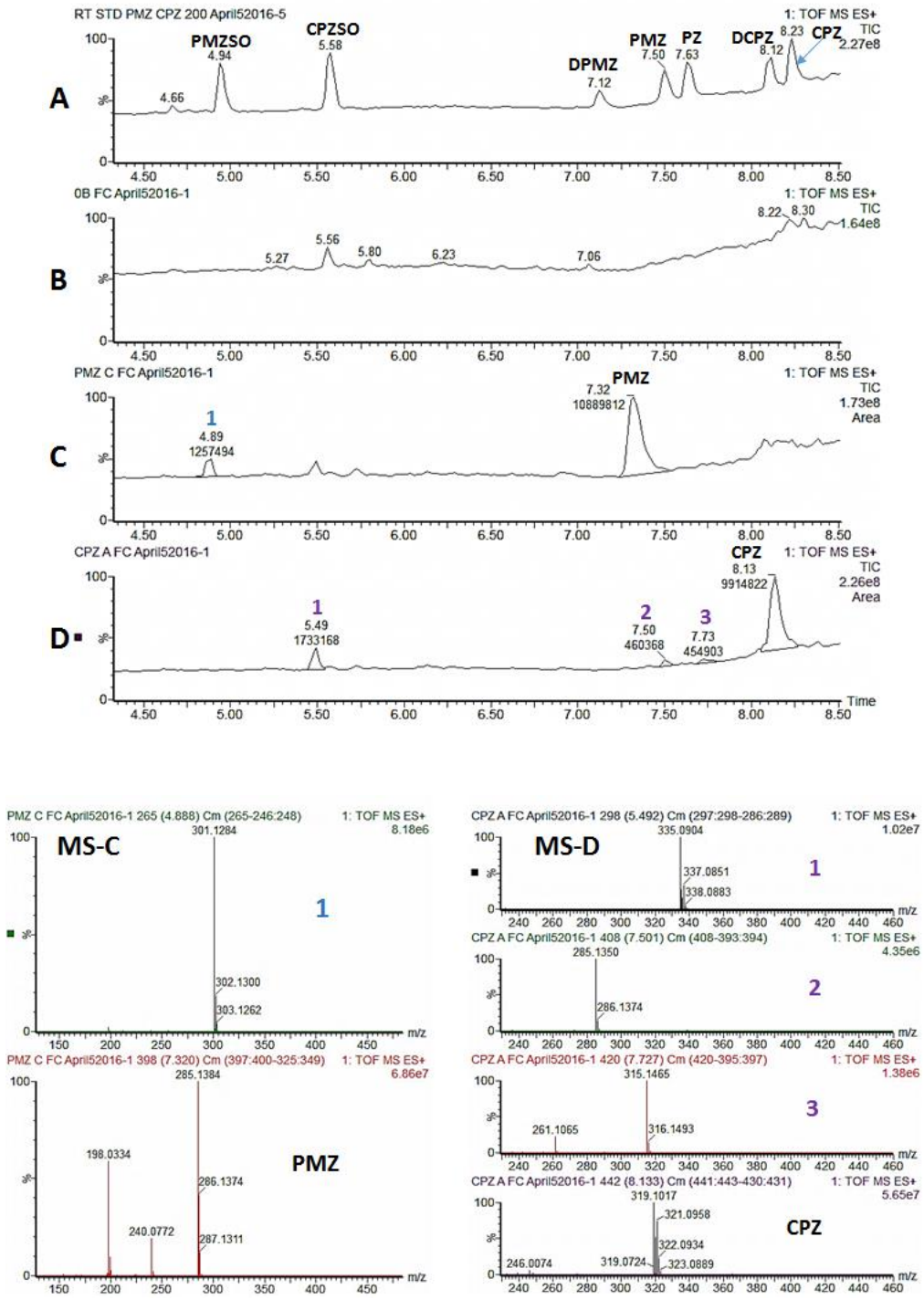

Figure 12: (A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. (C,D).UHPLC-q-TOF-MS total ion chromatograms of extracted promethazine and chlorpromazine standards at a concentration of 1000 ng/ml in BTE using filtration as the extraction method. Extraneous peaks are labeled 1-3. Below the TICs are the

corresponding accurate mass spectra (MS) for each labeled peak in chromatograms C and D (MS-C, MS-D).

