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THE DETECTION AND DISTRIBUTION OF ANTIDEPRESSANTS IN BIOLOGICAL SPECIMENS

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The Detection and Distribution of Antidepressants in Biological Specimens

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Abstract

This study developed and validated a method utilizing Ultra-Performance Liquid Chromatography (UPLC) paired with tandem mass spectrometry (MS-MS) to detect and quantify thirteen antidepressants and metabolites in blood and various biological tissue samples from deceased pilots whose specimens were sent to the Federal Aviation Administration (FAA) for toxicological analysis following autopsy. Validation was conducted using a modified version of American National Standards Institute/American Academy of Forensic Science Standards Board (ANSI/ASB) Standard 036, and included a calibration model, limit of detection, carryover, cross contribution, dilution integrity, drug interference, bias and precision, fluid and tissue controls, ion suppression/enhancement, recovery, process efficiency, stability, and measurement of uncertainty. Using a linear dynamic range of 200 times the lowest cutoff concentration, all analytes and matrices were successfully validated. Analytes were found to be stable at 4°C for at least four days, through at least three freeze/thaw cycles at -20°C, and on the instrument autosampler (10°C) for at least four days.

Once this method was successfully developed and validated, Phase II consisted of a postmortem distribution study that examined citalopram and its N-desmethyl metabolite to determine the feasibility of relating a tissue drug concentration to the blood concentration, as well as a metabolite to drug ratio. The only correlations that could be established were the citalopram brain:blood ratio at 8.3 and the citalopram muscle:blood ratio at 1.6. Though other correlations were not established, notable trends were observed. Liver and lung had the highest concentrations of drug and metabolite, while spinal fluid and vitreous had the lowest. No metabolite to drug ratio correlation was established, although bile appeared to have the highest

ratio at 1.3 while all other specimens were below 0.5, indicating a low concentration of metabolite present compared to the parent drug.

Introduction

To date, little research has been done on the postmortem distribution of antidepressants, especially selective serotonin reuptake inhibitors (SSRIs), which have a wide therapeutic index and rarely contribute to the cause of death when taken on their own (Levine et al., 2001). However, these drugs are of vital importance to organizations such as the FAA due to their side effects and potential impact on motor skills (Federal Aviation Administration, 2022). Antidepressants have common side effects that include fatigue, drowsiness, insomnia, tremors, dizziness, and lightheadedness amongst many others, all of which can affect pilot performance and potentially lead to more crashes (Lewis et al., 2013). Until 2010, the FAA prohibited the use of antidepressants and SSRIs (Durham and Bliss, 2019). Now that some of these drugs are allowed for pilot therapeutic use, it is important to include them in postmortem analyses to understand how many aviation accidents they play a role in, so that the safest regulations for air traffic can be implemented.

Postmortem distribution is also a focus of this two-part study because in forensic cases, there are many times when the desired gold standard of a peripheral blood sample is unavailable. Peripheral blood samples are preferred, as this blood is only affected by drug distribution from localized tissues, rather than the wider distribution from organs and tissues affecting other matrices (Cook et al., 2020). However, the FAA reports that they receive blood samples in only 70% of cases (Lewis et al., 2015; Øiestad et al., 2018). Even if a blood sample is unavailable, a sample of organ, muscular, or skeletal tissue may be possible. Being able to not only test these organ and tissue samples but relate the results back to blood concentrations is critical. One issue faced is that while analyzing these types of biological specimens is not uncommon in forensic practice, there is currently limited information correlating tissue concentrations to blood

concentrations. However, therapeutic and toxic drug concentration ranges are commonly listed only for blood and plasma, such as in Schulz et al., 2020. This presents a challenge if only tissue samples are available, as their relation to blood concentration is needed for the most accurate interpretation of effects given the analytical results (Yarema and Becker, 2005).

The purpose of this two-part research study was to develop and validate a method utilizing Ultra-Performance Liquid Chromatography (UPLC) paired with tandem mass spectrometry (MS-MS) to detect and quantify thirteen antidepressants and metabolites in blood and various biological tissue samples from deceased pilots whose specimens were sent to the FAA for toxicological analysis following autopsy. Phase II consisted of a postmortem distribution study that examined citalopram and its N-desmethyl metabolite to determine the feasibility of relating a tissue drug concentration to the blood concentration, as well as a metabolite to drug ratio, to aid in a more accurate interpretation of toxicological results.

Literature Review

Antidepressants

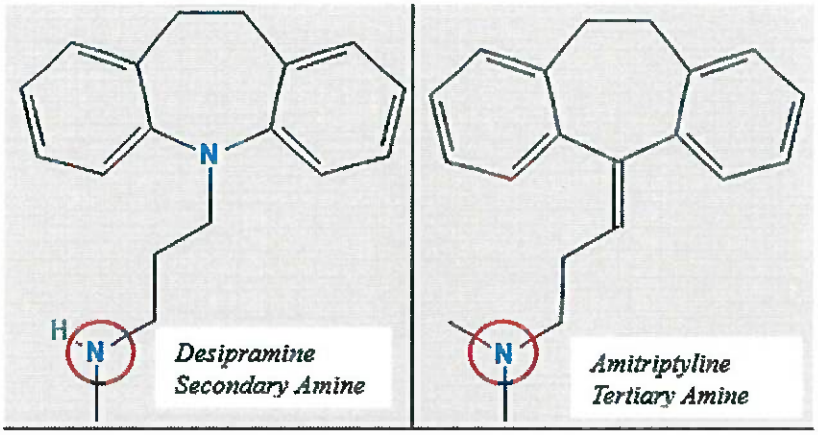
Depression and anxiety affect an estimated twenty-one million Americans (Durham and Bliss, 2019). Factors contributing to these disorders include genetics, stressful or traumatic life events, and environment (Durham and Bliss, 2019). Symptoms of anxiety and depression include fatigue, insomnia, worry, gastrointestinal upset, loss of appetite, grief, sadness, fear, irrational and harmful thoughts, and irritability (Durham and Bliss, 2019). Antidepressant medication is commonly prescribed not only to treat depression, but also anxiety disorders, obsessive-compulsive disorder (OCD), and post-traumatic stress disorder (PTSD) (Johnson et al., 2007). While antidepressants are among the most prescribed types of medication, to date most postmortem research has only focused on the analysis of toxic levels, rather than the detection and assessment of therapeutic levels. In addition, data regarding therapeutic levels is typically only reported for concentrations found in blood (Lewis et al., 2015). In forensic cases, there are many times when blood samples are not a viable option due to the lack of sample availability. The state of body decomposition and the traumatic nature involved in some forensic cases can eliminate the possibility of collecting a blood sample, leaving tissue specimens to be relied upon for drug concentrations. The FAA, which disallowed the use of these drugs until 2010, is working to alleviate the issues caused by this lack of research because, while the antidepressant medication may not directly be the cause of death, the side effects could lead to contributing factors of an accident (Rogers et al., 2017). Durham and Bliss (2019) reported that in one-third of the cases they studied, the pilot's usage of SSRIs or their underlying mental condition was a contributing factor in plane crashes. Common side effects of antidepressants include dizziness, insomnia, drowsiness, headaches, blurred vision, tremors, and lightheadedness, all of which can

affect pilot performance (Lewis et al., 2013). Additionally, antidepressants can be the source of many drug-drug interactions, due to their ability to induce or inhibit cytochrome P450 enzymes. Antidepressants are divided into several categories based on their mechanism of action. This literature review focuses on the classes of tricyclic antidepressants (TCAs) and SSRIs, both of which block the reuptake of serotonin (or norepinephrine in the case of some TCAs) in the brain. Serotonin (5-hydroxytryptamine or 5-HT), a neurotransmitter that also acts as a hormone, plays a part in multiple functions including hunger, memory, sleep, behavior, happiness, and even the constriction of blood vessels (Coleman and Gouaux, 2018).

Tricyclic antidepressants share a common core structure of three joined rings attached to a secondary or tertiary amine as seen in Figure 1.

Figure 1.

Structure of a Tricyclic Antidepressant.



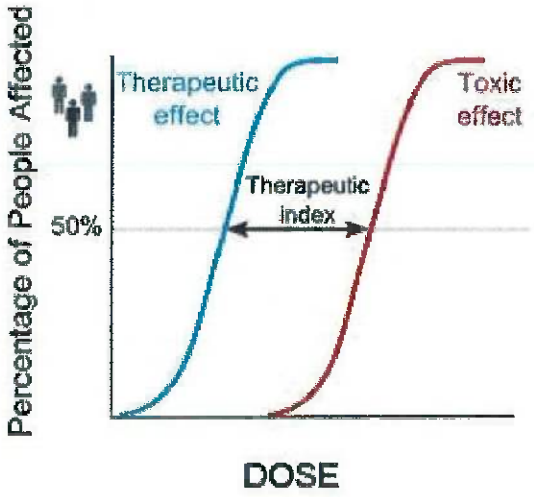
Note. Tricyclic antidepressants feature a three-ring structure connected to a secondary or tertiary amine, with "R" representing any chemical group. The structures were drawn using the PubChem Sketcher (U.S. National Library of Medicine [NLM], 2023).

These medications work by blocking serotonin or norepinephrine reuptake at presynaptic terminals; thus, increasing their concentration in the synaptic cleft (Moraczewski and Aedma, 2022). While tricyclics used to be the top treatment option for depression, they are now less

common because of their safety issues. In addition to typical antidepressant side effects, tricyclics have a narrow therapeutic index (TI), meaning that the line between effective treatment and toxicity is a fine one (Moraczewski and Aedma, 2022). Drugs with a larger TI are generally considered safer than those with a narrow TI because the amount of drug it takes to be effective is much smaller than the amount normally required to induce toxic effects (Hansen, 2020). An illustration of this principle is depicted in Figure 2.

Figure 2.

Therapeutic Index

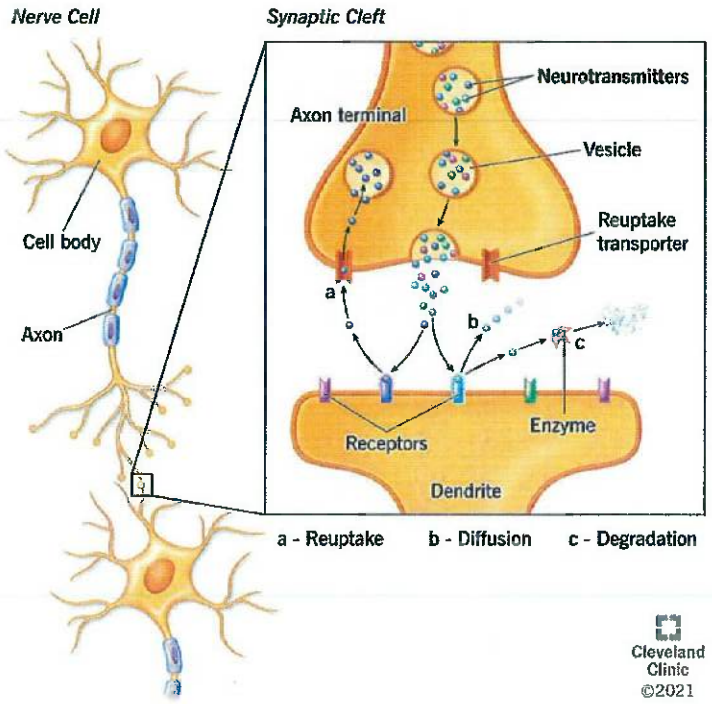


Note. The therapeutic index (TI) is the dosage window where a drug is considered both effective and safe. TI is determined by dividing the toxic dose by the effective dose seen in 50% of people (Hansen, 2020).

Clomipramine, marketed as Anafranil, is a tricyclic antidepressant that blocks the reuptake of serotonin, thereby increasing its concentration in the synaptic cleft, and providing a boost in mood and function (Avella et al., 2004). Figure 3 illustrates the receptors and reuptake transporters in the synaptic cleft where this process occurs.

Figure 3.

Synaptic Cleft Reuptake



Note. Inhibitors bind to the reuptake transporters in the synaptic cleft, disrupting the reuptake process and increasing the chemical concentration present (Cleveland Clinic, 2022).

Schulz et al. (2020) states that the therapeutic blood concentration range for clomipramine and its active metabolite, desmethylclomipramine, is 230-450 ng/mL combined, while toxicity can be observed at 450 ng/mL combined, demonstrating the dangerously narrow therapeutic index of clomipramine. These levels are only reported in the reviewed scientific literature regarding blood and plasma concentrations. However, clomipramine, with a large volume of distribution (V_d) of 17 L/kg, undergoes wide distribution throughout the body[1]. This,

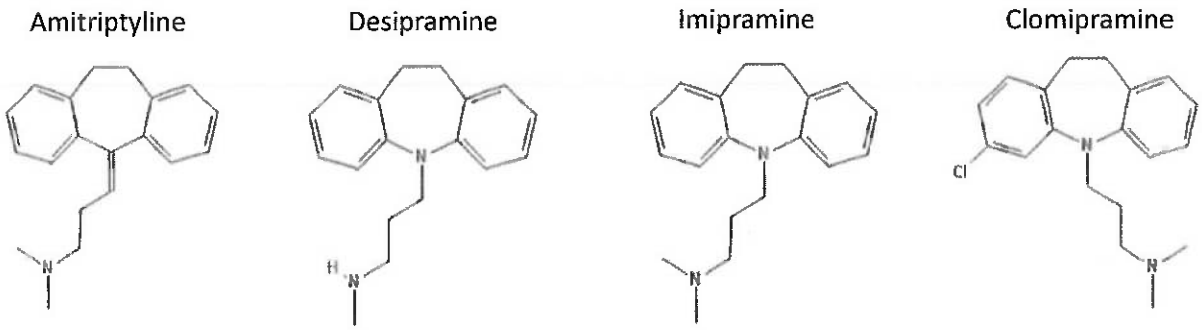
[1] Volume of distribution (V_d) refers to the apparent amount of body water a drug would be equally distributed in based on an observed blood or plasma drug concentration following a given dose and is a measure of the distribution of drug throughout the body. Drugs which are lipophilic ($V_d > 1$) undergo greater distribution from the plasma into surrounding structures in the body, requiring a higher dosage to maintain the desired plasma concentration. Several factors affect V_d including gender, molecular size, charge, and pH (Berezhkovskiy, 2013)

paired with the narrow therapeutic index, means that blood may not be a reliable postmortem source for determining toxicity (Avella et al., 2004). Avella et al. (2004) suggests using blood samples in conjunction with liver and brain tissue, when possible, for a more accurate concentration determination.

