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Simultaneous Determination of Fourteen Antipsychotic Drugs in Whole Blood by Solid Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry

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This thesis has been presented to and accepted by the office of Graduate Studies, John Jay College of Criminal Justice in partial fulfillment of the requirements for the degree of Master of Science in Forensic Science.

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Abstract

Anti-psychotic drugs are commonly prescribed to patients to treat several mental conditions, such as bipolar, schizophrenia, and manic-depressive disorder. The analysis of anti-psychotic drugs in blood is a common practice in clinical and forensic toxicology, to monitor drug treatment (therapeutic drug monitoring) or to explain the cause of the impairment or intoxication in human performance and in postmortem cases. However, most of the current studies have been performed in plasma, and a limited number in blood. We developed and validated a method to confirm and quantify a panel of commonly prescribed anti-psychotic drugs in whole blood using solid phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MSMS). The anti-psychotic drugs in the panel were: aripiprazole, asenapine, clozapine, olanzapine, 9-hydroxyrisperdone (paliperidone), quetiapine, chlorpromazine, fluphenazine, perphenazine, risperidone, haloperidol, lurasidone, ziprasidone, and brexpiprazole. The blood samples were extracted by solid phase extraction using cation exchange cartridges. The chromatographic separation was performed in reversed-phase column using 0.1% formic acid in water and methanol for mobile phases, and in the mass spectrometer two MRM (multiple reaction monitoring) transitions were acquired in positive electrospray mode. The method was validated showing good linearity with a range of 1 to 1000ng/mL. Limit of quantification was established at 1ng/mL and the drug panel was shown to be both accurate and precise. Other validation studies completed were dilution, carryover, selectivity, specificity and stability. When an authentic donor was used to test the method, 9-hydroxyrisperidone was detected and quantitated. The method validation of

this panel of psychotropic medications is a step in a larger project that assesses the steady state levels of therapeutic dosages.

Introduction

In 2013, 4.8 million adults reported filling a prescription for and taking an antipsychotic drug (Moore & Mattison, 2017). Antipsychotic drugs, also known as neuroleptics, are prescribed by doctors to assist patients with managing their psychosis. Those that suffer from schizophrenia, bipolar disorder, and manic-depressive disorder are often prescribed antipsychotics because the drugs act as a major tranquilizer. This neuroleptic agent reduces confusion, delusions and hallucinations that are symptoms often encountered by these patients. As a result, the patient experiences reduced motor activity, decreased anxiety and a general altered state of consciousness. Neuroleptics also have a sedative effect. Antipsychotic drugs are classified as either typical or atypical. Typical antipsychotics, known as first generation antipsychotics (FGAPs), were synthesized in the 1950s. Unfortunately, typical antipsychotics have been observed to have severe side effects, such as tardive dyskinesia. This side effect is the observance of uncontrollable jerky facial and body movements that is oftentimes permanent (Tung & Procyshyn, 2007). In response to the severe side effects associated with typical antipsychotics, second generation antipsychotics (SGAPs), also known as atypical antipsychotics, with fewer side effects were developed in the 1990s. Some atypical antipsychotics have secondary uses; for example, aripiprazole being used to treat Tourette's disorder (Padala et al., 2005) and some of the symptoms of autism (Stachnik & Gabay, 2013); as well as chlorpromazine and perphenazine being used to treat severe nausea and vomiting (Schnabel et al., 2010).

It is uncertain the exact mechanism of how antipsychotics work but they are categorized based on whether they are agonist or antagonist of either dopamine or

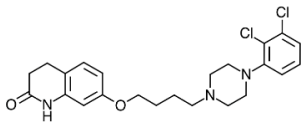
serotonin receptors in the brain (Rauly-Listienne et al., 2007). Many antipsychotics, both first and second generation, are antagonist with either dopamine or serotonin receptors, with the exception of aripiprazole and brexpiprazole. These two SGAPs are partial agonist which is an agonist that is not at maximal potency, but it prevents other more effective agonists from binding with the dopamine or serotonin cell receptors (Jackson, 2010). Overall, atypical antipsychotics generally have a higher affinity for serotonin receptors while typical antipsychotics generally have a higher affinity for dopamine receptors (Richtand et al., 2007).

The determination of antipsychotics in blood samples is of critical interest in clinical and forensic settings. Therapeutic ranges of most antipsychotics are narrow requiring sensitive and reliable analytical methods to identify and quantify the compounds in different biological samples (Zhong et al., 2016). Several studies have indicated that therapeutic drug monitoring (TDM) for SGAPs have recently come back into the spotlight (Ruan et al., 2018). A patient only benefits from taking psychotropic medications when the drug concentration in blood is within the therapeutic drug range. The therapeutic drug range is used by doctors to determine that there is enough medication in the person's system to assist with managing their symptoms. When the drug concentration is below the range then it may not be effective, and if it above the range then there is the potential for toxicity (Grundmann et al., 2014). Antipsychotic measurements in biological samples can be requested in retrospect to assess appropriateness of dosage when a patient has committed a crime or has died in such a way that their treatment is questioned (Fisher et al., 2013). In addition, people who take antipsychotic drugs have an increased risk of rapid unpredicted death compared to those

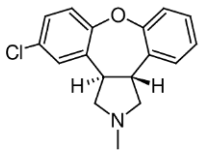
who do not take antipsychotic drugs (Saar et al., 2009). Due to the altered state of consciousness of antipsychotics, there is the potential for abuse. Since antipsychotics do not fall under the category of being controlled substances, many prescribers are unaware that these drugs can also be abused and used recreationally (Hanley & Kenna, 2008). It has been reported that quetiapine is one of the most commonly misused atypical antipsychotic, due to its sedative and/or antihistaminic effect that appeal to abusers (Malekshahi et al., 2014).

Previously published studies described analytical methods for the determination of typical and atypical antipsychotics in various matrices that include blood, serum, and plasma. Many of these methodologies employed liquid-liquid extraction (Patteet et al., 2014; Remane et al., 2011; Sistik et al., 2016), protein precipitation (Gradinaru et al., 2014; Juenke et al., 2013), solid phase extraction (Proenca et al., 2012; Saar et al., 2009) and online solid phase extraction (Ruan et al., 2018 & Zhong et al., 2016). All instrumental analyses were performed by LC-MSMS with the exception of the study conducted by Mercolini et al., (2007) in which they explored HPLC-UV in order to create a method using easily accessible and inexpensive instrumentation. Many studies have used gradient LC-MSMS while the study conducted by Aravagiri et al., (2007) and Merconlini et al., (2007) utilized isocratic LC-MSMS procedures in anticipation of creating an easier and simpler method. Most of these methodologies were developed in serum and plasma with few exploring whole blood (Amundsen et al., 2013; Fisher 2013; Saar et al., 2009). Whole blood is the only matrix available if hemolysis has occurred, and is the most common matrix in post mortem toxicology.

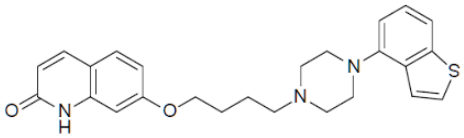
The goal of this project was to develop and validate a sensitive and specific analytical method for the determination of fourteen antipsychotic drugs (Figure 1) in whole blood by LC-MSMS. This method included 4 typical antipsychotics (chlorpromazine, fluphenazine, perphenazine and haloperidol) and 10 atypical antipsychotics (aripiprazole, asenapine, clozapine, olanzapine, 9-hydroxyrisperdone (paliperidone), quetiapine, risperidone, lurasidone, ziprasidone, and brexpiprazole), including the antipsychotics most recently approved by the Food and Drug Administration (FDA).