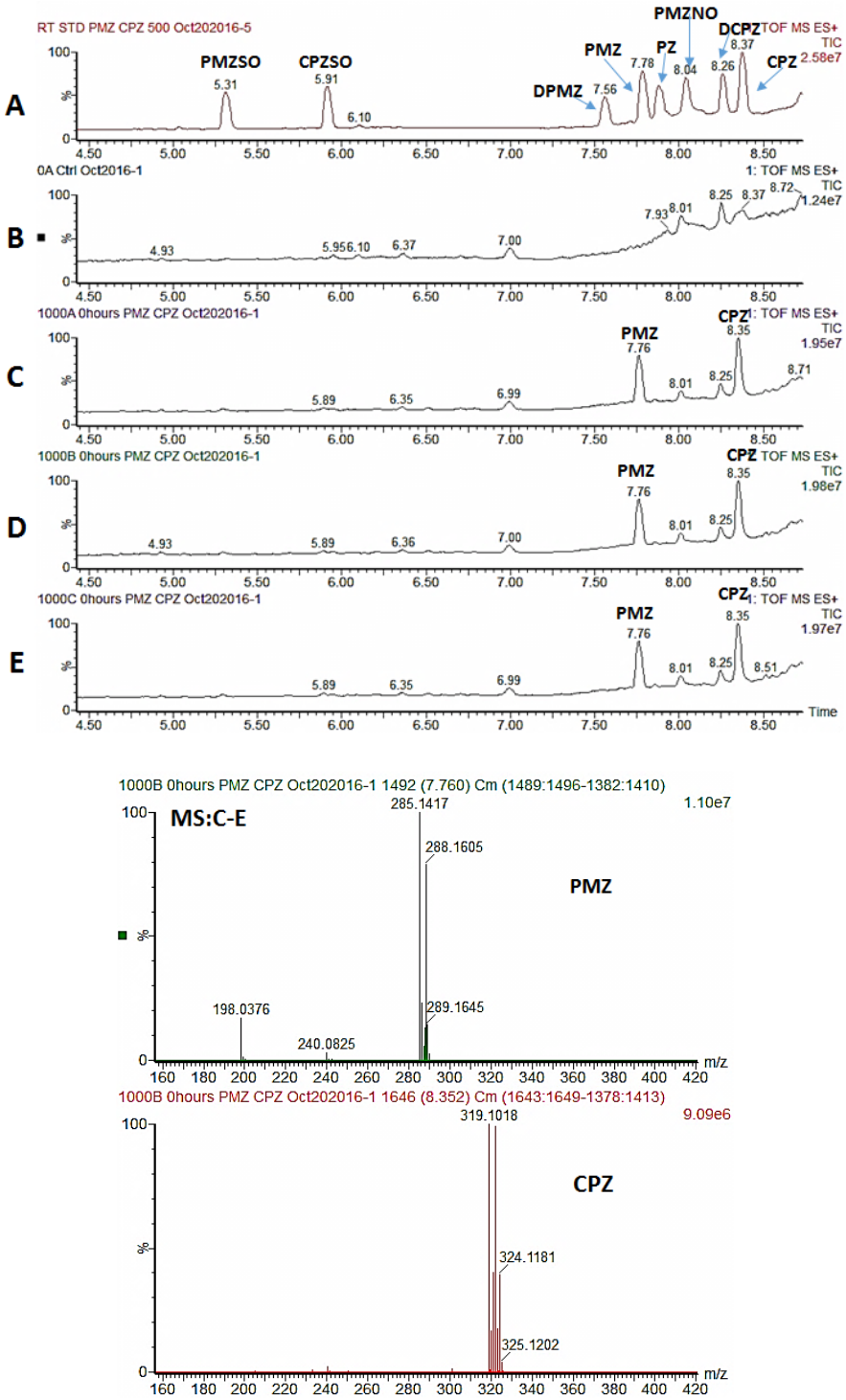

Figure 13: (A) Unextracted analyte standard mixture for retention time verification. (B) Drug-free negative control. (C,D,E) UHPLC-q-TOF-MS total ion chromatograms of extracted promethazine and chlorpromazine standards (n=3) at a concentration of 1000 ng/ml in BTE

using filtration without evaporation as the extraction method. Below the TICs are the corresponding accurate mass spectra (MS) for the peaks at 7.76 and 8.35, which correspond to promethazine and chlorpromazine, respectively.

2.3.5 Semi-Quantitative Comparison of Oxidation Products - Comparing Different Extraction Conditions

Table 6 compares the relative formation of the oxidized species produced, based on the sample preparation method. As the method changes, the relative formation of the PMZSO changes as indicated by a decrease in the peak area ratio as we remove steps in the sample preparation method. FPTE converts less of the analyte to its corresponding oxidized form than solid-phase extraction and, if the evaporation step is removed, oxidation products are not formed. Furthermore, it is important to note that the deuterated internal standards also convert to their corresponding oxidized form to the same extent as the analytes in the unextracted standards (~4%).