TCAs such as amitriptyline, desipramine, imipramine, and clomipramine, the structures of which are shown in Figure 4, are still prescribed, but the use of SSRIs are now more favored among physicians.

Figure 4.

Molecular Structures of Amitriptyline, Desipramine, Imipramine, and Clomipramine

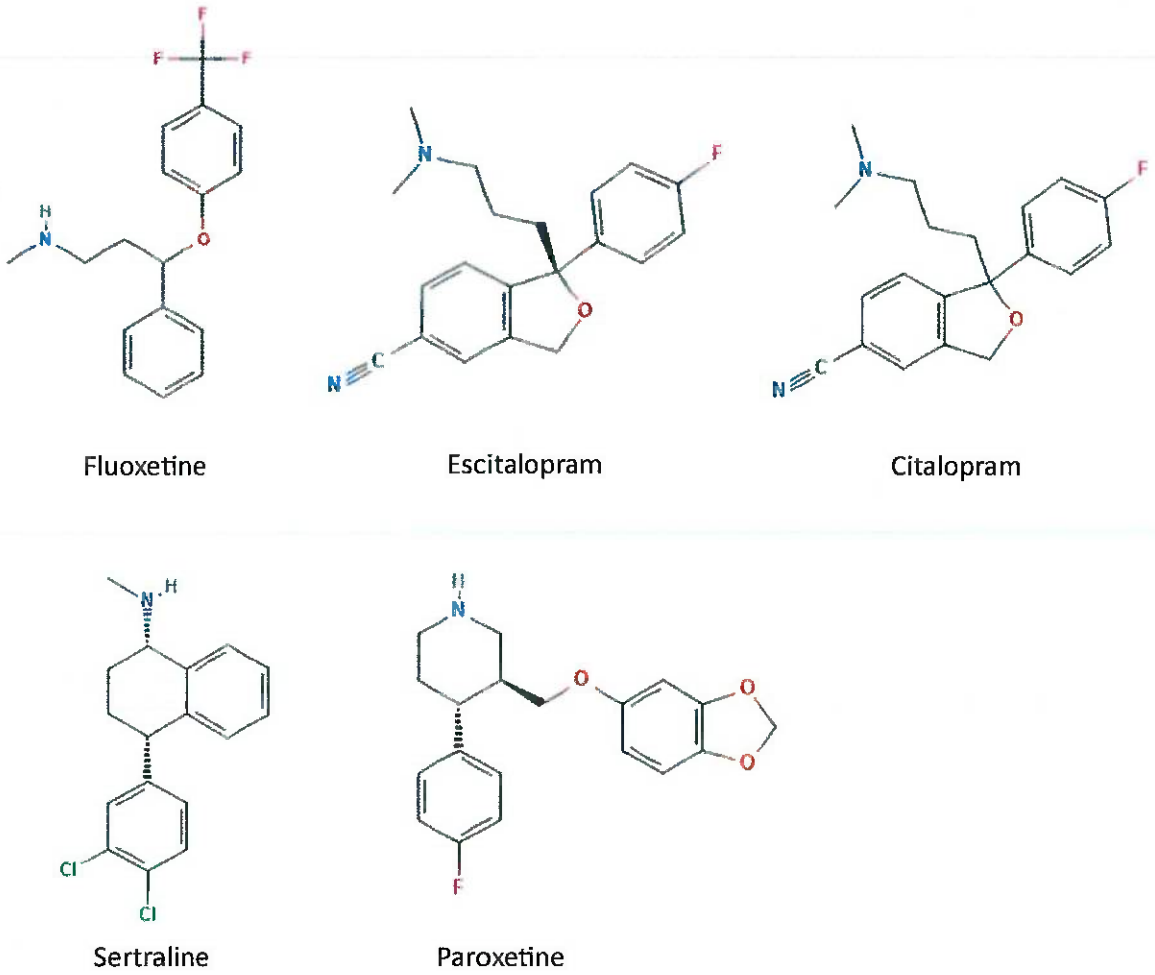


Note. Molecular structures were sourced from NLM, 2023.

SSRIs have been found to be as effective as the older generation TCAs, but they have a wider therapeutic index, allowing safer treatment (Green, 2003). They also have fewer reports of the severe side effects associated with TCAs, such as weight gain and cardiovascular issues (Green, 2003). However, abruptly stopping treatment can lead to withdrawal symptoms, so discontinuation should be tapered off and closely monitored (Green, 2003). The FAA currently approves the four SSRIs shown in Figure 5 for usage, fluoxetine, escitalopram, citalopram, and sertraline, though research focuses on other drugs of potential use such as paroxetine as well (Durham and Bliss, 2019).

Figure 5.

Molecular Structures of Fluoxetine, Escitalopram, Citalopram, Sertraline, and Paroxetine.



Note. Molecular structures were sourced from NLM, 2023.

Pilots are required to have six months of evaluations at a consistent dosage to be eligible for flights (Durham and Bliss, 2019). While it is beyond the scope of this review, more information regarding pilot usage of SSRIs can be found in Title 14 CFR Part 67 (Durham and Bliss, 2019).

Unlike TCAs, SSRIs do not share a common core structure, though they share the same behavior. The mechanism of action involved with SSRIs is blockage of the serotonin transporter (SERT) resulting in an outward-open conformation, which then increases the concentration of 5-HT in extracellular spaces and the synaptic cleft by inhibiting transport and reuptake (Coleman and Gouaux, 2018). Since the transporters for norepinephrine and dopamine are related to the 5-HT transporter by amino acid sequence, inhibition of these transporters works in much the same way (Coleman and Gouaux, 2018).

Citalopram [(+)-l-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile], often recognized by the brand name Celexa, is distributed to consumers as the hydrobromide salt (Levine et al., 2001). Schulz (2020) lists citalopram's therapeutic range as 50-110 ng/mL in blood, in contrast to Levine et al. (2001) which notes a typical therapeutic concentration of around 200 ng/mL in blood. Citalopram has a V_d of 12 L/kg and is highly protein bound (Levine et al., 2001). In all postmortem cases studied by Levine et al. (2001), even when citalopram was found in much higher than therapeutic blood concentrations, it was not considered a factor in cause of death, because of its wide TI. Concentrations were found to be higher in liver and kidney samples than in blood, likely due to metabolism and citalopram's large V_d (Levine et al., 2001). Interestingly, with chronic therapeutic use, citalopram was found to have a higher concentration than its metabolite, N-desmethylcitalopram, which differs from most other SSRIs like sertraline where a 1:1 ratio is expected due to metabolism (Levine et al., 2001).

Sertraline [(1S,4S)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-N-methyl-1-naphtalenamine], marketed as Zoloft has a therapeutic range of 50-250 ng/mL in blood, though some toxic effects have been noted at the upper level (Lewis et al., 2013). With a wide

therapeutic index, lethal levels are six times the upper therapeutic limit at 1,500 ng/mL (Lewis et al., 2013). Sertraline's chemical structure features a secondary amine linked to a tetrahydronaphthalene ring system and a dichlorophenyl group (Coleman and Gouaux, 2018). Being a secondary amine indicates sertraline may play a role in norepinephrine reuptake inhibition in addition to serotonin (Coleman and Gouaux, 2018). With a large V_d of 20-76 L/kg, sertraline undergoes first-pass metabolism via the liver and forms its metabolite norsertraline via demethylation (Lewis et al., 2013)[2]. Since sertraline exhibits high protein binding and is too big for renal filtration, biliary excretion is the main route of elimination.

Fluoxetine (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine), brand name Prozac, has a therapeutic blood concentration range of 120-500 ng/mL (Schulz et al., 2020). Toxic and lethal levels are reported as 1000 ng/mL and 2200 ng/mL, respectively, but it has been noted that overdoses were rare due to the safety of the drug (Johnson et al., 2007; Schulz et al., 2020). Like sertraline, fluoxetine also has a large V_d at 20-42 L/kg indicating wide distribution into tissues throughout the body (Johnson et al., 2007). The half-life of fluoxetine, the time it takes for the drug concentration to decrease by half due to metabolism or excretion, is four days, so any detected concentration may not be from recent ingestion (Johnson et al., 2007). Fluoxetine affects not only serotonin, but at high concentrations can also affect norepinephrine and dopamine reuptake in the frontal cortex with its trifluoronated aromatic ring joined to a phenylpropylamine group (Coleman and Gouaux, 2018).

[2] First-pass metabolism is the process in which a drug undergoes transformation prior to entering systemic circulation in an effort to reduce its bioavailability. Depending on the acidity of the drug, when ingested orally it will either be absorbed in the stomach or intestines. Via the hepatic portal blood system, the drug will undergo metabolism in the liver where it is broken down in an effort to excrete the foreign substance either in urine (if water soluble) or through the bile and feces (if lipid soluble). This effect has the ability to drastically reduce the amount of drug reaching systemic circulation. Intravenous administration bypasses this effect, therefore reducing the dosage required for effectiveness (Herman and Santos, 2022).

Paroxetine [3S,4R-3-[(2H-1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl) piperidine] is commonly known as Paxil. Distributed in the hydrochloride (HCl) form, Paroxetine HCl has a phenylpiperidine structure commonly seen in opioids (Green, 2003). Paroxetine exhibits weak inhibition of norepinephrine and dopamine, but it has also been found to be an inhibitor of nitric oxide synthase, decreasing nitrate levels which can lead to vasoconstriction and reduced flow of oxygenated blood to the heart (Green 2003). One key feature of paroxetine is that it may induce apoptosis, regulated cell death, enhancing the immune system that is often weakened as a side effect of depression (Green, 2003). Paroxetine is noted as one of the more potent SSRIs and has a V_d of 17 L/kg with 95% protein binding ability (Lewis et al., 2015). Paroxetine undergoes extensive metabolism and 62% is excreted as inactive metabolites in urine (Green, 2003). Therapeutic concentrations are reported at 10-120 ng/mL with toxic and lethal concentrations reported at 350 ng/mL and 3,700 ng/mL, respectively (Lewis et al., 2015). While the safety and effects of antidepressant concentration in blood are well documented as seen above, the nature of forensic casework requires reliable information regarding postmortem drug concentrations in a variety of biological matrices.

Postmortem Redistribution (PMR) and Suitable Sampling Practices

Postmortem redistribution (PMR) refers to the way drugs move throughout the body after death, resulting in a change of concentration. This occurs due to gradient diffusion, metabolism, drug degradation, and the bacteria involved in the decomposition process (Mantinieks et al., 2021). Several factors affect how a drug is redistributed throughout the body including the route of administration, the acidity of the drug, lipophilicity, and the V_d, as well as the particular individual's unique body chemistry (Yarema and Becker, 2005).

Drugs that are basic, lipophilic, and have a V_d greater than 3 L/kg tend to undergo greater PMR (Mantinieks et al., 2021). Basic, lipophilic drugs which are highly concentrated in organs become ionized inside a cell's postmortem acidic conditions, creating a concentration gradient which causes distribution of unionized drugs through passive diffusion into cells for ionization (Yarema and Becker, 2005). Ischemic cell damage can occur within minutes in the brain and hours in the liver, indicating PMR can take place quickly in many cases, which should be kept in mind when collecting samples for testing (Yarema and Becker, 2005). In addition to considering the time factor when collecting samples, specimens should be kept cold to help inhibit the bacteria formed during putrefaction from metabolizing the drugs, lowering their concentrations in turn (Yarema and Becker, 2005).

While time and temperature are crucial factors in determining drug concentration, forensic scientists must also take care when choosing sample matrices and collection methods. One major issue with currently available studies is that there is no standardization for how samples are obtained nor for suitable matrices other than peripheral blood. While peripheral blood is the current gold standard for toxicological analysis, it is not available in all cases. The FAA reports that they only receive blood samples in approximately 70% of cases, meaning that other biological specimens are heavily relied upon (Lewis et al., 2015). Many current studies examine the possibility of utilizing matrices such as central blood, vitreous humor, liver, lung, brain, skeletal, and muscle tissues, but a correlation with peripheral blood concentration has not been established. Interestingly, Yarema and Becker (2005) note that cardiac (central) blood is one of the least useful samples for quantitation because it gains such a high concentration of drugs from other organs during PMR. Agonal aspiration can also lead to elevated concentration levels in cardiac blood (Yarema and Becker, 2005). Despite this, cardiac blood is still a popular matrix

and research shows that there is no significant difference in concentration between samples taken from different sides of the heart (Zilg et al., 2017).