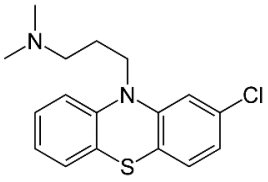
Aripiprazole



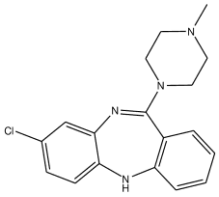
Asenapine



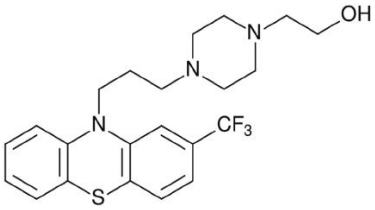
Brexpiprazole



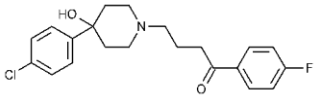
Chlorpromazine



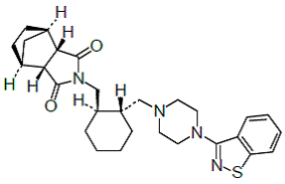
Clozapine



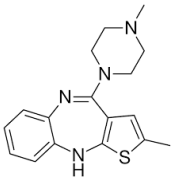
Fluphenazine



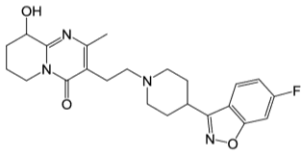
Haloperidol



Lurasidone

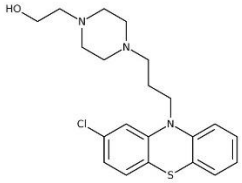


Olanzapine

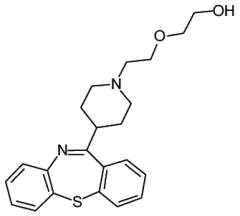


Paliperidone

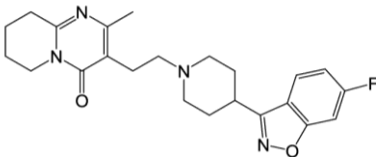
(9-Hydroxyrisperidone)



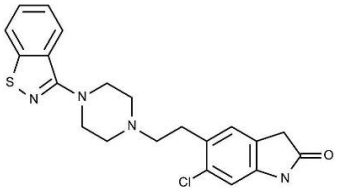
Perphenazine



Quetiapine



Risperidone



Ziprasidone

Figure 1: Chemical structure of target analytes in the present research panel.

1. Materials and Methods:

1.1 Standards, Chemicals and Reagents:

The drug standards were purchased from Cerilliant (Round Rock, TX): quetiapine fumarate 1.0 mg/mL in 1mL methanol, ziprasidone HCl 1.0 mg/mL in 1mL methanol, lurasidone HCl 1.0 mg/mL in 1mL methanol, risperidone 1.0 mg/mL in 1mL methanol, chlorpromazine HCl 1.0 mg/mL in 1mL methanol, 9-hydroxyrisperdone 1.0 mg/mL in 1mL methanol, olanzapine 1.0 mg/mL in 1mL acetonitrile, clozapine 1.0 mg/mL in 1mL methanol, fluphenazine dihydrochloride 1.0 mg/mL in 1mL methanol, haloperidol 1.0 mg/mL in 1mL methanol and aripiprazole 1.0 mg/mL in 1mL 1:1 methanol/water with 1% 1N HCl. Drug standards in powder form, perphenazine 1g and brexpiprazole 10mg,

were purchased from Cayman Chemical Company (Ann Arbor, MI) and asenapine 5mg from Toronto Research Chemicals Inc (Ontario, Canada).

Internal standards were purchased from Cerilliant (Round Rock, TX); clozapine-d_4 100µg/mL in 1mL methanol , aripiprazole-d_8 100µg/mL in 1mL acetonitrile, haloperidol-d_4 100 µg/mL in 1mL methanol, olanzapine-d_8 100 µg/mL in 1mL acetonitrile, 9-hydroxyrisperdone-d_4 100µg/mL in 1mL methanol, quetiapine-d_8 hemifuramate 100µg/mL in 1mL methanol, chlorpromazine-d_3 maleate 100µg/mL in 1mL methanol, and lurasidone-d_8 100µg/mL in 1mL methanol.

The following chemicals and reagents were purchased from V.W.R. International (Radnor, PA): HPLC grade water, methanol, acetic acid, ammonium hydroxide, dichloromethane, isopropanol, and formic acid. Sodium phosphate buffer (pH 6.0) pouches were purchased from UCT: Forensics (Bristo, PA). Pouches contained 1.70g disodium hydrogen phosphate and 12.14g sodium dihydrogen phosphate hydrate. Instructions for preparation were to add 600mL of deionized water to 1000mL volumetric flask, add in the contents of the buffer pouch and mix/stir. The mixture was diluted to the mark with deionized water to yield 1000mL solution.

1.2 Materials

Materials used included Cerex: Trace B 711-335 cation cartridges purchased from Tecan SP, Inc (Baldwin Park CA). LC-MSMS vials were purchased from Phenomex (Torrance, CA) and caps for vials were purchased from Agilent (Santa Clara, CA). SPEware sample concentrator Cerex 48 purchased from Cera Inc (Baldwin Park, CA).

1.3 Intermediates and Calibrators:

Intermediates were created from the standard stock solutions in order to be used as working solutions. All individual drug standards were combined in up to 10mL of methanol to form a stock panel mixture. Serial dilutions were performed from 1,000,000ng/mL (1mg/mL) to create 100,000ng/mL, 10,000ng/mL, 1,00ng/mL and 100ng/mL. This was done by taking original 10mL vial of drug containing 1mg/mL, pipetting 1mL from it into a new vial and adding up to 10mL of methanol. This created the 100,000ng/mL stock solution. Then taking 1mL of 100,000ng/mL stock and adding up to 10mL of methanol to create 10,000ng/mL. This process is continued serially to create 1,000ng/mL and 100ng/mL stock panel solutions.

Calibrators were prepared at 1ng/mL, 5ng/mL, 10ng/mL, 100ng/mL, 250ng/mL, 500ng/mL and 1000ng/mL. Quality Control were prepared at 10ng/mL, 100ng/mL and 250ng/mL. Calibrators were prepared by spiking known volumes of stock panel solution into blood.

1.4 LC-MSMS Instrument

An Agilent 1290 Infinity Series LC System equipped with an autosampler combined with an Agilent 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) was used. A Phenomenex Aeris Widepore C4 200 column (4.6mmx100mm, 3.6 μm particle size) held at a temperature of 50˚C in the column compartment was used for chromatographic separations. The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B) at a flow rate of 0.5 mL/min. The gradient conditions began at 0% B lasting for a duration of 1.15 mins, increasing to 85% B by 3 min, then increasing to 95% B by 3.50 min, then finally

conditions returned to 0%B by 3.51 min with a stop time at 4 min total. The instrument had a cycle time of 1 min to equilibrate back to initial conditions making total sample run 5 min. The mass spectrometer (MS) analyzed the compounds using Agilent jet stream-electrospray ionization source (AJS-ESI) under positive mode. The MS parameters included a gas temperature of 350˚C, gas flow at 10 L/min, nebulizer at 50 psi, and capillary voltage at 3.5 kV. All data was recorded and processed using Agilent MassHunter Quantitative Analysis for QQQ (Version B.06.00/Build6.0.388.0). The two most abundant product ions of MRM transitions were chosen for each analyte, the first for quantitation and the second for ion ratio comparison as confirmation as shown in Table 1.