Table 6: A semi-quantitative comparison of the relative extent of formation of PMZSO and CPZSO from the corresponding parent compound, based on the sample preparation method. The unextracted drug standard is prepared in mobile phase A and analyzed. The peak area ratio is the ratio of the area of PMZSO or CPZSO relative to that of the parent analyte originally added (i.e., PMZ or CPZ). The peak area ratio is represented as a mean percentage (n=3).

	Promethazine	**Chlorpromazine**
Method	**Mean Peak Area Ratio (A_{PMZSO}/A_{PMZ} ×100%)**	**Mean Peak Area Ratio (A_{CPZSO}/A_{CPZ} ×100%)**
SPE EA Elution Solution	67.0	89.2
SPE DCM Elution Solution	38.9	48.9
Filtration with Evaporation	16.3	13.8
Filtration without Evaporation	3.6	3.0
Unextracted Standards	3.9	3.6

2.3.6 Method Validation Results

Using FTPE without evaporation, the validation process was undertaken, with concentration dependence, LOD, LOQ, precision, accuracy, recovery and matrix effects assessed according to SWGTOX guidelines (68). All analytes were fit with quadratic regression lines and concentration dependence was assessed over the range 10 to 2000 ng/mL. Correlation coefficients of r^2>0.99 were observed over 5 different days. The LOD and LOQ were determined to be 10 ng/mL for all analytes. Summarized in Table 7 are the precision and accuracy data which proved to be acceptable ranging from 0.04%-14%, and 0.09% - 20%, respectively. Recovery ranged from 90%-110% and the matrix effects calculations revealed that the average suppression or enhancement did not exceed 25% for all analytes. Analyte stability while resident on the autosampler tray in extracted samples revealed there was no loss in analyte response in excess of 20% of the initial response ratio at t=0 hr for all analytes except for the PMZ-D3 internal standard. At the 24 hr time interval, PMZ-D3 demonstrated loss in response exceeding the 20% threshold. However, this instability did not affect the precision or accuracy of the standard curves, this could be because the time required to analyze all curve samples on the instrument doesn't commonly exceed 15 hr.

Table 7: Summary of validation parameter results (LOD, LOQ, precision, linearity, bias). Filtration without evaporation was used as the sample preparation method and a UPLC-QTOF-MS as the analytical instrument. Data were collected over 4 different sets of extractions of analyte standard mixtures ranging from 10-2,000 ng/mL, where each standard concentration was analyzed in triplicate.

Analyte	Limit of Detection (LOD, ng/mL)	Limit of Quantitation (LOQ, ng/mL)	Precision (CV, %) (Acceptance Criteria: ≤ 20%) [# failed]	Linearity (Acceptance Criteria: $R^2 \geq 0.99$)	Bias (%) (Acceptance Criteria: ≤ 20%) [# failed]
PMZ	10	10	0.04-8.2[0/34]	0.998	1.4-(-17.4) [0/10]
PMZSO	10	10	0.37-13.9[0/34]	0.998	-1.4-(18.5) [0/10]
DPMZ	10	10	0.8-10.8 [0/34]	0.998	-1.1-(8.4) [0/10]
CPZ	10	10	0.9-13.79[0/34]	0.999	0.55-(19.9) [0/10]
CPZSO	10	10	0.7-11.9[0/34]	0.998	0.66-(13.6) [0/10]
DCPZ	10	10	0.2-12.7 [0/34]	0.998	0.09-(18.1) [0/10]

2.4 Discussion

The initial purpose of this research was to develop and validate a semi-quantitative method to evaluate the relative distribution of selected phenothiazine drugs (PMZ and CPZ), and their N-desmethyl and sulfoxide metabolites in skeletal remains. The method was intended for application to studies examining different PMZ and CPZ exposure patterns to understand the significance of drug and metabolite levels in toxicological analysis of bone, and to assess whether their small differences in chemical structure were associated with significant differences

in the patterns of drug and metabolite distribution. During the characterization of analytical figures of merit, precision and bias were not consistently meeting the required criteria (≤ 20%). UPLC-PDA chromatograms from extracted standards showed extraneous peaks and further experiments revealed that the analytes were degrading during sample preparation. Consequently, the main objective of the work shifted to the characterization of analyte degradation.