Another specimen of interest is skeletal tissue. Skeletal tissue can be categorized into two groups, bone tissue and bone marrow (Vandenbosch et al. 2020). Bone marrow can be further separated into red marrow which produces blood cells and cells for bone formation, and yellow marrow which produces fat cells and adipocytes and composes about 70% of an adult's marrow (Vandenbosch et al. 2020). Vandenbosch et al. (2020) found that long or trabecular bone tissue tends to reflect a higher drug concentration than short bones which is due to the higher vascularization rate. Bone marrow shows potential as a screening matrix and skeletal tissue is known to be highly resistant to putrefaction; however, very few studies have been done to determine suitability (Vandenbosch et al. 2020).

A controversial sample option found through research is vitreous humor (VH). While most current literature indicates that VH is an unsuitable substitute for peripheral blood due to sampling and analytical difficulties, Yarema and Becker (2005) and Ntoupa et al. (2020) disagree. VH, made up of 98% water and found in the posterior chamber of the eye, has no vascularization and is therefore highly protected from PMR and other decomposition processes (Ntoupa et al., 2020). During the Ntoupa et al. (2020) study, all drug-positive blood cases also tested positive during the analysis of the VH, however, concentrations were extremely low. Whether this was due to drug characteristics such as protein binding ability and lipophilicity, or testing methods is unclear (Ntoupa et al., 2020). However, undetectable amounts were labeled as a common issue in multiple other studies involving VH and was one reason it was deemed unsuitable. With a more sensitive method, VH may be an acceptable alternative matrix for qualitative analysis, if not quantitative (Ntoupa et al., 2020).

One specimen type that researchers are quick to agree is useful for postmortem drug quantitation is organ tissue, especially liver and kidney tissue. Consensus in current literature indicates that drugs are easily detected and quantified in liver and kidney samples, though concentrations are usually markedly higher than in blood and no direct correlation has been able to be established (Yarema and Becker, 2005). These trends are reasonable considering the liver and kidneys play major roles in drug elimination during first and second-pass metabolism.

Understanding the correlation between postmortem tissue and blood concentrations is crucial. PMR can lead to higher concentrations in tissues such as the liver which must be accounted for (Yarema et al., 2005). Ultimately, developing more inclusive screening tests to allow for smaller samples, identifying correlations between concentrations in peripheral blood and other sample matrices, establishing postmortem and antemortem concentration correlations, and standardizing collection protocols will allow for more accurate analyses and reliable interpretations.

Testing Methods and Results

Specimen Collection and Preservation

Samples from human liver, heart, lung, kidney, brain, muscle, VH, skeletal tissue, cardiac blood, and peripheral blood were all featured in the reviewed literature. Both the collection site and sampling method affect the measurable concentration of drugs, which is why standardized protocols are necessary though not always possible due to varying agency procedures (Cook et al., 2000). It is suggested that for blood samples, peripheral be used instead of cardiac due to the higher distribution found in the chest cavity (Cook et al., 2000). Peripheral blood is only affected by distribution from localized tissue, whereas cardiac, or central, blood is subject to distribution from multiple organs and tissue (Cook et al., 2000). The femoral vein is the most popular

collection site for peripheral blood sampling and Cook et al. (2000) note that the vein should be ligated as soon as possible to prevent contamination from central blood flow. Most research methods involved storing the blood samples at -20°C with 1% sodium fluoride-potassium oxalate (Lewis et al., 2013). Other biological samples were reportedly stored at -20°C without further preservation, though the collection methods were not detailed (Lewis et al., 2013). Blood samples were initially screened for the drug of interest, and upon further testing, the concentrations were found to be within 10% of the original value, indicating that no drug degradation had occurred in samples during storage using this method, which was up to five years in some cases (Johnson et al., 2007). It should be noted that reported research only discussed samples from bodies with no to moderate putrefaction, and samples were typically collected within a few days of death and analyzed within a few weeks (Johnson et al., 2007; Øiestad et al., 2018).

Instrumentation and Analysis

Recent years have shown many advances in analytical instrumentation and techniques, resulting in the ability to test for several drugs and metabolites of interest at one time. Not only does this save time and money, but this also allows for the analysis of small sample sizes and matrices like VH where there is often only a total volume of 2-3 mL from which to draw a sample (Øiestad et al., 2018). Currently, most toxicological analysis is performed via liquid chromatography (LC) or gas chromatography (GC) coupled with mass spectrometry (MS).

Chromatography involves the principle of separating components from a mixture. The sample flows through a column in the instrument, interacting with a mobile phase and a stationary phase. Depending on the interactions between the analytes and the phases, analytes will elute and reach the detector at different times. The time spent from entering the column to

reaching the detector is called the retention time and is shown as peaks on the instrument output called a chromatogram (Ross-Carr et al., 2017). Using mass spectrometry, these retention times and the mass-to-charge ratios (m/z) of unknown samples are compared to an established database to identify the component as described below (Ross-Carr et al., 2017). LC involves liquid mobile phases such as methanol to push the sample through the column and help separate the components while GC involves a carrier gas such as helium for the mobile phase and relies on a temperature gradient to help separate the components. While GC is best suited for thermally stable, non-polar compounds, LC is versatile enough to analyze these compounds as well as thermally active, polar compounds (Ross-Carr et al., 2017).

Mass spectrometry requires ionizable compounds because it fragments compounds into ions and sorts them based on their m/z (Ross-Carr et al., 2017). Once these fragments are sorted, their abundance is detected and the spectrum is produced (Ross-Carr et al., 2017). These results are compared to a database in the case of GC/MS or ion transition monitoring for LC/MS to elucidate the components of the compound. Selective ion monitoring (SIM) allows for the filtration of molecules of a certain mass allowing greater selectivity and the ability to scan a single mass multiple times over a given timeframe (Ross-Carr et al., 2017). Multiple reaction monitoring (MRM) has multiple filtration steps where the larger m/z parent ion is allowed through the first quadrupole, then this ion is fragmented into daughter ions and filtered further by m/z (Ross-Carr et al., 2017). Both methods improve selectivity and allow for lower limits of detection (LOD), the smallest concentration detectable, and quantitation (LOQ), the lowest concentration able to be established with accuracy.

For analysis via liquid chromatography, antidepressants are extracted from the biological specimen using either solid phase extraction (SPE) with non-polar and strong cation exchange

sorbents, or a liquid-liquid extraction, typically with ethyl acetate or n-butyl chloride (Lewis et al., 2013). Prior to extraction, tissue samples are routinely homogenized in a 1:2 dilution with 1% sodium fluoride (Johnson et al., 2007). When GC-MS is the chosen analysis method, samples are often derivatized with pentafluoropropionic anhydride (PFPA) due to the polar nature of antidepressants (Lewis et al., 2013).

Scientific literature shows the following parameters for analysis. Sertraline had a LOD of 0.78 ng/mL with the LOQ being set at this limit, while the LOD and LOQ for its primary metabolite, norsertraline, were 1.56 ng/mL (Lewis et al., 2013). The linear dynamic range (LDR) where the signal is proportional to the analyte concentration was 0.78-800 ng/mL for sertraline and 1.56-800 ng/mL for norsertraline (Lewis et al., 2013). The correlation coefficient (R^2) was 0.99 with a value of one indicating a perfect linear correlation (Lewis et al., 2013). GC-MS analysis revealed qualifier ions at 276 m/z and 451 m/z for sertraline and 239 m/z and 437 m/z for norsertraline, though ions may vary slightly with LC-MS analysis (Lewis et al., 2013). The quantifier ion for both was 274 m/z (Lewis et al., 2013). For fluoxetine, SIM detected qualifier ions at 115 m/z and 117 m/z with a quantifier ion at 294 m/z (Johnson et al., 2007). Its metabolite, norfluoxetine, also had qualifier ions at 115 m/z and 117 m/z, but the quantifier ion was detected at 280 m/z (Johnson et al., 2007). The LOD was 1.56 ng/mL and the LOQ was 3.13 ng/mL for both fluoxetine and norfluoxetine (Johnson et al., 2007). The LDR was 3.13-800 ng/mL and the R^2 was 0.994 (Johnson et al., 2007). Paroxetine had a LOD and LOQ of 3.13 ng/mL and an LDR of 3.13-1,600 ng/mL with $R^2 = 0.99$ (Lewis et al., 2015). Qualifier ions were at 338 m/z and 216 m/z and the quantifier ion was at 475 m/z (Lewis et al., 2013). Clomipramine's quantifier ion was at 58 m/z and the qualifiers were at 85 m/z and 269 m/z (Ntoupa et al., 2020). The LOD for clomipramine was 1.50 ng/mL and the LOQ was 5.00 ng/mL,

but no LDR was reported (Ntoupa et al., 2020). Apart from clomipramine, all LDRs above encompass a concentration range from subtherapeutic to lethal levels. While analysis of the above antidepressants and their metabolites were conducted individually in the articles reviewed, this research seeks to combine analysis into one method, saving time, costs, and reducing the sample size required.

Results and Discussion

The consensus found in the literature is that the selected antidepressants were detectable and quantifiable in all analyzed matrices. However, no correlation has been established between blood concentrations and the concentration in other biological samples. Øiestad et al. (2018) point out that urine is an acceptable sample for screening, but blood concentration cannot be estimated from this because time since the last urination, hydration levels, residual volume and drug excretion all play a role in the detected concentration. Zilg et al. (2017) established that central blood concentrations are higher than peripheral blood and maintains that peripheral blood should continue as the gold standard for sampling. It is important to ligate the femoral vein before sampling to prevent contamination (Cook et al., 2000). There was little difference between right and left-side sampling of the heart, and though arterial blood was found to have higher concentrations than venous, this was attributed to PMR rather than antemortem concentrations (Zilg et al., 2017).

An area of study where there is disagreement is regarding the use of VH. While many studies had issues consistently detecting analytes in VH, Ntoupa et al. (2020) suggests that it is a suitable matrix. Antidepressants were found in all cases where the blood tests were positive, but the detected concentration and ratio to blood varied greatly (Ntoupa et al., 2020). This suggests that VH is suitable for qualitative analysis, but not quantitative. Ntoupa et al. (2020) reported that

if the VH concentration is higher than blood, drug consumption likely occurred a long time before death. Other studies such as Øiestad et al. (2018) state that VH concentration is typically much lower than blood concentrations and is often undetectable if blood concentration is low or the drug exhibits high protein binding. While VH is resistant to postmortem changes, it does not seem that reliable concentrations are consistently detectable.

Skeletal tissue showed promising results and a linear trend was observed between concentrations in blood and bone marrow and blood and bone tissue (Vandenbosch et al., 2020). General concentration trends from high to low were bone marrow, blood, and bone tissue (Vandenbosch et al., 2020). Vandenbosch et al. (2020) reported that bone tissue is a depository for drugs of chronic usage. There is currently little data available on the analysis of these tissues, so more studies are needed to confirm these results.

General trends for the drugs analyzed show that the highest concentrations were found in the liver and lungs, followed by the brain and blood (Lewis et al., 2015). Ratios varied greatly, but concentrations were several times higher in the various biological specimens than in the blood. For example, Lewis et al. (2013) reported that the average liver-to-blood concentration ratio for sertraline was 74 ± 59, yet for paroxetine, it was 5.77 ± 1.37 (Lewis et al., 2015). These ratios depend on many factors including protein binding, the volume of distribution, the postmortem interval, and whether the drug is known to increase or decrease in concentration during postmortem processes. In addition to the distribution ratios, it is important to consider their coefficient of variation (CV).

The CV shows dispersion by calculating the ratio of the standard deviation to the mean. Johnson et al. (2007) claimed that if the CV is less than 25%, the distribution ratio of alternative specimens can be cautiously used to estimate the blood concentration. The citalopram and

fluoxetine studies, as well as the liver and spleen specimens from the paroxetine studies, fall into this category (Johnson et al., 2007; Lewis et al., 2015; Øiestad et al., 2018). However, most paroxetine specimens and the sertraline study all had a high CV ranging from 27-99% (Lewis et al., 2013, 2015). Multiple factors affect CV including the time between drug consumption and death, the postmortem interval, extent of PMR, specimen collection site and method, drug metabolism, and the overall health of the individual (Lewis et al., 2013). Current literature mainly features cases with a short postmortem interval, typically preserving the body for specimen collection within a few hours. More research is needed involving a longer time span between death and specimen collection, since this will most accurately reflect a large portion of forensic casework, where bodies are often times in a greater state of decomposition before arriving to a pathologist.

Current research faces some limitations. As mentioned above, studies with longer postmortem intervals are needed. Bodies with a higher level of putrefaction should also be analyzed to reflect real-world scenarios. Another issue affecting this limited research is that most subjects were male. There are many noted physiological differences between males and females, and it is known that the pharmacokinetics of drugs differ between genders. With current literature focused primarily on males, it should not be assumed that the trends observed can be generalized back to female decedents. Most importantly, it bears remembering that due to PMR the postmortem concentration does not necessarily correspond with the antemortem concentration depending on how long after death the sample was taken. Research should also include the ability to distinguish between acute concentration spikes and levels resulting from chronic use. As Mantinieks et al. (2021) states, it is difficult to interpret the significance PMR has on antidepressant concentration. The drug concentration measured after an autopsy may not be the

concentration present at the time of death, therefore, the interpretation of its role in the cause of death may be distorted (Yarema and Becker, 2005). Mantinieks et al. (2021) found that the median postmortem to antemortem concentration ratio for antidepressants was greater than one, indicating that PMR raises the concentration as expected, though the exact relationship between these ratios could not be established. Cook et al. (2000) performed a study on individuals where the antemortem concentration was known and found that the postmortem concentration was as high or higher than the antemortem in all cases. The sampling site and technique also greatly affected the concentration measured postmortem (Cook et al., 2000). It is also worth noting that drug metabolites are often more polar than the parent drug and will therefore undergo different PMR (Cook et al., 2000). Cook et al. (2000) suggests that the parent-to-metabolite ratio may be more useful than trying to calculate the postmortem-to-antemortem ratio, especially in cases where acute consumption is suspected. With these limitations in mind, research is still promising concerning the analysis and interpretation of drug concentrations in alternative matrices.