Optimization of mass spectrometry, chromatography and extraction was required prior to validation. Optimization of analytes begin with obtaining the values for mass spectrometric analysis. Both analytes and their internal standards were optimized independently of each other. Direct injection of analytes into the mass spectrometer and observing the highest peak intensity determined the precursor ion. By manipulating the values of the fragmentor and collision cell levels, the two product ions were determined. The values for cell accelerator voltage remained at 7 and polarity was set to positive.

Table 1: MRM transitions monitored and energy conditions for target analytes and their deuterated analogs. Highlighted in bold the quantifier transition.

Compound name	Precursor ion *m/z*	Product ion *m/z*	Fragmentor V	Collision Energy V
Aripiprazole	448.1	285	120	24
	448.1	**218**	**120**	**20**
Chlorpromazine	319.1	86.1	100	20
	319.1	**58.2**	**130**	**60**
Clozapine	327.2	296	100	20
	327.2	**270.1**	**100**	**20**
Fluphenazine	438.2	171.1	120	26
	438.2	**143.1**	**120**	**26**
Haloperidol	376.2	165.1	140	20
	376.2	**123.1**	**140**	**40**
Lurasidone	493.3	166.1	170	40
(5β/6β Hydroxylurasidone)	**493.3**	**120.0**	**170**	**60**
Olanzapine	313.2	256	150	20
	313.2	**84.1**	**150**	**20**
Paliperidone	427.2	207	140	20
(9-Hydroxyrisperidone)	**427.2**	**110**	**140**	**44**
Perphenazine	404.1	171.2	140	20
	404.1	**143.1**	**130**	**30**
Quetiapine	384.2	279	140	20
	384.2	**253**	**140**	**16**
Risperidone	411.2	191.1	140	28
	411.2	**110**	**150**	**60**
Ziprasidone	413.1	194.1	160	36
	413.1	**159**	**160**	**44**
Asenapine	286.1	229.1	140	26
	286.1	**166.2**	**140**	**32**
Brexpiprazole	434.2	273.2	120	22
	434.2	**98.2**	**120**	**42**
Internal Standards				
Aripiprazole-d_8	456.2	293	140	24
Chlorpromazine-d_3	322.1	89.2	100	15
Clozapine-d_4	331.2	272	140	20
Haloperidol-d_4	380.2	169.1	140	20
Lurasidone-d_8	501.3	166	120	45
Olanzapine-d_8	331.2	261.1	150	20
Paliperidone-d_4 (9-Hydroxyrisperidone-d_4)	431.2	211.1	140	26
Quetiapine-d_8	392.2	258	140	20

Optimization of chromatography was completed using an Aeris reversed phase column and binary pumps. The Aeris column was determined to be best for separation of antipsychotic drugs in blood when compared with other columns, such as the Bi-Phenyl column. The two mobile phases were 0.1% formic acid (mobile phase A) and methanol (mobile phase B). The mobile phase B solvent increased the elution strength of mobile phase. Optimization was initially completed in isocratic mode with 90% mobile phase B in order to observe peaks and the order in which they appeared. Then the percent of mobile phase B was varied along with the length of time, in order to separate the peaks of the different drugs. Table 2 shows the final mobile phase compositions and times that showed clean, separate chromatographic peaks of each drug.

Table 2: Mobile phase gradient (flow 0.5 mL/min, column temperature 50˚C)

Time (min)	**A(%)**	**B (%)**
0.00	100	0
0.75	100	0
3.00	15	85
3.01	5	95
3.50	5	95
3.51	100	0

1.5 Solid Phase Extraction

Solid phase extraction was completed on an SPEware Automated Liquid Dispenser (ALD) manufactured by Cera Inc (Baldwin Park, CA). The SPE cartridges, Cerex: Trace B 711-335, were conditioned with 1mL of HPLC grade methanol, and then 1mL of HPLC grade water. An aliquot of 50µL of internal standard and 250µL of sample

with 1mL of 0.1M sodium phosphate buffer was loaded in the cartridge. The cartridges were washed with 3mL of HPLC grade water, 3mL of 0.1M acetic acid and 3mL of 25% methanol in water. The samples were then dried with heated nitrogen for 14 min before being eluted with 0.75mL of elution solvent Dichloromethane:Isopropanol:Ammonium Hydroxide prepared at a ratio of 70:26:4 The elutants were dried in the SPEware: Cerex 48 Sample Concentrator (Baldwin Park, CA) at 40℃ for up to 20 min with a steady flow of N_2 gas. The samples were reconstituted with 100µL 0.1% formic acid (mobile phase A) and 10µL were injected into the LC-MSMS.

2. Ethical issues

This method development required human whole blood specimens. The materials in this research were acquired by Cordant health solutions' R&D lab. The blood used for development of the method was expired blood donated by the New York Blood Bank. The blood samples that contained drugs of interest used for various experiments such as matrix effects and selectivity were donated by Cordant Health Solutions' production lab. These blood samples were expired and followed The Health Insurance Portability and Accountability Act (HIPAA) privacy rule in that they were not identified by patient name or contained any personal information.

3. Method validation:

Method validation (linearity, precision, accuracy, limit of quantification, matrix effects, extraction efficiency, process efficiency, interferences, dilution integrity, carryover and autosampler stability) for analyzed antipsychotic drugs in whole blood

were carried out following the guidelines of the Scientific Working Group for Forensic Toxicology (SWGTOX, 2013).

3.1 Linearity, Accuracy & Precision

Linearity depicts the straight-line relationship between the analyte response and analyte concentration. This was investigated by using 7 different calibrators over 5 different days. Each calibration curve was created using stock blood obtained from the blood bank and was generated by the Masshunter data analysis software. The ratio of analyte response to internal standard response was plotted against concentration. The calibration curve was set by software to ignore the origin and was evaluated using three curve weightings (none, $1/x$, $1/x^2$). Calibration curves we additionally evaluated using linear or quadratic fit models. Linearity was acceptable if the individual calibration points were within ±20% of the expected concentration value. The correlation coefficient must be greater than 0.985 to be acceptable.

Accuracy and precision are a series of tests that ensures that the results of the analytical method are close to the known value (accuracy) and also close to each other (precision). This was calculated by observing the inter and intraday values of the quality control (QC) samples at 10ng/mL (low), 100ng/mL (med) and 250ng/mL (high). These samples were observed in triplicate over five different days. Accuracy was measured by determining the percent error of the QC samples, which should be less than 20%. Precision was measured by determining the coefficient variation between the samples, also known as the relative standard deviation of the QC samples, which should be less than 15%. The formulas used to study these parameters are summarized in Table 3.

Table 3: Formulas for accuracy and precision

Accuracy =	average concentration over 5 days / expected concentration value	*100%
Precision =	standard deviation of concentration over 5 days / average concentration over 5 days	*100%

3.2 Limit of Quantitation

Limit of quantitation is determined by observing the results of 10 different samples in which the drug panel is spiked into donor blood at the lowest level concentration (1ng/mL) to determine that the limit has been reached in analyte response. The percent error must be below 20% in order for the data to be acceptable.

3.3 Matrix Effects, Analyte Recovery and Process Efficiency

Matrix effects experiment is used to determine if the matrix components (blood in this case) have any effects on the ionization of the analytes of interest. Matrix effects can include ion suppression, ion enhancement or neither when observing the response of the analytes. Fifteen different samples were observed in duplicate with first set consisting of drug panel standards prepared in matrix and then extracted as per SPE extraction discussed earlier. The samples were at mid-level concentration (500ng/mL) and no internal standard was added. The second set consisted of drug panel standards spiked into the matrix after extraction of blank blood samples. The samples were also at mid-level concentration (500ng/mL) and no internal standard was added. A third set of samples consisted of three neat samples that were prepared by directly injecting 125µL of 1000ng/mL drug panel standard into LC-MSMS vials, drying down in sample concentrator and reconstituting in 0.1% formic acid. Analyte recovery involved

determining what percent of known amount of drug concentration was detected. Process efficiency involved determining how efficient the whole method was. These were both calculated by the formulas listed below. The formulas used to study matrix effect, process efficiency and analyte recovery are summarized in Table 4.