As demonstrated by the results, the parent drugs (PMZ, CPZ) underwent oxidation during sample preparation and extraction. Through analysis of analytical reference standards, we confirmed that PMZ was oxidized to its corresponding sulfoxide and N-oxide while CPZ was oxidized to its corresponding sulfoxide. Thus, some of the oxidation products included naturally occurring metabolites of the drugs. In addition to the identified oxidation products, other products were formed for which the putative identity include the sulfone, sulfoxide-N-oxide, chlorpromazine N-oxide or hydroxylated form of the parent drug. The oxidation was also observed in analysis of whole blood, and to a greater extent, which indicates the phenomenon does not occur only in bone tissue extract. Furthermore, the autosampler stability results provide strong evidence that the oxidation took place during the extraction , before the sample was placed on the instrument, and did not occur over time as the samples remained on the autosampler tray.

2.4.1 Sample Preparation of Basic Drugs

The complexity of biological samples has led to the development of numerous sample preparation techniques, and the selection of a suitable technique is based on chemical properties of the drug, the biological matrix and the limitations of the analytical instrumentation. Solid phase extraction (SPE) has become a common extraction technique used in forensic toxicology

because of the advantages with respect to amenability to automation, high selectivity, and cleanliness of extracts. Methods for SPE of basic drugs has been described (84,88-93) which employ solvents such as those utilized in this work (i.e., phosphate buffer, water, methanol, acetic acid, ethyl acetate, ammonium hydroxide and isopropanol). Work in our laboratory has successfully demonstrated the use of SPE for analysis of various basic drugs, including amitriptyline (94), dextromethorphan (86), meperidine (95), ketamine (96) and their metabolites in skeletal tissues using standard protocols. Research in our laboratory continues with the aim of assessment of the distribution patterns of drugs of forensic relevance with various physicochemical characteristics (e.g, acid-base character, volume of distribution, half-life, etc). The phenothiazines investigated here are relevant to post-mortem casework (97-105), and thus presented an interesting opportunity to compare skeletal drug and metabolite distribution patterns between structural analogues.

2.4.2 Oxidation of Phenothiazines

The susceptibility of phenothiazines to oxidation has been reported (106-31), although the majority of such work was done between 1950-1990. The most commonly reported oxidation occurs at the sulfide linkage which first forms an unstable radical cation that leads to the generation of the sulfoxide. After the sulfoxide is formed, subsequent oxidation can occur which results in the formation of the sulfone (113-115). Various factors have been noted to influence the oxidation reaction including acidity, concentration of oxidizing agents, time, temperature and the side-chain of the molecule (110-112). Much of the research describing phenothiazine oxidation has focused on the generation of oxidation products by chemical, electrochemical, enzymatic and catalytic means (112,114,116-118). The oxidation of phenothiazines remains a

complex subject, largely depending on the experimental conditions and debate about the mechanisms and products formed continues.

To the authors' knowledge, no published articles in the analytical toxicology literature have reported oxidation of the phenothiazine drugs during the process of preparation of biological samples for analysis. It is possible that the formation of oxidation products may go unnoticed, depending on the sample preparation methods and analytical instrumentation employed. For example, with the use of targeted methods such as GC/MS in SIM mode, or LC/MS/MS in MRM mode, ions corresponding to the oxidation products may not arise in the appropriate time window, or they may be completely excluded from the list of ions or transitions used. For those methods that monitor sulfoxide metabolites, the presence of sulfoxide in any given calibrant or sample chromatogram is expected. Provided that the oxidation reaction occurs in a reproducible and concentration dependent manner, acceptable standard curves could be generated in a given assay.

The results of this work suggest that the extent of oxidation is highly reproducible under these sample preparation conditions, as the measured CV values in replicate analyses of all analytes were less than 20% in over 90% of extractions done (Table 3). If the extent of oxidation was not reproducible, a much wider variability in measured precision and bias values would be expected. This reproducibility represents another reason that analyte oxidation during sample preparation might go unnoticed.

2.4.3 Effect of Matrix on Phenothiazine Oxidation

The data presented here suggests that the extent of oxidation and the products formed may vary between sample matrices, as shown in comparing data from samples prepared in bone tissue

extract (Fig. 8) to those prepared in blood (Fig. 9). This is consistent with studies reporting the oxidation of sulfide functionalities due to various reactive molecules present in blood (50,51). The authors noticed that CPZ is converted to CPZSO in whole blood, but a large conversion is due to the set-up of the analytical procedure (50,51). Given the wide variation in the nature of sample matrices in post-mortem toxicology, it is likely that the extent of phenothiazine oxidation could differ from sample to sample. Thus, even though the vast majority of bias estimations from the various extractions performed were within acceptable limits, this was likely because the same blank matrix was used in preparation of calibrants and the positive control samples used in measurement of accuracy, resulting in a similar extent of oxidation between them. In casework, the matrix used for preparation of standard curves necessarily differs from that of a given sample, and the extent of oxidation may be expected to also differ. Hence, measured phenothiazine and metabolite concentrations may be inaccurate, interfering with toxicological interpretation of the results.