Conclusions

Current literature indicates that tricyclic antidepressants and SSRIs can be detected and quantified in a variety of matrices including blood, brain, liver, lung, kidney, muscle, skeletal tissues, and VH. With some controversy surrounding suitability, VH may be better suited as a qualitative method until further research is done (Øiestad et al., 2018). While the distribution ratios for blood and other biological specimens varied between drugs, general trends were seen with concentrations. Concentrations were usually highest in the liver and lungs followed by the brain, blood, urine, and muscle tissues (Johnson et al., 2007; Lewis et al., 2013, 2015). With caution, there is potential for ratios with a CV under 25% to be used to calculate the blood concentration based on the concentration measured in the alternative specimen (Johnson et al.,

2007). Since PMR can greatly affect concentration, distribution studies are needed because the concentration measured after autopsy may not correspond to the antemortem concentration, making the interpretation of its significance difficult (Yarema and Becker, 2005). The parent-to-metabolite ratio is one way to potentially address this issue, especially in cases with acute rather than chronic usage (Cook et al., 2000). The largest limitation of current studies is that most cases involved a short postmortem interval and male subjects with little to no putrefaction. Since putrefaction, the time between death and sample collection, and differing pharmacokinetics between genders can all affect PMR, these issues should be addressed prior to being generalized back to the majority of forensic casework. Additionally, it is critical to establish standardized protocols since sampling location and technique can affect concentration. Ligation is needed before the collection of femoral blood samples, and it is suggested that venous blood be used, though there is currently no protocol requiring this (Cook et al., 2000). Sample preservation and storage should also be standardized for optimal results.

Since blood samples are not available in all cases, as shown by the 70% availability during analysis at the FAA, it is vital to not only be able to detect and quantify concentrations in alternative specimens but to be able to relate that concentration back to blood (Lewis et al., 2013). While research is typically only concerned with toxic levels of antidepressants, the list of side effects associated with these drugs shows the potential to be contributing factors to fatality accidents, illustrating the need for analysis at therapeutic levels. With depression and anxiety disorders being amongst the leading diseases and SSRIs being some of the most prescribed medications in the United States, further research on the detection and quantitation of these drugs in alternative biological matrices is worthwhile.

Methods and Materials

Chemicals and Reagents

Methanolic standards of each antidepressant at a concentration of 1,000,000 ng/mL, except norsertraline at 100,000 ng/mL, were purchased from Cerilliant for calibrators. Isotopically labeled methanolic standards were purchased from Cerilliant at a concentration of 100,000 ng/mL for use as internal standards. For control solutions, 1,000,000 ng/mL methanolic standards for each drug and metabolite were purchased from Lipomed Pharmaceuticals and a 100,000 ng/mL Amitriptyline N-beta-D-glucuronide was purchased from Cerilliant. A list of these drugs along with their therapeutic ranges in blood/plasma can be found in Table 1. Therapeutic drug values were taken from Schulz (2020) Critical Care guide. In some cases, the therapeutic concentration of the active metabolite was not listed or was listed in conjunction with its parent metabolite. Type 1 deionized water (DW) from a Milli-QT_{plus} Water System was used for all aqueous solutions. Bovine whole blood was used as a diluent in all calibrators and controls. LC/MS grade Methanol, formic acid and acetonitrile (ACN) were purchased from Fisher Scientific.

Table 1.

List of Antidepressants and Isotopically Labeled Standards

Compound	Concentration (ng/mL)	Manufacturer	Therapeutic Range (ng/mL in blood/plasma)
Citalopram	1,000,000	Cerilliant/Lipomed	50-110
N-Desmethylcitalopram	1,000,000	Cerilliant/Lipomed	N/A
Sertraline	1,000,000	Cerilliant/Lipomed	10-150
Norsertraline	100,000	Cerilliant/Lipomed	10-150
Bupropion	1,000,000	Cerilliant/Lipomed	10-20
Hydroxybupropion	1,000,000	Cerilliant/Lipomed	550-1500
Amitriptyline	1,000,000	Cerilliant/Lipomed	50-300

List of Antidepressants and Isotopically Labeled Standards

Compound	Concentration (ng/mL)	Manufacturer	Therapeutic Range (ng/mL in blood/plasma)
Amitriptyline N-beta-D-glucuronide	100,000	Cerilliant	N/A
Nortriptyline	1,000,000	Cerilliant/Lipomed	70-170
Clomipramine	1,000,000	Cerilliant/Lipomed	90-250
Desmethylclomipramine	1,000,000	Cerilliant/Lipomed	160-180
Trazodone	1,000,000	Cerilliant/Lipomed	700-1000
Chlorophenylpiperazine (mCPP)	1,000,000	Cerilliant/Lipomed	N/A
Paroxetine	1,000,000	Cerilliant/Lipomed	2-65
Citalopram-d_6	100,000	Cerilliant	_____
N-Desmethylcitalopram-d_3	100,000	Cerilliant	_____
Sertraline-d_3	100,000	Cerilliant	_____
Buproprion-d_9	100,000	Cerilliant	_____
Hydroxybuproprion-d_6	100,000	Cerilliant	_____
Amitriptyline-d_3	100,000	Cerilliant	_____
Nortriptyline-d_3	100,000	Cerilliant	_____
Clomipramine-d_3	100,000	Cerilliant	_____
Desmethylclomipramine-d_3	100,000	Cerilliant	_____
Traxodone-d_6	100,000	Cerilliant	_____
Chlorophenylpiperazine-d_8 (mCPP-d_8)	100,000	Lipomed	_____
Paroxetine-d_6	100,000	Cerilliant	_____

Note. Internal standards do not have a listed therapeutic range as they are used to combat matrix effects during analysis.

Optimization

The antidepressants and internal standards were optimized for Ultra-Performance Liquid Chromatography and Tandem Mass Spectrometry (UPLC/MS-MS) analysis using direct infusion. For each antidepressant, a concentration of 100 ng/mL in methanol (MeOH) was created. For internal standards, a concentration of 1000 ng/mL in MeOH was created. These samples were placed on the Waters Acquity UPLC/MS-MS and data was processed using

MassLynx software searching for a quantifier ion and up to four qualifier ions. When possible, high mass ions were selected to reduce interference. The retention times and ions determined by this process were used as the beginning parameters for this research experiment and are shown in Table 2.

Table 2.

Mass Spectrometer Parameters

Compound	Retention Time (min)	Cone Voltage (V)	Precursor Ion (m/z)	Product Ions (m/z)	Collision Energy (eV)
Amitriptyline	2.06	28	278.1	91.0 104.9 116.9 232.9*	10 11 13 13
Amitriptyline-D_3	2.06	24	281.3	90.8* 104.9 116.9	22 22 22
Bupropion	0.96	14	240.1	130.9 166.2 184.0*	24 16 10
Bupropion-D_9	0.96	22	249.2	131.1 166.7 185.0*	26 18 12
Citalopram	1.45	30	325.1	109.0 115.9* 262.1	10 17 11
Citalopram-D_6	1.45	30	331.1	108.9* 116.0 262.1	24 24 18
Clomipramine	2.44	14	315.2	58.1* 86.1 191.8 227.0 242.0	22 40 52 40 24
Clomipramine-D_3	2.44	20	320.2	191.9 229.0* 244.0	50 40 26
N-Desmethylcitalopram	1.39	24	311.0	108.9* 116.0 262.1	10 15 9
N-Desmethylcitalopram-D_3	1.39	24	314.2	108.9* 115.9 262.1	20 22 16

Compound	Retention Time (min)	Cone Voltage (V)	Precursor Ion (m/z)	Product Ions (m/z)	Collision Energy (eV)
Desmethylclomipramine	2.36	10	301.2	191.9 227.0 242.1*	46 38 24
Desmethylclomipramine-D_3	2.36	20	306.2	191.9 229.0 244.1*	46 38 22
Hydroxybupropion	0.69	4	256.1	131.4 139.0 238.1*	26 28 12
Hydroxybupropion-D_6	0.69	24	262.2	131.1 139.0* 166.9	28 26 20
mCPP (Chlorophenylpiperazine)	0.70	2	197.0	44.1* 56.0 197.4	20 20 25
mCPP (Chlorophenylpiperazine)-D_8	0.70	6	205.0	48.5 123.0 158.0*	20 26 18
Norsertraline	2.21	24	275.1	91.0 123.2 129.0*	12 44 14
Nortriptyline	1.99	36	264.2	90.9 104.9 117.2* 233.1	10 10 20 7
Nortriptyline-D_3	1.99	32	267.2	90.8 104.9* 116.9	20 18 20
Paroxetine	1.76	44	330.2	70.1 151.0 192.2*	28 22 20
Paroxetine-D_6	1.76	20	336.2	153.0 182.0 198.1*	22 24 20
Sertraline	2.31	28	306.0	128.9 159.0* 275.01	20 30 10
Sertraline-D_3	2.31	20	311.1	128.9 160.9* 277.0	20 26 12
Trazodone	1.04	20	372.2	95.9 148.0* 176.0	45 15 10

Compound	Retention Time (min)	Cone Voltage (V)	Precursor Ion (m/z)	Product Ions (m/z)	Collision Energy (eV)
Trazodone-D_6	1.04	20	378.2	149.9 154.0 182.1*	34 32 24

* Transition ion used for quantitation

Calibrators and Controls

Due to the wide range of therapeutic values for the drugs in this method, a mixed concentration calibration stock solution was created using Cerilliant antidepressant standards in DW. This solution was created to encompass drug concentrations down to at least half of the lowest therapeutic level and at least ten times the upper therapeutic level. The initial concentration of the stock solution was created at 2000 times the lowest (cutoff) concentration desired (2000c). This process was repeated using Lipomed standards to create a control stock solution. An internal standard mixed concentration stock solution was created in DW. Initial stock solution concentrations for each drug and internal standard are given in Table 3. Bovine whole blood was used as the diluent in all calibrators and controls. An antidepressant calibration curve was built using serial dilutions of the 2000c concentrations as shown in Table 4, to give an LDR of 200c. Low, medium, high and negative quality control (QC) samples were created by serial dilution as seen in Table 5.

Table 3.

Stock Solution Concentrations

Drug	Initial Water Stock Concentration (ng/mL)	LDR (ng/mL)	Paired Internal Standard	Internal Standard Stock Concentration (ng/mL)
Amitriptyline	20,000	10-2,000	Amitriptyline D_3	1,000
Nortriptyline	20,000	10-2,000	Nortriptyline D_3	1,000
Bupropion	2,000	1-200	Bupropion D_9	100

Drug	Initial Water Stock Concentration (ng/mL)	LDR (ng/mL)	Paired Internal Standard	Internal Standard Stock Concentration (ng/mL)
Hydroxybupropion	2,000	1-200	Hydroxybupropion D_6	100
Citalopram	20,000	10-2,000	Citalopram D_6	1,000
N-Desmethylcitalopram	20,000	10-2,000	N-Desmethylcitalopram D_3	1,000
Clomipramine	20,000	10-2,000	Clomipramine D_3	1,000
Desmethylclomipramine	20,000	10-2,000	Desmethylclomipramine D_3	1,000
Norsertraline	10,000	5-1,000	Sertraline D_3	500
Sertraline	10,000	5-1,000	Sertraline D_3	500
Paroxetine	2,000	1-200	Paroxetine D_6	100
Trazodone	100,000	50-10,000	Trazodone D_6	5,000
mCPP	20,000	10-2,000	mCPP D_8	1,000

Table 4.

Antidepressant Calibration Curve

Calibrator (multiple of cutoff)	**Volume of Calibrator (µL)**	**Volume of Blood (µL)**
200	200	1800
100	1000	1000
50	1000	1000
25	1000	1000
10	800	1200
5	1000	1000
2	800	1200
1	1000	1000

Note. Begin with 2000c calibrator stock. Use next highest calibrator for serial dilution.

Table 5.

Antidepressant Quality Controls (QC)

QC (multiple of cutoff)	Volume of QC (µL)	Volume of Blood (µL)
80	80	1920
20	250	750
3	150	850
Negative	0	500

Note. Begin with 2000c control stock. Use next highest quality control for serial dilution

To prepare the samples, separate 500 µL aliquots of each calibrator and control were taken and 50 µL of the internal standard solution was added to each tube. A "crash and shoot extraction" was then performed by adding 3 mL of ice cold 50:50 ACN:MeOH to each sample, vortexing for thirty seconds, allowing samples to sit at room temperature for ten minutes, and centrifuging for ten minutes at 1500 x *g*. Following centrifugation, a 300 µL aliquot of each supernatant was added to a 0.2 µm Thomson PTFE filter vial for analysis.

Ultra-Performance Liquid Chromatography and Tandem Mass Spectrometry (UPLC/MS-MS) Conditions

Analysis was performed using a Waters Xevo TQ-S Acquity UPLC. An Acquity UPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm) was used at a temperature of 60°C. Mobile Phase A (MPA) consisted of 0.1% formic acid in DW, while Mobile Phase B (MPB) was 0.1% formic acid in ACN. The flow rate was set to 0.70 mL/min with a six second needle pre-wash and a 10 second post-wash. Ramp parameters were tested using a 50 ng/mL dilution of the antidepressant stock solution in MeOH. Method parameters were initially set at 20% MPB to a gradient of 40% MPB over 2.5 minutes. This was then raised to 95% MPB over 0.49 minutes before immediately

dropping back to the starting conditions for a run time of three minutes. An autosampler injection volume of 1 µL was chosen and the autosampler temperature was held at 10°C. Waters MassLynx MS software was used for analyte detection and quantitation.