Table 4: Mathematical calculations used to determine matrix effect, process efficiency and analyte recovery.

Formulas

A=Neat B=Pre-extraction C=Post extraction

Matrix Effect	[B/A] *100
Analyte Recovery	[C/B]*100
Process Efficiency	[C/A]*100

3.4 Endogenous and exogenous interferences

This study was used to determine method specificity. It was determined there were no interferences when the response of the samples in the study fall below half of the limit of quantification (LOQ).

3.4.1 Selectivity (endogenous interference)

Selectivity is used to ensure that when a negative control is analyzed, meaning there are no drugs in the sample, no peaks will be observed. This was done by comparing 15 samples that were negative control, 15 samples that contained the drug panel at a mid-level concentration (250ng/mL) with no internal standard added, and 15 samples that were negative control that contained only internal standard at a concentration of 50ng/mL.

3.4.2 Specificity (exogenous interferences)

Specificity is used to observe possible interferences from other compounds in the sample matrix. In this experiment, the sample contained the drug panel at low concentrations (10ng/mL) and was loaded with other drugs at the highest level concentration (1000ng/mL) to see if the other drugs had an effect on being able to determine the analytes of interest. The other drugs loaded consisted of a panel of popular drugs of abuse that included: 6-acetylmorphine, amphetamine, methamphetamine, MDMA, MDA, MDEA, benzoylecgonine, cocaine, cocaethylene, norcocaine, methadone, EDDP, morphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, noroxycodone, norhydrocodone and phencyclidine. This is important as this experiment checks for isobars which are compounds with the same molecular weight. In order to differentiate between analytes of interest and the other added analytes, chromatography was used to separate based on retention times.

3.5 Dilution Integrity

Dilution integrity ensures that a sample found at a concentration above the upper limit of linearity can be successfully diluted, analyzed and able to accurately provide the original concentration result. Dilution factors included were: 1:2, 1:5 and 1:10. All dilutions had to quantify within +/-20% of the expected concentration. Drug panel were fortified at the upper limit level of 1000ng/mL and dilutions were carried out.

3.6 Carryover

Carryover study is used to determine if any of the analytes in drug panel exhibit potential for carryover from previous injections. If a sample of high concentration is injected into the LC-MSMS, some of the analytes can remain in the injection system and

can cause contamination or interference of results in the next sample injection. Carryover was noted by the observation of analyte peaks in the 2 blank injections following the carryover sample injection. The highest level calibrator point (1000ng/mL) and 10x the highest level calibrator point (10,000ng/mL) were used in this study as carryover samples. If peaks were seen in the blank injections, the response value had to be less than half the LOQ response value. If the response values were greater than half the LOQ then additional washing steps are needed, such as including an extra autosampler cleaning step or a blank solvent run.

3.7 Autosampler Stability

Stability is used to observe if analytes degrade over a period of time while stored in the autosampler. The extracted samples were left out in their autosampler vials at room temperature over a period of 3 days. Each day the analytes were re-analyzed and compared with a calibration curve that was extracted that day to determine if the sample degraded over the course of 3 days. Accuracy was measured by percent error of samples.

3.8 Authentic Donors/ Proficiency Testing

This study was done to verify that an “unknown sample” can be analyzed with precision and accuracy. Samples were obtained from Cordant’s production lab containing the drugs in the panel. For those samples that did not have an authentic donor, a “blind sample” was prepared by Cordant’s R&D lab staff to act as a proficiency test.

4. Results

Linearity:

The requirement to pass linearity was r2=0.985. All drugs in this panel exhibited linearity above the required r2=0.985 for the 5 days of data. Linearity results are shown in Table 5.

Table 5: Mean, standard deviation and CV of r2, slope, and intercept for each drug in panel

Aripiprazole	**r2**	**Slope**	**Intercept**	**Asenapine**	**r2**	**slope**	**intercept**
Mean	0.995	34.443	0.034	**mean**	0.994	2.416	0.065
StdDev	0.002	24.463	0.045	**StdDev**	0.005	1.731	0.125
Brexpiprazole	**r2**	**slope**	**intercept**	**Chlorpromazine**	**r2**	**slope**	**intercept**
mean	0.994	16.824	0.012	**mean**	0.998	32.958	0.008
StdDev	0.003	12.732	0.016	**StdDev**	0.001	23.460	0.019
Clozapine	**r2**	**slope**	**intercept**	**Fluphenazine**	**r2**	**slope**	**intercept**
mean	0.996	27.206	0.010	**mean**	0.994	7.422	0.002
StdDev	0.002	25.608	0.011	**StdDev**	0.004	7.052	0.004
Haloperidol	**r2**	**slope**	**intercept**	**Lurasidone**	**r2**	**slope**	**intercept**
mean	0.997	16.808	0.014	**mean**	0.998	35.268	0.021
StdDev	0.001	17.380	0.020	**StdDev**	0.001	24.100	0.029
Olanzapime	**r2**	**slope**	**intercept**	**Paliperidone**	**r2**	**slope**	**intercept**
mean	0.997	31.802	-0.003	**mean**	0.998	16.747	0.004
StdDev	0.001	29.268	0.019	**StdDev**	0.001	16.223	0.004
Perphenazine	**r2**	**slope**	**intercept**	**Quetiapine**	**r2**	**slope**	**intercept**
mean	0.994	4.468	0.032	**mean**	0.997	37.079	0.062
StdDev	0.003	3.994	0.078	**StdDev**	0.001	36.822	0.100
Risperidone	**r2**	**slope**	**intercept**	**Ziprasidone**	**r2**	**slope**	**intercept**
mean	0.996	27.982	0.022	**mean**	0.996	7.709	0.004
StdDev	0.003	26.739	0.029	**StdDev**	0.002	5.464	0.006

Accuracy and Precision:

Accuracy was measured using the percent error of the concentration of analytes to the expected concentration. The percent error must be below 20% in order for the data to be acceptable. Precision was measured using the Coefficient Variation (CV), which must be below 15% for the data to be acceptable. Overall the drug panel exhibited good accuracy and precision results (Table 6). Aripiprazole, brexpiprazole, chlorpromazine, lurasidone, and ziprasidone all exhibited results above 20% error for intraday accuracy on day 4 for one of the QC levels. This was likely due to instrumentation error as all other results were within range. Fluphenazine and perphenazine also showed failures on interday accuracy on both day 4 QC 250ng/mL and day 5 QC 10ng/mL.

Precision as measured by the coefficient variation were all within acceptable range, with the exception of perphenazine (interday 24%), indicating that overall, the method is very precise. This indicates this method is able to identify and quantify the drugs in this panel with satisfactory results.

Table 6: Accuracy and precision validation results for the target analytes in blood.