2.4.4 Effect of Extraction Conditions on Phenothiazine Oxidation

The original purpose of this study was to develop and validate a method for the analysis of selected phenothiazine drugs and their respective desmethyl and sulfoxide metabolites in decomposed skeletal remains of rats. Initial attempts to validate the method, according to SWGTOX requirements, utilizing microplate solid-phase extraction using an elution solution based on ethyl acetate, and analysis by UPLC-PDA, were unsuccessful. Results showed that analytes were degrading by oxidation during sample preparation and some of the oxidation products included naturally occurring metabolites of the drugs being assayed. In some cases, the oxidation resulted in unacceptable bias and precision. Therefore, the focus of the work shifted towards identification of the source of the oxidation and redesign of the analytical method such

that the oxidation was minimized or eliminated. The first area that was investigated was the elution step in the SPE process. Experiments where PMZ and CPZ standards were dissolved directly in the ethyl acetate-based elution solvent and then evaporated to dryness (i.e., no extraction step from biological matrix) showed extensive oxidation. We theorized that the drugs were degrading due to elution solvent instability and as a result switched from dichloromethane to ethyl acetate. However, as Figures 10,11 and Table 6 depict ethyl acetate caused a greater number of oxidation products to form and the relative amount of oxidation product formed was higher. Next, numerous experiments were conducted such as reducing the temperature of the Centrivap from 70^0C to 40^0C, eliminating exposure to light, and evaporation under argon gas instead of vacuum centrifugation. None of these alterations eliminated oxidation. The subsequent sample preparation method that was employed removed the elution step completely by utilizing FPTE in place of SPE. The results were promising (Fig. 12 and Table 6), with reduced oxidation of PMZ and CPZ.

Based on the minimal presence of oxidation products in neat standards, the evaporation step of the FPTE process was removed. By removing the evaporation step, no additional oxidation was induced, and the amount of oxidation product formed was the same as in the unextracted neat standard (Fig. 13, Table 6). Given that this new sample preparation method was able to eliminate the degradation of the phenothiazines, we were able to successfully validate it for the analysis of PMZ, CPZ and their corresponding metabolites. In comparing the results of the four sample preparation methods we are able to conclude that sample preparation method highly influenced the extent of oxidation, specifically the type and relative formation of the oxidized species produced.

2.4.6 Conclusions

We have demonstrated that degradation of selected phenothiazines occurred by oxidation during sample preparation. The oxidation products observed included common metabolites of the parent drug and may confound toxicological interpretation. The incidence of oxidation may not be detected by certain analytical configurations. These results are of particular importance for laboratories employing tandem MS methods for analysis of phenothiazines based on MRM. Also, the variability in the extent of oxidation between different samples and calibrators may yield erroneous results. Our work also established the influence of changing the sample preparation method on the extent of oxidation. A new simple extraction method was developed and validated for the analysis of phenothiazines in skeletal tissues that did not measurably generate any oxidation products.

Chapter 3

3.1 Conclusion

In the present study, it was demonstrated that promethazine and chlorpromazine have stability issues due to their susceptibility to oxidation at the sulfur and nitrogen atoms. While using a method that was causing PMZ and CPZ to be converted to their corresponding oxidation products, respectable coefficient of variation values were achieved. Thus, there may be nothing to alert an unsuspecting analyst when data are erroneous during analysis of patient samples with PMZ or CPZ. Furthermore, the extent of oxidation and the products formed may vary between sample matrices and as a result will differ from sample to sample. The results of this work also established that the phenomenon of oxidation will vary between different analytical methods and this is a concern because not all laboratories will use the same method causing them to produce dissimilar results. In conclusion, the analysis of phenothiazines may lead to overestimation or

underestimation and lead to false toxicological interpretations. It is important to create an experimental design that includes various stability experiments and it is necessary to develop an analytical method where degradation does not occur.

3.2 Future Work

The data generated in this study will support future work applying this method to study the disposition of promethazine and chlorpromazine in bone and the effects of different drug exposure patterns such as acute vs. repeated and the delay between exposure and death. Other potential research would include examining the stability of other phenothiazines to determine their predisposition to oxidation. Lastly, applying the validated method to multiple phenothiazines to ensure its applicability and reproducibility.

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