Method Validation

Calibrators and blood controls were prepared as above. Bovine serum and human urine controls followed the blood control preparation protocol. Human tissue samples of brain, liver, lung, and muscle were weighed out at a 1:2 ratio of tissue to DW and homogenized using an Omni Bead Ruptor bead mill homogenizer. Antidepressant tissue controls were created by aliquoting 1.5 g of tissue homogenate (equivalent to 0.5 g of tissue), adding the Lipomed control stock solution referenced above to create low, medium, and high-level controls for each, then adding a 50 µL aliquot of the previously created internal standard stock solution. All calibrators and controls were prepared using the "crash and shoot" method described in the preceding sections. Method validation was completed according to FAA protocol, which is a modified version of ANSI/ASB Standard 036. This validation included the following tests using bovine blood unless otherwise noted and are briefly described below: calibration model, LOD for blood and tissues, bias and precision for blood and tissues, carryover, refrigerator stability, freeze/thaw stability, process stability, ion suppression, recovery, process efficiency, hydrolysis efficiency, dilution integrity, cross contribution, drug interference, and determination of the measurement of uncertainty.

For the calibration model, calibration curves were created as above on five separate days and analyzed. An LOD study was performed by creating blood controls from 0.0625c – 1c and analyzing three samples of each concentration in duplicate on three separate days. Bias and precision were tested by analyzing five controls of each tissue, fluid, and blood at the

concentrations listed in Table 4; bias and precision was examined over 5 days for blood and on 1 day for all other matrices. Carryover was tested by analyzing each drug five times using the highest calibrator, followed by a water blank, then low calibrator and observing the analyte peak areas. This process was then repeated using a concentration ten times the highest calibrator.

The refrigerator, freeze/thaw, and process stability studies were performed for five consecutive days. A calibration curve and five blood controls at low, middle, and high concentrations were created and analyzed on Day 1. The blood controls from Day 1 were kept on the instrument throughout the study and analyzed each day against a fresh curve. Process stability was tested by comparing these daily concentrations against their initial value on Day 1. Refrigerator stability was tested by adding the antidepressant control stock to create 20 blood controls at each level and storing them in the refrigerator. Starting on Day 2, five samples of each control concentration were pulled from the refrigerator, IS was then added and the "crash and shoot" extraction was performed as above. These samples were analyzed using a fresh calibration curve each day. For the freeze/thaw study, the refrigerator study protocol was performed with the exception of storing samples in the freezer and completely thawing all remaining samples each day before refreezing. Concentrations from each day of the refrigerator and freeze/thaw study were compared to the initial Day 1 control concentrations to determine stability.

Ion suppression and enhancement, recovery, and process efficiency were tested at the high and low control concentrations. For each concentration, ten samples of each type and five "neat" samples containing 50:50 ACN:MeOH were aliquoted. The appropriate amount of antidepressant stock solution and IS were added (pre-spiked) to five samples of each type. An extraction was performed on all ten samples, spiked and unspiked, of each type. Following the

extraction, antidepressant and IS were added (post-spiked) to the remaining five unspiked samples, as well as the five "neat" samples. Peak areas of the analyte and IS quantitation ions of the post-spiked and neat samples were analyzed to determine any ion suppression or enhancement due to matrix effects. To test recovery, analyte and IS quantitation ion peak areas of the pre and post-spiked samples were compared to each other. Process efficiency was analyzed by comparing the analyte and IS quantitation peak areas of the neat and pre-spiked samples.

Urine hydrolysis was performed for amitriptyline only, by aliquoting 500 µL of a 200 ng/mL amitriptyline N-beta-D-glucuronide in urine solution, adding 100 µL of IMCSzyme recombinant β-glucuronidase (activity > 50 kU/mL), 200 µL of IMCS Rapid Hydrolysis Buffer (pH=6.8), 50 µL of 1 µg/mL IS, vortexing for 30 seconds, and incubating at 60°C for one hour. Following incubation, this method's extraction process was carried out as normal.

Dilution integrity was analyzed at ratios of 1:1, 1:10, and 1:100 for all analytes by creating and analyzing six blood samples of each ratio. A cross contribution study was performed by analyzing all drug and internal standards individually at a concentration of approximately 100 ng/mL in 50:50 ACN:MeOH and monitoring all drug ions for false-positives. Drug interference was tested by creating a high concentration stock solution of commonly encountered drugs not found in this study including acetaminophen, alprazolam, amlodipine, caffeine, cotinine, diazepam, diphenhydramine, ibuprofen, lamotrigine, naproxen, quetiapine, and THC. This stock solution was added to five mid-level blood controls and compared to five controls without the interference mix. Based on the data obtained during the validation process, the measurement of uncertainty for each analyte was calculated using Excel.

Phase II – Postmortem Distribution Study

Sample Selection and Storage

A case search was performed using the FAA Civil Aerospace Medical Institute (CAMI) toxicology laboratory database in Oklahoma City, OK. Aviation accident cases in the last five years (Sept. 2017- Sept. 2022) with known positive blood results for the antidepressants of interest were selected. To further narrow down results, cases were filtered by the number of desired tissue specimens (liver, lung, kidney, spleen, muscle, brain, heart, urine, vitreous humor (VH) and bile) available. Based on these results, cases featuring known positive blood samples for citalopram and N-desmethylcitalopram were selected for the distribution study. All personal identifying information was kept anonymous from the researchers. Due to the lack of personal identifying information, and the samples being from deceased individuals, the University of Central Oklahoma Institutional Review Board determined this study did not contain human research and was not subject to their oversight. Blood samples were stored in 1.0% and 2.0% (w/v) sodium fluoride and potassium oxalate at -20°C until analysis. All tissue specimens were stored unaltered at -20°C until analysis.

Sample Preparation and Extraction

Calibrators and controls were prepared as described in the above sections. For each unknown case sample, 50 µL of internal standard was added to 0.5 mL of liquid sample or 1.5g of tissue homogenate. A "crash and shoot" extraction was performed using 3 mL of cold 50:50 ACN:MeOH. Calibrators, controls, and samples were vortexed for 30 seconds, allowed to sit at room temperature for 10 mins., then centrifuged at 1500 x *g* for 10 mins., and 300 µL were transferred to a Thomson PTFE filter vial for UPLC/MS-MS analysis using the instrument parameters listed in the previous sections.

Data Analysis

During optimization, a high mass quantitation ion with a minimum signal-to-noise ratio of 10 was selected for each drug and internal standard. A minimum of two qualification ions with minimum signal-to-noise ratios of 10 were also selected for each. Response factors were determined using quantitation ions by dividing the area of the analyte quantitation peak by the internal standard peak. Calibrators were required to be within ± 20% of the target concentration value to be accepted. Controls were also required to be within ± 20% of the target concentration value and were analyzed with all specimens at the beginning and end of runs to ensure accuracy and precision. Quantitation was performed using an internal standard calibration method. A calibration curve was created by plotting the response factor versus the calibrator analyte concentration and determining the best line of fit. This calibration curve was used to determine the concentration of each control and specimen sample. For the distribution portion of the study, the response factor from each fluid and tissue sample was compared against the response factor of the blood calibration curve for that case and the concentration ratio was determined. The ratios for each like matrix amongst the cases were compared and analyzed for any trend that could relate alternate matrix drug concentration back to blood.

Results and Discussion

Method Validation

Calibration curves for all analytes had a quadratic fit type with a $1/x^2$ weighting. Average concentrations, %CV, and R^2 values from the calibration curve model for each analyte are given in Table 6 and the LOD in blood is shown in Table 7. All R^2 values for the calibration model were over 0.999, indicating good fit. The overall %CV was required to be under 20% and the LOD was required to be no greater than the lowest calibrator concentration. All analytes met this requirement except for Norsertraline in the LOD study. On day two of the LOD study, the intrarun peak areas for norsertraline were consistent, but were noticeably lower than the other two days, indicating instrument variability for that day. However, since norsertraline's %CV for all three days of the study were well under 20% (10.78, 15.18, 16.92), it was deemed acceptable to use 1 ng/mL (1c) as the LOD.

Table 6.

Average Concentration and % CV of Five-Day Calibration Curve Model

Analyte	200c		100c		50c		25c		10c		5c		2c		1c		Curve
	Avg. Conc. (ng/mL)	%CV	Avg. Conc. (ng/mL)	%CV	Avg. Conc. (ng/mL)	%CV	Avg. Conc. (ng/mL)	%CV	Avg. Conc. (ng/mL)	%CV	Avg. Conc. (ng/mL)	%CV	Avg. Conc. (ng/mL)	%CV	Avg. Conc. (ng/mL)	%CV	R^2
Amitriptyline	1987.1	0.5	1023.8	1.5	495.7	0.8	244.5	2.7	97.9	1.6	49.1	2.8	20.2	2.7	10.4	2.9	0.9998
Nortriptyline	1954.2	4.6	1003.4	0.4	495.0	1.2	251.1	0.8	101.5	3.1	49.7	2.4	20.7	1.4	9.6	2.9	0.9999
Bupropion	199.9	0.5	99.9	2.3	50.4	1.4	25.0	3.8	9.8	3.8	4.9	3.4	2.0	7.3	1.0	4.3	0.9998
Hydroxybupropion	199.6	1.0	101.1	4.5	48.3	5.4	25.6	7.9	10.2	8.4	5.3	0.7	2.0	6.0	0.9	6.9	0.9991
Citalopram	1997.0	0.2	1008.4	1.1	494.0	1.7	250.4	2.0	100.1	2.0	50.1	3.0	20.2	3.6	9.9	1.9	0.9999
N-Desmethylcitalopram	1988.4	-0.6	1015.3	1.5	500.1	0.0	247.0	-1.2	97.8	-2.2	48.4	-3.3	20.3	1.7	10.4	4.0	0.9996
Clomipramine	1998.1	-0.1	1003.7	0.4	496.9	-0.6	252.1	0.8	99.5	-0.5	49.1	-1.9	21.0	4.7	9.7	-2.9	0.9998
Desmethylclomipramine	1995.6	-0.2	1014.4	1.4	480.5	-3.9	259.9	5.5	98.6	3.3	51.2	8.8	20.0	9.9	9.8	12.0	0.9991
mCPP	2003.0	0.2	992.2	0.8	502.3	1.5	251.1	3.3	101.1	2.3	50.6	2.2	20.0	1.0	9.8	5.3	0.9999
Paroxetine	200.6	0.4	98.5	2.4	50.3	2.5	25.3	1.4	10.3	4.6	5.0	5.2	1.9	3.2	1.0	4.2	0.9998
Sertraline	1003.7	0.4	491.4	2.1	251.1	3.1	126.2	5.4	52.7	2.7	25.2	6.6	10.0	6.0	4.7	7.0	0.9997
Norsertraline	199.8	0.7	100.1	3.4	50.7	3.4	225.1	6.6	9.4	5.6	4.9	2.6	2.0	8.7	1.1	8.0	0.9995
Trazodone	9936.1	1.0	5109.5	2.9	2481.8	0.9	1225.4	3.7	492.1	3.1	240.9	3.0	104.9	4.8	50.7	9.7	0.9997

Table 7.