Aripiprazole		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	14.33	0.22	2.15	2.12	3.97
		100ng/mL	3.79	-1.95	-4.85	5.89	8.46
		250ng/mL	-4.51	-6.44	-2.45	**-25.60**	9.87
	Precision (%)	**10ng/mL**	3.13	3.60	4.87	4.38	5.17
		100ng/mL	2.05	0.69	2.54	4.64	5.80
		250ng/mL	0.81	1.28	3.09	1.49	0.94
Interday n=15	**Accuracy (%)**	**10ng/mL**	4.56				
		100ng/mL	2.27				
		250ng/mL	-5.82				
	Precision (%)	**10ng/mL**	6.43				
		100ng/mL	6.13				
		250ng/mL	12.24				

Asenapine		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	-13.83	15.49	15.88	13.87	12.39
		100ng/mL	6.38	7.49	16.22	-1.10	19.95
		250ng/mL	-17.48	-7.34	9.34	0.94	-10.67
	Precision (%)	**10ng/mL**	3.27	3.21	5.89	0.94	3.73
		100ng/mL	1.40	1.96	1.76	2.73	1.18
		250ng/mL	1.59	2.19	1.69	2.26	1.48
Interday n=15	**Accuracy (%)**	**10ng/mL**	8.76				
		100ng/mL	9.79				
		250ng/mL	-5.04				
	Precision (%)	**10ng/mL**	11.13				
		100ng/mL	7.06				
		250ng/mL	10.00				

Brexpiprazole		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	7.33	5.64	6.09	14.91	-6.80
		100ng/mL	10.09	16.27	7.75	**23.23**	-21.58
		250ng/mL	-7.25	19.50	3.27	-18.26	-5.97
	Precision (%)	**10ng/mL**	1.32	1.79	1.50	0.35	1.93
		100ng/mL	0.52	1.58	1.14	1.91	0.96
		250ng/mL	1.26	1.08	0.87	1.22	1.58
Interday n=15	**Accuracy (%)**	**10ng/mL**	5.44				
		100ng/mL	7.16				
		250ng/mL	-1.74				
	Precision (%)	**10ng/mL**	6.78				
		100ng/mL	14.38				
		250ng/mL	12.91				

Chlorpromazine		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	7.80	10.28	1.32	-5.48	1.31
		100ng/mL	1.18	-2.40	-6.23	-3.22	6.90
		250ng/mL	-0.82	1.61	-4.53	**-26.44**	-19.69
	Precision (%)	**10ng/mL**	0.81	0.96	0.87	0.54	0.85
		100ng/mL	1.29	1.03	1.10	1.96	1.90
		250ng/mL	1.30	0.13	1.09	1.12	1.37
Interday n=15	**Accuracy (%)**	**10ng/mL**	3.05				
		100ng/mL	-0.76				
		250ng/mL	-9.97				
	Precision (%)	**10ng/mL**	5.44				
		100ng/mL	4.78				
		250ng/mL	12.35				

Clozapine		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	16.76	6.17	8.93	4.45	-0.47
		100ng/mL	2.22	6.78	6.17	1.58	9.67
		250ng/mL	-12.39	-4.13	-1.19	0.88	-15.85
	Precision (%)	**10ng/mL**	0.86	1.01	1.05	2.19	1.42
		100ng/mL	0.60	0.73	1.57	0.23	1.06
		250ng/mL	0.56	0.64	0.98	2.76	1.23
Interday n=15	**Accuracy (%)**	**10ng/mL**	7.17				
		100ng/mL	5.28				
		250ng/mL	-6.53				
	Precision (%)	**10ng/mL**	5.48				
		100ng/mL	3.02				
		250ng/mL	7.11				

Fluphenazine		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	17.13	6.57	-3.25	3.38	**-28.12**
		100ng/mL	-9.74	1.79	0.02	6.94	-11.89
		250ng/mL	-20.80	-20.90	17.10	**-29.12**	-13.37
	Precision (%)	**10ng/mL**	4.04	2.85	0.92	2.84	6.21
		100ng/mL	2.22	3.17	2.74	0.86	2.18
		250ng/mL	2.48	5.34	0.33	1.35	4.54
Interday n=15	**Accuracy (%)**	**10ng/mL**	-0.86				
		100ng/mL	-2.58				
		250ng/mL	-13.42				
	Precision (%)	**10ng/mL**	15.67				
		100ng/mL	7.69				
		250ng/mL	**18.82**				

Haloperidol		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	13.60	-2.88	3.26	-1.39	7.01
		100ng/mL	1.39	0.19	0.37	-3.45	7.66
		250ng/mL	-6.20	-8.36	-1.30	-4.80	-6.50
	Precision (%)	**10ng/mL**	0.17	0.87	0.78	0.92	1.29
		100ng/mL	0.63	0.71	0.91	0.66	0.38
		250ng/mL	0.49	0.99	0.93	0.34	0.94
Interday n=15	**Accuracy (%)**	**10ng/mL**	3.92				
		100ng/mL	1.23				
		250ng/mL	-5.43				
	Precision (%)	**10ng/mL**	5.81				
		100ng/mL	3.63				
		250ng/mL	2.61				

Lurasidone		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	14.83	2.86	-3.16	7.27	4.61
		100ng/mL	1.98	-4.18	-2.63	1.58	-1.03
		250ng/mL	-3.95	-8.37	-2.39	**-30.37**	6.69
	Precision (%)	**10ng/mL**	2.61	2.21	1.61	1.71	1.54
		100ng/mL	0.66	1.25	1.21	1.90	2.22
		250ng/mL	0.93	1.01	1.58	1.38	1.54
Interday n=15	**Accuracy (%)**	**10ng/mL**	5.28				
		100ng/mL	-0.86				
		250ng/mL	-7.68				
	Precision (%)	**10ng/mL**	5.93				
		100ng/mL	2.85				
		250ng/mL	13.46				

Olanzapine		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	7.94	5.07	-4.51	8.57	1.65
		100ng/mL	-1.61	-1.97	4.63	9.57	-0.26
		250ng/mL	-10.38	-14.55	-8.19	-7.16	-19.12
	Precision (%)	**10ng/mL**	0.90	1.10	2.15	0.49	3.48
		100ng/mL	0.84	1.47	1.16	3.27	2.99
		250ng/mL	0.59	0.99	2.25	2.87	1.99
Interday n=15	**Accuracy (%)**	**10ng/mL**	3.74				
		100ng/mL	2.07				
		250ng/mL	-11.88				
	Precision (%)	**10ng/mL**	5.00				
		100ng/mL	4.88				
		250ng/mL	5.39				

Paliperidone		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	6.18	-2.18	-0.29	-3.92	2.73
		100ng/mL	2.34	0.96	1.61	-1.51	3.39
		250ng/mL	-6.07	-6.03	-5.11	-3.68	3.33
	Precision (%)	**10ng/mL**	0.74	1.86	1.11	0.63	1.33
		100ng/mL	0.90	0.32	1.30	0.40	2.22
		250ng/mL	0.19	1.07	0.69	1.19	0.78
Interday n=15	**Accuracy (%)**	**10ng/mL**	0.50				
		100ng/mL	1.36				
		250ng/mL	-3.51				
	Precision (%)	**10ng/mL**	3.78				
		100ng/mL	2.05				
		250ng/mL	3.76				

Perphenazine		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	**26.72**	10.65	8.32	11.98	**-45.29**
		100ng/mL	-11.08	-3.87	0.43	9.31	-4.86
		250ng/mL	-19.13	-20.44	14.57	**-27.42**	-10.36
	Precision (%)	**10ng/mL**	4.07	3.55	2.05	1.67	8.83
		100ng/mL	2.02	3.19	2.37	1.88	2.11
		250ng/mL	2.83	4.67	0.78	1.43	3.47
Interday n=15	**Accuracy (%)**	**10ng/mL**	2.48				
		100ng/mL	-2.01				
		250ng/mL	-12.55				
	Precision (%)	**10ng/mL**	**24.43**				
		100ng/mL	7.28				
		250ng/mL	16.94				