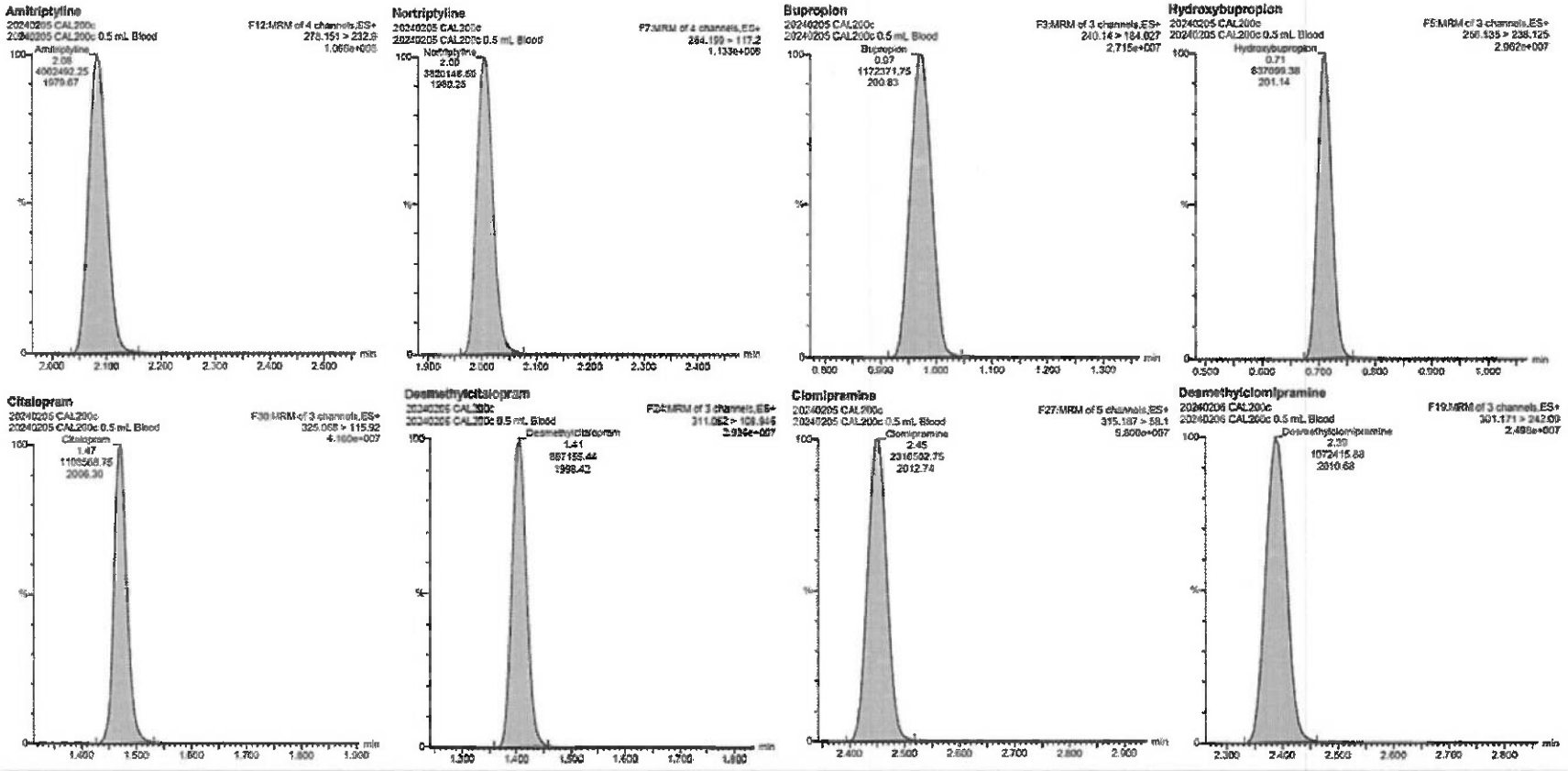
Limit of Detection (LOD) in Blood

Analyte	LOD Concentration (ng/mL)	Average Peak Area	% CV
Amitriptyline	5	9043.00	9.60
Nortriptyline	5	12,496.64	16.05
Bupropion	1	4313.87	15.63
Hydroxybupropion	1	3236.28	17.30
Citalopram	5	3120.60	16.15
N-Desmethylcitalopram	2.5	1233.84	16.45
Clomipramine	10	10,568.89	17.64
Desmethylclomipramine	10	6054.89	16.86
mCPP	10	6000.46	18.36
Paroxetine	1	1852.75	16.03
Sertraline	5	5036.04	18.35
Norsertraline	1	1764.09	25.41
Trazodone	6.25	1208.38	19.22

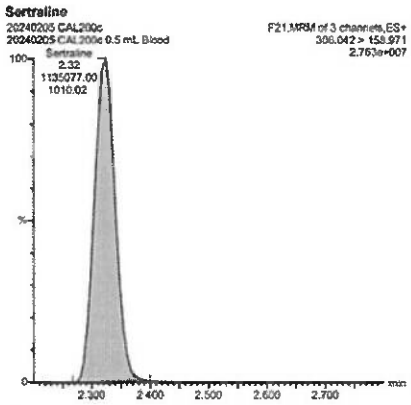
Figure 6 illustrates the chromatography of the quantitation ion peak for each drug, showing that no drugs in the method overlap in their elution. While hydroxybupropion and mCPP have similar retention times, this was not an issue due to the large difference in the m/z of the transition ions with hydroxybupropion having a m/z 256.1 > 238.1, and mCPP having a m/z 197.0 > 44.1.

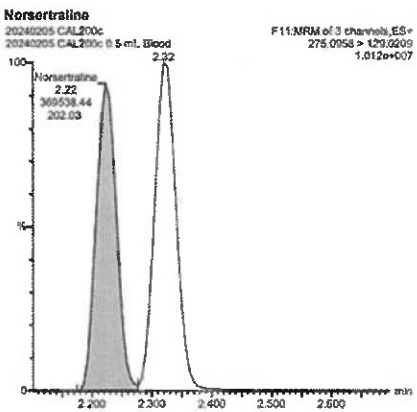
Figure 6.

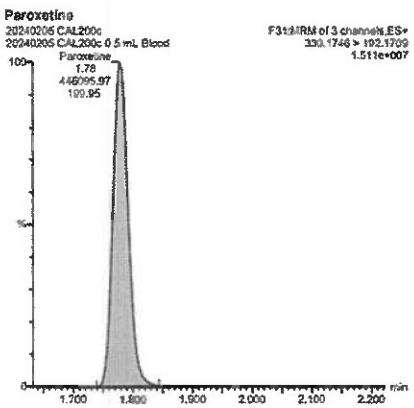
Chromatography of Quantitation Ions

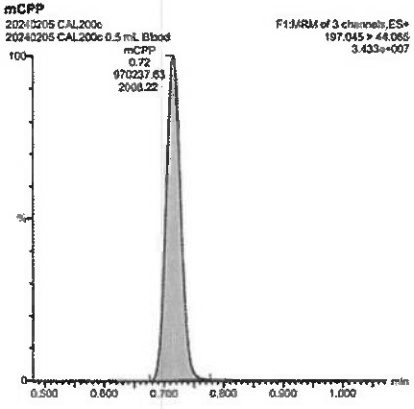


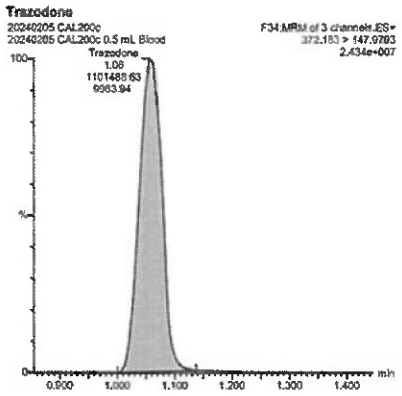
Chromatography of Quantitation Ions











Carryover was calculated based on the percentage of the peak area detected in the blank compared to the peak area of the low calibrator, with <20% being deemed acceptable. No analytes had any detectable peak areas in the water blanks and therefore had no carryover. Cross contribution of analytes in this method were determined by running each drug and internal standard separately and monitoring all other drug ions for peaks as signs of potential contamination or cross contribution. Due to in-source fragmentation, the quantitation peak for sertraline is detected along with norsertraline. However, there is a clear distinction between the two peaks, with a difference in the fragment masses and retention times, and was therefore deemed not to be an issue. No drugs or internal standards showed true peaks for any other ions, so it was determined that cross contribution was not a factor in this method. The dilution integrity study found that all analytes produced accurate concentrations at dilutions up to 1:100 as shown in Table 8. Drug interference results are listed in Table 9 and showed that the only drug to experience effects was sertraline, which had slightly enhanced concentration in the presence of the interference mix, indicated by a % error greater than 20. No false positives were observed for any drug, including sertraline.

Table 8.

Dilution Integrity

Analyte	1:1 Dilution			1:10 Dilution			1:100 Dilution		
	Target (ng/mL)	Avg. Conc. (ng/mL)	%CV	Target (ng/mL)	Avg. Conc. (ng/mL)	%CV	Target (ng/mL)	Avg. Conc. (ng/mL)	%CV
Amitriptyline	2000	2087	1.7	2000	2134.3	2.5	20,000	20,474.20	2.4
Nortriptyline	2000	2016.1	3.7	2000	2120.2	2.9	20,000	17,840.60	2.2
Bupropion	200	203.1	1.2	200	213.8	2.5	2000	2008.8	2.8
Hydroxybupropion	200	201.5	1.8	200	211.5	4.1	2000	1992.6	3
Citalopram	2000	2025.3	1.5	2000	2116.4	3.3	20,000	21,797.40	1.9
N-Desmethylcitalopram	2000	2037.9	1.9	2000	2132.7	3.4	20,000	19,918.40	3.5
Clomipramine	2000	2097.2	4	2000	2006.5	2.2	20,000	18,793.40	3.1
Desmethylclomipramine	2000	2127.3	4.8	2000	2295.6	3.1	20,000	19,147.80	10.7

Analyte	1:1 Dilution			1:10 Dilution			1:100 Dilution		
	Target (ng/mL)	Avg. Conc. (ng/mL)	%CV	Target (ng/mL)	Avg. Conc. (ng/mL)	%CV	Target (ng/mL)	Avg. Conc. (ng/mL)	%CV
mCPP	2000	2042.1	2	2000	2127.2	2.4	20,000	19,332.20	3
Paroxetine	200	203.5	2.8	200	199.7	4.2	2000	1827.8	4.7
Sertraline	1000	1016.2	2.8	1000	1040.9	4.5	10,000	9082.4	6.1
Norsertraline	200	195.1	2.2	200	211.6	2.5	2000	1805	6.4
Trazodone	10,000	10,317.80	1.4	10,000	11,001.60	2.2	100,000	109,876.40	5.1

Table 9.

Drug Interference Study

Analyte	Target Concentration (ng/mL)	Concentration w/Interference Mix (ng/mL)	% CV	% Error
Amitriptyline	200	214.70	0.90	7.35
Nortriptyline	200	186.85	1.84	-6.58
Bupropion	20	16.99	0.84	-15.05
Hydroxybupropion	20	18.59	6.13	-7.07
Citalopram	200	193.81	1.92	-3.10
N-Desmethylcitalopram	200	237.81	0.79	18.90
Clomipramine	200	201.09	2.34	0.55
Desmethylclomipramine	200	178.89	5.66	-10.55
mCPP	150	147.56	1.33	-1.63
Paroxetine	20	19.32	6.51	-3.39
Sertraline	100	123.90	3.90	23.90
Norsertraline	20	17.71	3.29	-11.44
Trazodone	1000	858.23	1.45	-14.18

Bias and precision were analyzed over five separate runs with all results having a % CV and % error <20% as summarized in Table 10. Table 11 includes the results for the fluid and tissue controls. For urine, serum, and tissues, if the low QC concentration (3c) could not be reliably replicated, an apparent LOD study was conducted at a higher concentration as noted. Analytes with apparent LODs for tissues and fluids included bupropion at 5 ng/mL for liver, lung, and muscle, hydroxybupropion at 10 ng/mL for lung and 20 ng/mL for all other tissues and

fluids, N-desmethylcitalopram at 50 ng/mL for brain, lung, and muscle, and 100 ng/mL for liver, mCPP at 50 ng/mL for lung and muscle, and 100 ng/mL for brain and liver, sertraline at 25 ng/mL for lung, and norsertraline at 5 ng/mL for brain, liver, lung, and muscle.

Table 10.

Bias and Precision

Analyte	QC - 3c		QC - 20c		QC - 80c	
	Average Conc. (ng/mL)	% CV	Average Conc. (ng/mL)	% CV	Average Conc. (ng/mL)	% CV
Amitriptyline	28.46	8.75	197.46	6.43	840.47	5.30
Nortriptyline	28.41	10.19	198.91	7.29	793.86	7.99
Bupropion	3.18	6.27	22.14	5.75	89.36	4.46
Hydroxybupropion	2.67	6.11	18.85	7.54	74.78	9.52
Citalopram	28.05	5.11	198.31	4.69	816.02	3.35
N-Desmethylcitalopram	37.63	1.52	169.73	1.41	956.47	0.18
Clomipramine	28.49	6.32	202.76	9.79	800.11	7.05
Desmethylclomipramine	27.30	9.51	190.77	6.52	760.93	7.35
mCPP	25.15	3.62	179.67	4.23	722.22	4.29
Paroxetine	2.66	7.89	19.06	5.51	77.70	4.74
Sertraline	14.48	8.46	102.18	9.02	416.03	8.32
Norsertraline	2.72	10.23	18.14	8.70	72.75	7.26
Trazodone	143.10	5.94	986.08	7.98	3971.54	5.71

Table 11.

Fluid and Tissue Control Study

Analyte	QC - 3c											
	Brain		Liver		Lung		Muscle		Serum		Urine	
	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV
Amitriptyline	33.09	3.18	33.48	2.79	33.10	2.79	33.25	1.38	32.03	1.05	32.18	1.89
Nortriptyline	30.82	2.96	30.75	2.95	30.11	4.51	30.82	2.96	30.84	1.97	30.82	2.10
Bupropion	3.40	1.65	See Apparent LOD		See Apparent LOD		See Apparent LOD		3.24	2.46	3.29	2.39
Hydroxybupropion	See Apparent LOD		See Apparent LOD		See Apparent LOD		See Apparent LOD		3.12	3.45	See Apparent LOD	
Citalopram	31.08	2.60	33.05	2.31	32.31	3.37	32.52	1.22	31.48	1.80	31.70	1.69
N-Desmethylcitalopram	See Apparent LOD		See Apparent LOD		See Apparent LOD		See Apparent LOD		32.58	2.30	32.85	1.84
Clomipramine	31.10	3.18	31.53	7.02	29.87	5.92	30.29	3.18	31.25	1.73	31.39	3.20
Desmethylclomipramine	31.47	3.22	31.94	4.61	30.96	6.68	32.46	5.06	31.32	7.25	32.55	3.55
mCPP	See Apparent LOD		See Apparent LOD		See Apparent LOD		See Apparent LOD		30.13	1.44	29.30	2.28
Paroxetine	2.98	7.55	3.06	6.73	3.12	11.77	3.19	5.69	3.21	10.48	3.08	8.26
Sertraline	17.38	4.78	17.33	7.44	See Apparent LOD		14.44	8.46	15.30	5.05	16.18	4.45
Norsertraline	See Apparent LOD		See Apparent LOD		See Apparent LOD		See Apparent LOD		3.38	6.27	3.05	8.01
Trazodone	165.72	8.56	176.41	7.49	170.23	4.82	176.50	0.96	157.50	0.73	163.59	3.11

QC - 20c

Analyte	Brain		Liver		Lung		Muscle		Serum		Urine	
	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV
Amitriptyline	171.95	1.07	168.25	2.65	170.78	1.83	167.00	2.64	170.99	1.33	171.71	1.78
Nortriptyline	162.21	0.79	161.33	1.38	162.63	1.55	161.39	0.54	161.88	1.94	161.06	0.38
Bupropion	23.91	12.30	22.52	3.88	21.62	0.34	21.56	0.40	21.36	2.09	21.26	1.61
Hydroxybupropion	20.76	5.33	19.05	11.68	19.81	4.40	19.31	8.88	19.33	2.88	20.39	10.00
Citalopram	180.62	0.39	160.76	0.50	161.56	0.62	185.16	1.37	178.55	2.77	162.11	1.01
N-Desmethylcitalopram	177.41	1.25	175.04	2.03	170.27	1.91	169.73	1.41	177.55	2.66	176.88	2.12
Clomipramine	177.08	3.59	174.56	2.23	185.15	5.36	177.62	6.05	175.81	2.76	171.57	4.82
Desmethylclomipramine	204.37	10.68	194.68	5.62	205.61	4.96	200.88	7.08	203.52	5.38	205.46	5.39
mCPP	194.84	1.39	192.42	2.07	193.90	1.52	192.02	2.73	189.69	0.57	193.24	2.97
Paroxetine	16.57	3.93	17.28	4.58	16.54	3.71	19.50	5.70	19.84	2.33	16.49	3.31
Sertraline	88.96	5.98	89.98	7.25	91.34	4.38	84.39	4.77	85.86	4.02	86.18	4.89
Norsertraline	17.69	5.30	18.65	6.23	18.69	3.78	16.58	3.96	19.07	3.80	18.71	6.49
Trazodone	930.28	1.59	903.70	2.01	888.87	1.38	883.06	1.63	925.10	1.80	933.66	1.77

QC - 80c

Analyte	Brain		Liver		Lung		Muscle		Serum		Urine	
	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV
Amitriptyline	859.39	1.61	858.60	0.80	859.21	0.72	874.48	1.33	852.59	0.95	856.88	1.41
Nortriptyline	836.97	1.32	842.13	2.38	831.92	1.22	823.28	2.29	838.56	0.82	835.67	1.53
Bupropion	84.51	1.54	83.17	2.01	84.76	1.65	81.30	2.90	83.85	0.77	84.87	1.55
Hydroxybupropion	86.50	0.71	86.49	6.60	83.19	2.95	76.25	10.53	84.31	4.16	91.23	1.80
Citalopram	843.46	0.84	788.86	2.22	852.93	1.09	869.56	0.78	848.92	0.75	848.50	1.07
N-Desmethylcitalopram	904.92	0.18	943.32	0.93	946.62	0.65	956.47	0.18	878.00	0.72	885.13	0.29

Clomipramine	836.09	3.25	832.61	7.04	852.37	2.94	847.63	3.78	859.65	2.76	847.04	3.80
Desmethylclomipramine	765.38	1.81	837.51	7.89	861.34	5.51	830.31	5.45	797.79	2.56	802.28	2.46
mCPP	835.98	2.33	798.79	1.20	819.29	2.33	811.66	0.86	825.37	0.91	826.20	1.87
Paroxetine	83.27	7.13	67.61	3.77	81.04	6.11	68.73	3.77	82.15	4.19	83.14	3.67
Sertraline	402.97	4.57	391.04	3.75	405.05	3.50	403.24	2.77	420.10	3.55	407.32	4.27
Norsertraline	70.98	8.88	67.95	3.19	75.82	2.78	67.31	3.15	83.67	2.21	85.23	5.43
Trazodone	4457.79	1.17	4604.64	1.37	4634.36	1.07	4647.35	0.81	4241.89	0.72	4377.04	5.99

Apparent LOD

Analyte	Brain		Liver		Lung		Muscle		Serum		Urine	
	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV	Average Conc. (ng/mL)	%CV
Amitriptyline	NA		NA		NA		NA		NA		NA	
Nortriptyline	NA		NA		NA		NA		NA		NA	
Bupropion	NA		5.75	1.11	4.83	2.72	5.27	2.33	NA		NA	
Hydroxybupropion	20.76	5.33	19.05	11.68	10.51	9.19	19.31	8.88	19.33	2.88	20.39	10.00
Citalopram	NA		NA		NA		NA		NA		NA	
N-Desmethylcitalopram	54.43	1.73	118.55	0.64	53.45	5.32	59.64	0.31	NA		NA	
Clomipramine	NA		NA		NA		NA		NA		NA	
Desmethylclomipramine	NA		NA		NA		NA		NA		NA	
mCPP	107.25	2.06	98.84	1.08	46.53	2.39	47.79	2.70	NA		NA	
Paroxetine	NA		NA		NA		NA		NA		NA	
Sertraline	NA		NA		26.23	5.10	NA		NA		NA	
Norsertraline	4.78	5.32	5.14	6.83	4.41	3.34	5.13	10.28	NA		NA	
Trazodone	NA		NA		NA		NA		NA		NA	

Ion suppression/enhancement, also known as matrix effects (ME), was analyzed to determine how the sample matrix (blood, different tissues, etc.) may impact the analysis process, leading to suppressed or enhanced ions, potentially affecting the LOD, bias, and falsely altering determined concentrations. Peak areas for the drug and internal standard were analyzed for each matrix and compared to the determined peak ratio of neat samples, to establish a corrected ion suppression/enhancement and corrected % CV. For this method, the corrected ion suppression/enhancement had to be <25% and the % CV had to be <20% unless acceptance criteria were met during the fluid and tissue control study. A result of 0 indicates there is no matrix effect, while negative values indicate ion suppression and positive values indicate ion enhancement. Recovery at the low and high QC concentrations was tested by comparing peak areas of pre and post spiked samples to determine what percentage of the drug present is extracted in each matrix using this method. Recovery of 100% is not to be expected from an analytical method, and a corrected recovery of 75-125% with a CV <20% was deemed acceptable unless criteria was met during the tissue control study, and 100% indicating perfect recovery. Process efficiency (PE) compares the peak areas of pre-spiked samples to neat samples, and examines the overall efficiency of the method when accounting for the results of the ME and recovery studies. A result of 100% is considered perfect efficiency. If a drug had an apparent LOD for a specific tissue, the low QC was not included in the ME, Recovery, and PE studies, but may be evaluated at a later date. ME, Recovery, and PE results for all other drugs and tissues were deemed acceptable and are summarized in Table 12 as the corrected percentage, with tissues having an apparent LOD being listed as N/A for the low QC.

Table 12.

Matrix Effects, Recovery, and Process Efficiency

Matrix Effect

Analyte	Blood		Brain		Liver		Lung		Muscle		Serum		Urine	
	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)
Amitriptyline	2.40%	0.8%	4.20%	-0.3%	10.00%	1.9%	13.00%	-0.5%	11.00%	1.0%	3.50%	0.2%	1.00%	0.80%
Nortriptyline	1.7%	0.40%	0.60%	0.90%	-2.60%	1.20%	-1.30%	0.90%	-2.20%	1.80%	2.10%	-0.40%	-0.30%	-0.50%
Bupropion	0.40%	0.0%	6.00%	-1.50%	N/A	-1.50%	N/A	-0.50%	N/A	-2.30%	1.50%	-1.10%	0.40%	-1.60%
Hydroxybupropion	5.30%	0.20%	N/A	-1.10%	N/A	4.80%	N/A	5.70%	N/A	-9.30%	N/A	-0.70%	N/A	6.90%
Citalopram	-0.80%	-0.40%	3.20%	-1.10%	-1.80%	-6.80%	5.90%	0.00%	6.30%	2.30%	2.10%	-1.20%	-1.40%	-1.40%
N-Desmethylcitalopram	3.70%	-2.20%	N/A	1.20%	N/A	5.60%	N/A	2.40%	N/A	6.20%	0.4%	-2.00%	4.50%	-0.50%
Clomipramine	8.40%	-0.10%	7.30%	-1.80%	10.30%	-2.10%	10.70%	0.80%	6.50%	-4.80%	5.20%	-0.70%	10.20%	-0.60%
Desmethylclomipramine	-2.30%	-6.00%	-1.70%	-3.70%	-5.30%	-2.90%	-6.50%	0.10%	-4.00%	-4.50%	-3.20%	-5.90%	1.60%	-5.00%
mCPP	1.20%	-1.2%	N/A	-0.60%	N/A	-3.50%	N/A	-1.70%	N/A	-3.00%	4.10%	-2.70%	1.70%	-0.60%
Paroxetine	-0.30%	0.00%	4.60%	-5.70%	1.60%	-10.00%	4.30%	-4.20%	2.10%	-16.70%	3.20%	-3.50%	-1.00%	-3.00%
Sertraline	-0.50%	3.10%	7.80%	2.00%	5.70%	1.80%	N/A	5.40%	-5.20%	1.30%	-5.20%	-1.20%	2.90%	0.70%
Norsertraline	0.4%	-0.30%	N/A	-1.70%	N/A	-7.10%	N/A	0.30%	N/A	-66.10%	-4.10%	-1.30%	-6.00%	-5.10%
Trazodone	2.00%	0.40%	12.20%	3.30%	34.80%	6.10%	42.40%	4.90%	45.40%	6.40%	0.90%	-1.40%	0.40%	0.20%

Recovery Efficiency

Analyte	Blood		Brain		Liver		Lung		Muscle		Serum		Urine	
	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)
Amitriptyline	96.90%	95.5%	98.80%	96.70%	94.80%	94.50%	91.20%	96.90%	93.20%	97.10%	96.40%	95.40%	99.20%	95.40%
Nortriptyline	96.8%	99.7%	70.80%	98.90%	102.00%	99.30%	98.60%	98.50%	113.50%	96.60%	97.50%	100.50%	99.90%	100.20%
Bupropion	97.8%	97.4%	97.8%	100.80%	N/A	99.20%	N/A	100.10%	N/A	97.90%	97.20%	99.60%	99.60%	101.40%
Hydroxybupropion	103.1%	102.6%	N/A	104.80%	N/A	99.20%	N/A	93.90%	N/A	103.10%	N/A	101.20%	N/A	101.70%
Citalopram	99.60%	96.1%	95.40%	97.50%	107.30%	97.00%	96.50%	97.40%	96.80%	97.10%	97.70%	98.10%	101.90%	98.30%
N-Desmethylcitalopram	95.8%	93.2%	N/A	94.00%	N/A	93.60%	N/A	96.90%	N/A	94.30%	101.10%	94.50%	98.00%	93.50%
Clomipramine	96.20%	97.4%	95.90%	97.30%	94.40%	97.40%	89.40%	96.60%	94.20%	101.80%	98.30%	98.90%	94.50%	97.50%
Desmethylclomipramine	99.0%	102.5%	96.40%	92.70%	102.20%	100.00%	101.00%	100.00%	101.60%	101.40%	97.60%	98.80%	96.40%	98.50%
mCPP	99.90%	100.3%	N/A	101.00%	N/A	99.5%	N/A	100.10%	N/A	100.60%	99.80%	101.80%	99.70%	99.90%
Paroxetine	98.4%	94.1%	91.10%	102.20%	96.60%	87.30%	96.10%	98.20%	100.20%	96.00%	100.20%	98.90%	100.90%	99.50%
Sertraline	98.60%	96.1%	97.80%	94.70%	99.60%	92.00%	N/A	92.20%	94.10%	95.50%	99.00%	102.00%	95.90%	96.9%
Norsertraline	102.1%	94.6%	N/A	81.70%	N/A	83.00%	N/A	85.60%	N/A	224.30%	108.70%	9.90%	97.90%	100.90%
Trazodone	96.10%	93.2%	98.20%	93.90%	97.30%	94.30%	80.30%	96.00%	77.80%	94.80%	97.00%	93.90%	101.50%	95.20%

Matrix Effects, Recovery, and Process Efficiency

Analyte	Process Efficiency													
	Blood		Brain		Liver		Lung		Muscle		Serum		Urine	
	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)	Low (3c)	High (80c)
Amitriptyline	99.20%	96.2%	102.90%	96.40%	104.20%	96.30%	103.00%	96.40%	103.50%	98.10%	99.60%	95.60%	100.10%	96.10%
Nortriptyline	98.4%	100.00%	71.20%	99.80%	99.40%	100.40%	97.20%	99.30%	110.90%	98.20%	99.50%	100.00%	99.60%	99.70%
Bupropion	98.10%	97.2%	103.50%	99.30%	N/A	97.70%	N/A	99.50%	N/A	95.50%	98.60%	98.50%	100.00%	99.70%
Hydroxybupropion	108.1%	102.60%	N/A	103.00%	N/A	103.00%	N/A	99.30%	N/A	91.10%	N/A	100.40%	N/A	108.60%
Citalopram	98.60%	95.7%	98.50%	96.40%	104.50%	90.20%	102.20%	97.40%	102.90%	99.30%	99.70%	97.00%	100.40%	96.90%
N-Desmethylcitalopram	99.5%	91.10%	N/A	95.10%	N/A	98.80%	N/A	99.20%	N/A	100.10%	101.50%	92.40%	102.30%	93.10%
Clomipramine	104.10%	97.30%	102.90%	95.50%	104.10%	95.10%	98.90%	97.30%	100.30%	96.80%	103.40%	98.10%	103.70%	96.70%
Desmethylclomipramine	96.8%	96.5%	94.50%	89.30%	95.90%	97.20%	92.80%	100.00%	97.60%	96.50%	94.30%	92.90%	97.60%	93.40%
mCPP	101.00%	99.10%	N/A	100.40%	N/A	96.00%	N/A	98.40%	N/A	97.50%	103.90%	99.10%	101.40%	99.20%
Paroxetine	98.00%	94.00%	95.20%	96.50%	98.40%	78.30%	100.10%	93.90%	102.30%	79.70%	103.10%	95.50%	99.10%	96.40%
Sertraline	98.00%	99.00%	105.50%	96.50%	105.40%	93.60%	N/A	97.10%	88.50%	96.60%	93.50%	100.80%	98.60%	97.60%
Norsertraline	99.20%	94.20%	N/A	80.10%	N/A	76.80%	N/A	85.70%	N/A	76.10%	101.50%	94.50%	91.2%	96.30%
Trazodone	98.00%	93.60%	110.10%	97.00%	131.10%	100.00%	114.10%	100.60%	112.70%	100.90%	97.90%	92.60%	101.70%	95.30%

Stability was assessed to determine the amount of time that samples reliably remain unchanged in situations such as refrigeration (4°C), repeated freezing and thawing (-20°C), and remaining on the instrument (10°C). It is important to know the accuracy resulting from these cycles as instrument malfunction or power outages may occur overnight causing the samples to need to be rerun, situations may arise necessitating storage in the refrigerator, samples often arrive frozen to the FAA for analysis necessitating at least one freeze/thaw cycle, and if dilutions are necessary after the original analysis, more freeze/thaw or refrigeration cycles will likely be needed. The results of these stability studies are listed in Table 13. If samples had a % CV and % error under 20%, they were considered stable. All drugs were found to be stable at 4°C for at least four days, stable for at least three freeze/thaw cycles, except amitriptyline and clomipramine at two cycles, and stable in the instrument autosampler post-extraction for at least four days.