Quetiapine		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	11.41	3.92	6.53	1.49	7.83
		100ng/mL	2.02	3.00	5.74	-0.20	6.53
		250ng/mL	-10.34	-7.32	-2.63	-2.03	2.88
	Precision (%)	**10ng/mL**	2.32	0.62	2.74	0.69	0.62
		100ng/mL	0.23	0.23	1.01	0.53	2.90
		250ng/mL	0.29	1.75	0.89	1.34	1.47
Interday n=15	**Accuracy (%)**	**10ng/mL**	6.24				
		100ng/mL	3.42				
		250ng/mL	-3.89				
	Precision (%)	**10ng/mL**	3.62				
		100ng/mL	2.78				
		250ng/mL	4.92				

Risperidone		**Concentration**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Intraday	**Accuracy (%)**	**10ng/mL**	7.68	5.29	5.36	-5.32	-14.84
		100ng/mL	4.29	0.60	14.56	0.86	5.45
		250ng/mL	-9.61	-8.52	-1.43	0.32	-9.17
	Precision (%)	**10ng/mL**	0.03	1.13	1.23	1.07	2.26
		100ng/mL	1.00	0.27	0.55	1.11	1.39
		250ng/mL	0.42	0.79	0.69	1.64	0.49
Interday n=15	**Accuracy (%)**	**10ng/mL**	-0.37				
		100ng/mL	5.15				
		250ng/mL	-5.68				
	Precision (%)	**10ng/mL**	8.65				
		100ng/mL	4.91				
		250ng/mL	4.59				

Ziprasidone		Concentration	Day 1	Day 2	Day 3	Day 4	Day 5
Intraday	**Accuracy (%)**	**10ng/mL**	6.59	2.82	-6.74	6.86	13.75
		100ng/mL	5.27	11.53	-4.71	5.27	6.76
		250ng/mL	-12.73	2.40	-4.61	**-22.32**	-3.17
	Precision (%)	**10ng/mL**	1.86	2.10	1.21	1.04	3.01
		100ng/mL	0.91	1.92	0.96	1.26	2.56
		250ng/mL	1.32	1.25	1.56	1.39	3.52
Interday n=15	**Accuracy (%)**	**10ng/mL**	4.66				
		100ng/mL	4.83				
		250ng/mL	-8.08				
	Precision (%)	**10ng/mL**	6.72				
		100ng/mL	5.32				
		250ng/mL	9.59				

Values greater than accepted criteria are highlighted in bold.

Matrix Effect, Recovery, Process Efficiency

The matrix effects study indicated that there was ion suppression in the drug panel as seen in Table 7. This was significant in the case of olanzapine, chlorpromazine, perphenazine, fluphenazine and ziprasidone with values as low as 45%. Olanzapine showed a CV of 32.2% indicating that there was significant variation among the different sources. In the case of process efficiency, chlorpromazine, perphenazine, and fluphenazine showed values lower than 75%. However, all drugs showed analyte recovery above 100%.

Table 7: Matrix effect (M.E.), analyte recovery and process efficiency at 500ng/mL in blood (n=15).

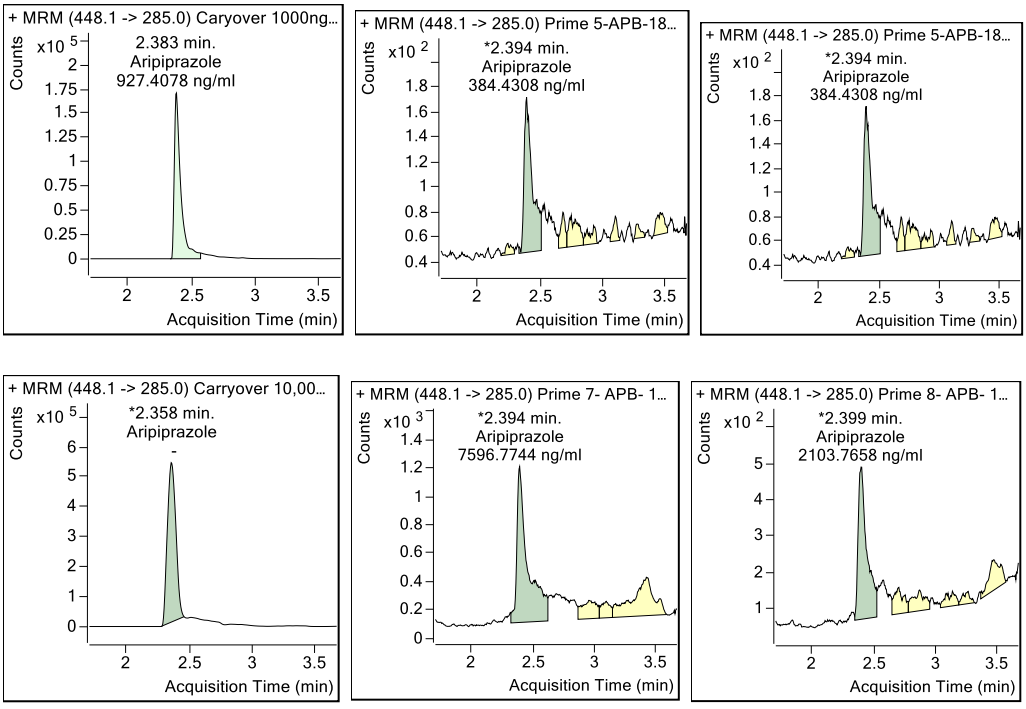
Drug	Matrix Effects	Analyte Recovery	Process Efficiency	CV M.E. (%)
Aripiprazole	76.19	107.25	81.71	5.15
Asenapine	90.49	103.74	93.88	2.81
Brexpiprazole	73.58	118.61	87.27	3.56
Chlorpromazine	58.99	116.29	68.60	4.35
Clozapine	82.69	118.37	97.88	2.65
Fluphenazine	46.22	120.23	55.58	11.19
Haloperidol	79.57	109.45	87.08	2.03
Lurasidone	75.71	122.36	92.64	2.47
Olanzapine	67.59	149.99	101.38	**32.20**
Paliperidone	96.04	107.04	102.80	1.82
Perphenazine	45.66	126.19	57.62	9.92
Quetiapine	90.00	107.70	96.93	2.90
Risperidone	99.12	107.95	107.00	1.51
Ziprasidone	69.58	116.10	80.78	2.09

Values greater than accepted criteria are highlighted in bold.

Carryover:

Samples were spiked at highest level calibrator point (1000ng/mL) and 10x the highest level calibrator point (10,000ng/mL) and followed normal extraction procedures. When completing worklist for LC-MSMS, two blank solvent runs were used after the 1,000ng/mL and two blank solvent runs were used after 10,000ng/mL. In this drug panel, none of the analytes exhibited carryover greater than half the LOQ when the blanks following the 1,000ng/mL calibrator point was observed. Only two drugs in the panel did not exhibit responses higher than half the LOQ in the blank solvent runs following highest level calibrator and 10x highest level calibrator- Quetiapine and Olanzapine (Figure 2). Several drugs exhibited carryover in the first solvent blank following the

10,000ng/mL injection. Asenapine, clozapine, risperidone, and 9-hydroxyrisperidone exhibited a response higher than half the LOQ in the first solvent blank following 10x highest level but in second solvent blank the response fell below half the LOQ. Fluphenazine, perphenazine, chlorpromazine, aripripazole (Figure 2) and ziprasidone exhibited responses in both the first and second solvent blanks significantly higher than that of the LOQ. For these drugs, when observed at 10,000ng/mL, additional autosampler cleaning step will need to be used. However, it should be noted that these drugs are rarely observed at this concentration level.



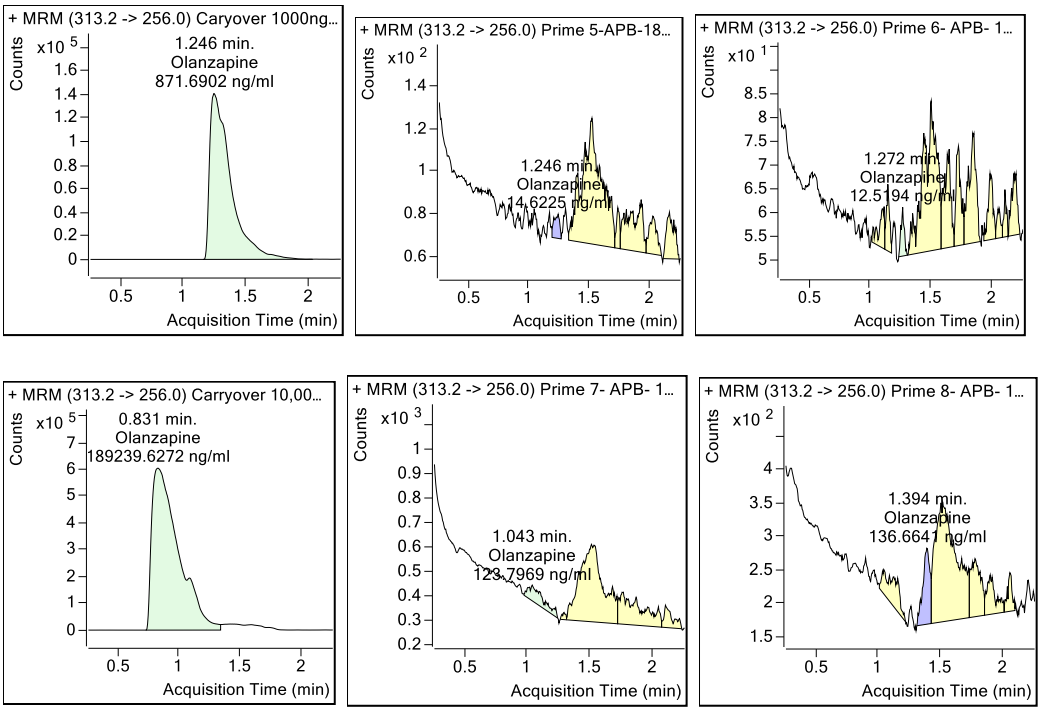


Figure 2: MRM chromatogram of aripiprazole exhibiting carryover in solvent blanks vs olanzapine not exhibiting carryover in solvent blanks. Reported concentrations of blanks are not accurate due to not containing internal standard.

Autosampler Stability:

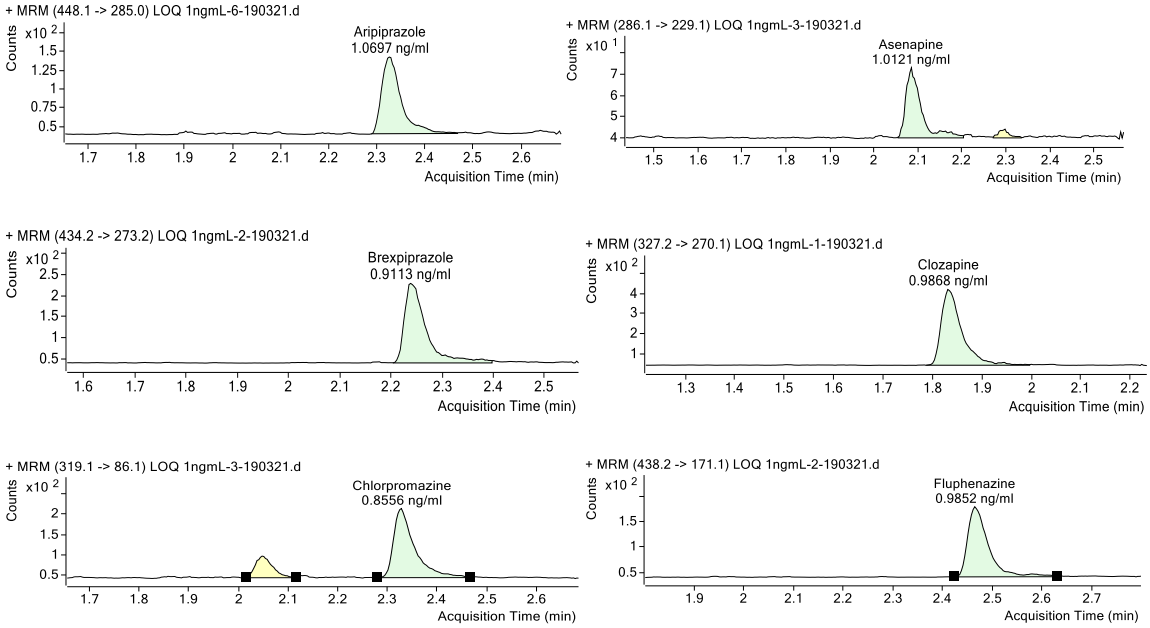
Many of the drugs in the panel did not show stability over the course of the three days in the autosampler. Olanzapine, Clozapine and Quetiapine all showed accuracy within 80-120% range. Lurasidone showed an accuracy slightly above 120% (123%). Risperidone, haloperidol, 9-hydroxyrisperidone, asenapine, fluphenazine and perphenazine all showed significant accuracy errors above 120%. Ziprasidone, brexpiprazole, chlorpromazine and aripiprazole showed accuracy errors below 80%. Over the course of the 3 days, some drugs showed a slight decrease in concentration while others showed a slight increase in concentration. Perphenazine and fluphenazine showed a significant increase in concentration by the third day which indicated that if reanalysis is necessary, the samples have to be extracted again.

Interferences (selectivity and specificity)

In the drug panel, there were no endogenous interferences observed. However, for exogenous interferences, several drugs- brexpiprazole, clozapine and ziprasidone showed some enhancement or suppression in the presence of the other added illicit drugs. The percent error for all the drugs were within the required 20% range with the exception of brexpiprazole, clozapine and ziprasidone showing a percent error of 34%, 34% and 39% respectively.

Limit of Quantification & Dilution:

The drugs in this panel had a limit of quantification at 1ng/mL and percent error were all within 20% (Figure 3). In this drug panel, 13 out of 14 drugs passed the dilution integrity study with accuracy within the 20% requirement. One drug, Olanzapine had results that exceeded the 20% limit with a percent error of 21.6% for the 1:5 dilution.