Table 13.

Refrigeration, Freeze/Thaw, and Instrument Stability

Analyte	Refrigerator (Days)	Freeze/Thaw (Cycles)	Instrument (Days)
Amitriptyline	4	2	4
Nortriptyline	5	3	5
Bupropion	5	3	5
Hydroxybupropion	5	3	5
Citalopram	5	3	5
N-Desmethylcitalopram	5	3	5
Clomipramine	4	2	4
Desmethylclomipramine	4	3	4
mCPP	5	3	5
Paroxetine	4	3	4
Sertraline	5	3	5
Norsertraline	5	3	5
Trazodone	5	3	5

The measurement of uncertainty for each drug in the method was determined by evaluating the error of reproducibility of the method, the maximum error determined for the pipettes, glassware, and balances, and the error of the drug standards. Uncertainty accounts for all known sources of error based on their standard deviations and was determined at the 95% confidence level, with an expanded uncertainty below 20 deemed acceptable. Table 14 shows an example of how the measurement of uncertainty was calculated, while Table 15 shows the measurement of uncertainty for each drug.

Table 14.

Measurement of Uncertainty Calculation

Uncertainty Budget Form: Citalopram				*Date Updated:*	3/8/2024		
Method: Antidepressants by LCMS							
Item #	**Sources of Uncertainty**	**Serial #**	**Type**	**% Standard Deviation**	**Distribution Method**	**Divisor**	**Standard Uncertainty (1σ)**
1	Positive Control Reproducibility	N/A	A	4.69%	Normal	1	4.69%
2	Drug Standard	FN07272154	B	0.60%	Normal	2	0.30%
3	Calibration Preparation - Pipette with largest σ	3460012	B	2.25%	Normal	2	1.13%
4	Calibration Preparation - Volumetric flask with largest σ	CP16697	B	0.27%	Normal	2	0.14%
5	Specimen Aliquoting - Pipette with largest σ	3460012	B	2.25%	Normal	2	1.13%
6	Specimen Aliquoting - Gravimetric	043601931	B	0.02%	Normal	2	0.01%
7	Specimen dilution - Volumetric flask with largest σ	CP00538	B	0.06%	Normal	2	0.03%
8	Internal Standard Aliquoting - Repeater with largest σ	M46469I	B	1.81%	Normal	2	0.91%
9	Bias in Positive Control	N/A	B	-0.84%	Rectangular	1.73	-0.49%
	Standard Uncertainty (1σ) = % Std Dev / Divisor						
	Type A or B Normal σ = % Std Dev / x where x=1 if k=1, x=2 if k=2				**Combined Uncertainty (1σ):**		5.07
	Type B Rectangular σ = % Std Dev/√3 where √3=1.73				**Confidence Level:**		k = 2 (95% CL)
	Combined Uncertainty: [√∑ (standard uncertainties^2)]*100						
Comments:							

Table 15.

Measurement of Uncertainty

Analyte	Measurement of Uncertainty
Amitriptyline	7%
Nortriptyline	8%
Bupropion	9%
Hydroxybupropion	8%
Citalopram	5%
N-Desmethylcitalopram	5%

Analyte	Measurement of Uncertainty
Clomipramine	10%
Desmethylclomipramine	7%
mCPP	7%
Paroxetine	6%
Sertraline	9%
Norsertraline	10%
Trazodone	8%

A hydrolysis efficiency study was only conducted for Amitriptyline, using Amitriptyline N-β-D-Glucuronide. A target concentration of 122.3 ng/mL was expected, with results indicating an average of 137.2 ng/mL for a 12.2% error and a 0.8% CV. Since both the % error and % CV were well under 20%, the glucuronidation process was considered successful.

Postmortem Distribution Study

Using the developed and validated method discussed above, blood-to-tissue ratios were analyzed for citalopram and N-desmethylcitalopram using biological samples from six FAA cases which had previously tested positive for the analytes. The ratio of N-desmethylcitalopram-to-citalopram was also analyzed. The ratios for each sample type in a case were determined based on the concentrations observed, as shown in Table 16, and all cases were averaged together to obtain the mean ratio. The mean ratio and standard deviation for this study can be found in Table 17. Based on FAA lab standards, a % CV under 25% was deemed acceptable criterion to establish a correlation between the tissue:blood ratio or N-desmethylcitalopram:citalopram ratio. Spinal fluid and vitreous were successfully analyzed but were excluded from the correlation criterion due to the low number of samples received. Citalopram in brain had a % CV of 23% and a mean brain:blood ratio of 8.3. Citalopram in muscle had a % CV of 24.9% and a mean

muscle:blood ratio of 1.6. No other results met the required criterion, and a direct ratio correlation could not be identified. Even though a direct correlation was not established for most specimen types, notable trends were observed. The liver and lungs had the highest concentrations for both citalopram and N-desmethylcitalopram with all concentrations being at least thirteen times higher in the liver and nine times higher in the lungs for both analytes. Spinal fluid, vitreous, and muscle had the lowest ratios, all being under 1.7. No N-desmethylcitalopram:citalopram ratios met the acceptable criterion, but trends indicated bile had the highest ratio (mean = 1.3), while every other sample type was under 0.5, indicating small amounts of the metabolite were present compared to the parent drug.

Table 16.

Case Study Concentrations

Citalopram Concentration (ng/mL)

Case	Bile	Blood	Brain	CSF	Heart	Kidney	Liver	Lung	Muscle	Spleen	Urine	Vitreous
01012466	2404.34	389.52	2500.95		2538.28	1720.30	5844.00	3901.50	538.57	5035.55	1780.00	368.02
01022466	793.82	490.53	3003.69		1953.40	1991.13		4277.00	616.10	2606.65	7546.65	
01032466	1542.75	100.12	1125.15		121.66	843.59	2311.66	12563.30	189.15	1747.63	464.35	
01042466		368.78	3306.51		5200.50	1215.11	4635.35	5565.75	418.55	5499.75	6971.10	
01052466		572.98	4114.05		2449.25	4272.55	17271.50	11650.50	830.38	8456.95	3356.00	
01062466	1252.48	118.14	1200.72	62.57	1478.41	10182.64	11106.35	1566.41	266.30	8303.20	1657.71	122.11
n	8	16	12	2	12	12	10	12	12	12	12	4
Mean	1498.3	340.0	2541.8	62.6	2290.2	3370.9	8233.8	6587.4	476.5	5275.0	3629.3	245.1
SD	587.3	176.5	1086.1	0.0	1529.3	3237.2	5360.3	4085.6	215.5	2548.4	2705.3	123.0
%CV	39.2	51.9	42.7	0.0	66.8	96.0	65.1	62.0	45.2	48.3	74.5	50.2

Desmethylcitalopram Concentration (ng/mL)

Case	Bile	Blood	Brain	CSF	Heart	Kidney	Liver	Lung	Muscle	Spleen	Urine	Vitreous
01012466	584.20	35.32	71.30		222.74	233.62	854.85	302.75	54.35	349.65	422.51	24.55
01022466	394.53	89.38	157.33		317.60	524.82		802.70	112.44	436.75	885.95	
01032466	5362.35	69.20	317.47		33.06	942.85	3314.49	7487.05	126.97	1264.93	369.22	
01042466		87.40	436.91		1530.65	837.95	1703.20	1834.85	102.90	1389.00	1517.00	
01052466		91.84	245.45		316.75	774.80	2379.50	1977.20	148.08	1027.35	541.95	
01062466	1441.37	49.89	263.30	21.48	631.69	778.07	3266.95	941.52	138.13	732.75	1011.78	37.32
n	8	16	12	2	12	12	10	12	12	12	12	4
Mean	1945.6	70.5	248.6	21.5	508.7	682.0	2303.8	2224.3	113.8	866.7	791.4	30.9
SD	2011.7	21.5	115.6	0.0	490.0	236.6	939.7	2424.5	30.5	393.1	400.1	6.4
%CV	103.4	30.4	46.5	0.0	96.3	34.7	40.8	109.0	26.8	45.4	50.6	20.6

Table 17.

Postmortem Distribution Study

Matrix	Citalopram		N-Desmethylcitalopram		N-Desmethylcitalopram:Citalopram Ratio	
	Mean Tissue:Blood Ratio	Standard Deviation	Mean Tissue:Blood Ratio	Standard Deviation	Mean Ratio	Standard Deviation
Bile	8.5	5.1	31.8		1.3	1.3
Brain	8.3	1.9	3.6	1.4	0.1	0.1
Cerebrospinal Fluid	0.5	(Single Sample)	0.4	(Single Sample)	0.3	(Single Sample)
Heart	7.1	4.7	7.3	5.9	0.2	0.1
Kidney	19	30.1	10	3.5	0.4	0.4
Liver	35	30.2	36.6	17.5	0.5	0.5
Lung	32.2	41.9	31.2	34.8	0.3	0.2
Muscle	1.6	0.4	1.7	0.5	0.3	0.2
Spleen	22.6	21.7	12.5	4.4	0.2	0.2
Urine	10.6	5.7	11.8	5.5	0.4	0.3
Vitreous	1	0.0 (n=2)	0.7	0.0 (n=2)	0.2	0.1 (n=2)

Conclusions

A UPLC/MS-MS method to detect thirteen antidepressants and metabolites in human biological specimens was successfully developed and validated according to a modified version of ANSI/ASB Standard 036. The overall % CV and % error were required to be within ± 20% to be deemed acceptable for validation. Due to the large number of analytes in this method, a mixed concentration calibration curve model was created over an LDR of 200 times (200c) the lowest calibrator (1c). The limit of detection in blood was successfully defined for all analytes in the method and was set no greater than the 1c calibrator. Carryover and cross contribution were evaluated, however, no drugs or internal standards showed any true unexpected peaks, so they were determined not to be a factor in this method. Dilution integrity was upheld in samples with dilutions from 1:1 to 1:100. Drug interference was measured using a high concentration mixture of drugs commonly taken in conjunction with those in this method. Sertraline was the only drug affected by this mixture, exhibiting a slightly enhanced concentration. No false positives for any analyte, including sertraline, were observed. Bias and precision were successfully tested over five separate runs at three different levels. Fluid and tissue controls at three levels were also analyzed, and apparent LOD studies were conducted for specific analytes and tissues that could not be routinely quantitated at the low QC concentration. Analytes with apparent LODs for tissues included bupropion for liver, lung, and muscle, hydroxybupropion for all tissues and fluids except blood, N-desmethylcitalopram for brain, lung, liver, and muscle, mCPP for liver, lung, brain and muscle, norsertraline for liver, lung, brain, and muscle, and sertraline for lung. With the exception of those with apparent LODs, ion suppression/enhancement (matrix effects), recovery, and process efficiency were studied for all tissues and fluids, including blood, with all samples meeting the set criteria. Stability was tested to determine how many days a sample will reliably remain unchanged while refrigerated, sitting on the instrument, and undergoing multiple freeze/thaw cycles. All analytes were stable at 4°C for at least four days, on the instrument for at

least four days, and for at least three freeze/thaw cycles at -20°C. Using the analyses discussed above, the measurement of uncertainty for all analytes was determined to be under the required measurement of 20. Hydrolysis efficiency was conducted on amitriptyline, using Amitriptyline N-β-D-Glucuronide and found to be well under the acceptable ± 20% CV and error.

A postmortem distribution study was conducted for citalopram and N-desmethylcitalopram to evaluate tissue:blood and metabolite:drug ratios. Acceptance criterion was set at ± 25% CV to reliably establish a correlation. Only citalopram in brain and muscle met this criterion, with ratios of 8.3 and 1.6, respectively. Though a correlation could not be established for the other fluids and tissues or N-desmethylcitalopram:citalopram, notable trends were observed. The liver and lungs had the highest concentrations of both drug and metabolite, while spinal fluid and vitreous had the lowest. For the N-desmethylcitalopram:citalopram ratio, bile had the highest ratio (1.3) while all other sample types were under 0.5, indicating small amounts of the metabolite present compared to the parent drug.

Future research suggestions include carrying out a matrix effect, recovery, and precision study on analytes in tissues with apparent LODs. Additionally, a postmortem distribution study on citalopram and N-desmethylcitalopram using a larger sample size would be beneficial. Overall, this novel analytical method was successfully developed and validated, and the postmortem distribution study was carried out successfully.

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