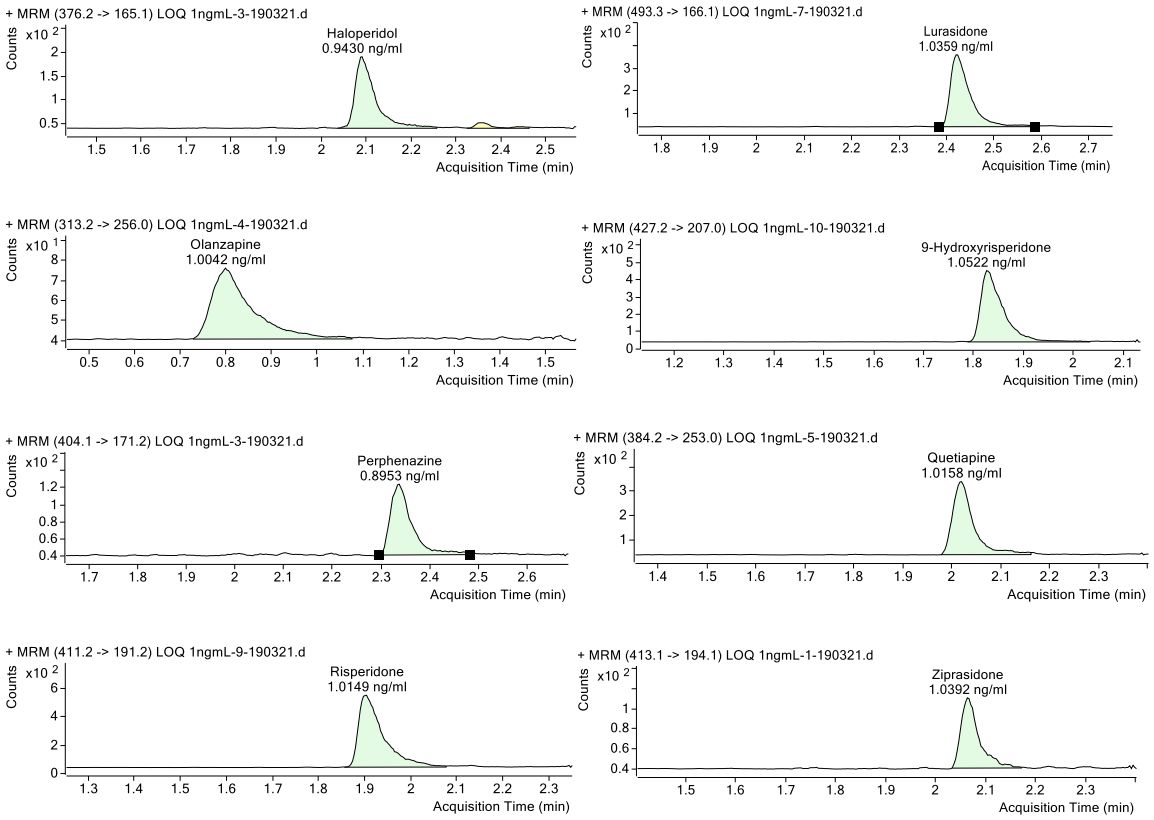


Figure 3: MRM chromatograms of drug panel at the limit of quantification (1ng/nL)

Authentic Donors/Proficiency Testing

Cordant's production lab provided one sample in which an authentic donor containing 9-hydroxyrisperidone of unknown concentration. This method was able to correctly identify the drug and provide a concentration of 8.16ng/mL (Figure 4). Several drugs in the panel were completed as a proficiency test in which lab staff spiked various samples at various concentrations. The drugs were able to be identified and quantified within the required 20% error range (Table 8).

Table: 8 Results of proficiency testing for 8 drugs in drug panel

Drug	spiked conc (ng/mL)	results (ng/mL)	percent error (%)
Asenapine	10	9.7742	-2.258
Brexpiprazole	200	176.346	-11.827
Clozapine	500	550.11	10.022
Fluphenazine	5	5.8076	16.152
Olanzapine	25	27.6294	10.5176
Quetiapine	125	119.58	-4.336
Risperidone	50	53.61	7.22
Ziprasidone	100	97.5953	-2.4047

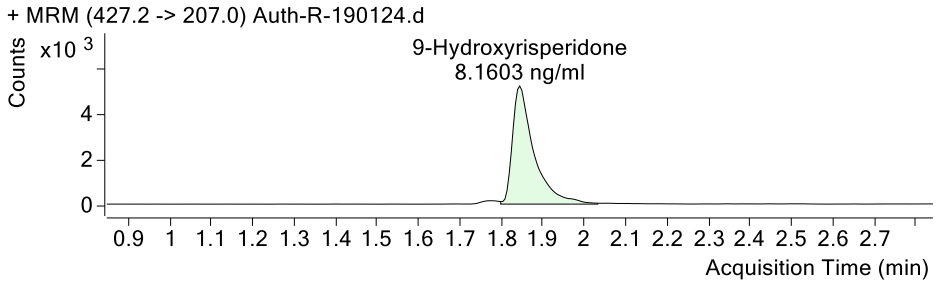


Figure 4: MRM chromatogram of the quantifier transition from an authentic donor sample containing 8.16ng/mL of 9-hydroxyrisperidone

5. Discussion

A method was developed and validated to simultaneously screen for and quantify fourteen antipsychotic drugs in whole blood. A small sample size of 0.25 mL was used in this method. The sample was diluted with 1mL sodium phosphate buffer, extracted by cation exchange solid phase extraction and analyzed by LC-MSMS. A linear range of 1-1000ng/mL in whole blood was obtained for all the drugs in the panel. The method was applied to 1 authentic sample that was positive for 9-hydroxyrisperidone (paliperidone), which the method was able to detect and quantify.

There have been many methods that have been developed analyzing antipsychotics in serum and plasma but much fewer studies explored whole blood as a matrix (Amundsen et al., 2013; Fisher et al., 2013; Montenarh et al., 2016; Roman et al., 2008; Saar et al., 2010). All of the studies that have used whole blood as the matrix completed the sample preparation via liquid-liquid extraction, as opposed to the current method that used solid phase extraction. While the previous studies have yielded successful results, liquid-liquid extraction may not always be preferred because of the increased risk of human error when removing the solvent layer needed for extraction, and the difficulty of automation. In the study completed by Saar et al., (2010) the sample volume was 100µL. While the current method requires 250µL, the total run time for each analyte in the panel is 5 minutes. The study conducted by Saar et al., (2010) has a run time method of up to 20 minutes with an additional 10 minutes of equilibrium time before analysis and 4 minutes between sample runs making this a significantly longer and unfavorable analysis time. In the study conducted by Montenarh et al., (2016), 25 neuroleptics were analyzed in whole blood, which is a larger panel than the current method. In addition, only 1 internal standard was used in order to save time and resources while the current method has 9 internal standards. Unfortunately, due to only having 1 internal standard, it was reported that a number of the analytes failed the validation criteria which could have been prevented by the use of additional internal standards.

None of the studies, Amundsen et al., (2013), Fisher et al., (2013), Montenarh et al., (2016), Roman et al., (2008) and Saar et al., (2010) included the recently FDA approved drug- brexpiprazole. In the current method, brexpiprazole showed good linearity and accuracy and precision. There was a higher than accepted percent error on

day 4 of accuracy and precision but as previously discussed this is likely due to instrumentation error as other analytes exhibited similar results. Brexpiprazole had some matrix effects with results at 73% but a CV of 3.56%. Brexpiprazole had optimal analyte recovery (118%) and the process efficiency was 87%. There was no significant carryover or interferences observed.

In two studies it was noted that olanzapine showed poor stability in whole blood (Fisher et al., 2013) or was dropped from the study due to instability in several validation experiments such as autosampler degradation and processed sample stability (Saar et al., 2010). In the current method presented, olanzapine showed good results for the autosampler stability study and also passed accuracy and precision studies within 20% error range. There was no carryover observed and when proficiency testing was completed, olanzapine was able to be identified and quantitated.

6. Conclusion

A method has been successfully developed and validated for the simultaneous detection of 14 antipsychotic drugs in blood using solid phase extraction and liquid chromatography tandem mass spectrometry. This method included the most recently FDA approved antipsychotic, brexpiprazole. The method required only 0.25 mL of whole blood sample achieving a LOQ of 1ng/mL. The method proved to be linear, accurate and precise for all drugs in the drug panel. The method validation of this panel of psychotropic medications has been found to be applicable for both clinical and forensic toxicology.

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