Validation of a GC/MS method for the determination of alkaline drugs in whole blood

Isabella Schember, B.S.[a*], Kristen Bailey, M.S.[b], James Kraner, Ph.D.[b], Lauren Richards-Waugh, Ph.D.[a]

[a]*Marshall University, 1401 Forensic Science Drive, Huntington, WV 25701*

[b]*Office of the Chief Medical Examiner, 619 Virginia St. W, Charleston, WV 25302*

Abstract

Forensic toxicologists analyze drugs and other toxicants found in bodily fluids or tissue to determine if the drugs present in the sample contributed to death or if their presence was relevant in the circumstances surrounding their death. Reliable analytical data are required for the correct interpretation and evaluation of toxicological findings. In an effort to ensure that reliable analytical data is being produced, methods and instruments need to undergo validation. A gas chromatography-mass spectroscopy method was optimized and validated for the determination of alkaline drugs (bupropion, meperidine, fluoxetine, diphenhydramine, doxylamine, tramadol, N-desmethyltramadol, chlorpheniramine, EDDP, venlafaxine, brompheniramine, dextromethorphan, methadone, O-desmethylvenlafaxine, amitriptyline, nortriptyline, doxepin, cyclobenzaprine, desmethyldoxepin, mirtazapine, promethazine, sertraline, citalopram, clomipramine, desmethylcitalopram, paroxetine, olanzapine, zolpidem, diltiazem, verapamil, norverapamil) in blood. Method validation was conducted utilizing the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines for method validation in forensic toxicology. These studies included evaluation of: matrix interference, interference from other commonly

encountered analytes, carryover, calibration model, bias, precision, limit of quantitation, and limit of detection.

Introduction

Unnatural deaths including suicide, motor vehicle crashes, homicide, suspicious, and drug-related fatalities are commonly encountered types of cases that are investigated. To help interpret the cause and manner of death, forensic toxicologists analyze drugs and other toxicants found in bodily fluids or tissue[1]. This analysis is necessary to determine if the drugs present in the sample contributed to death or if their presence was relevant in the circumstances surrounding their death. Blood is commonly used for detecting, quantifying, and interpreting these toxicants. Concentrations of these toxicants in the blood can be useful in establishing recent drug activity and to determine the effect that the drug had on the deceased at the time of death, or at the time the blood was taken.

For cases involving hospital treatment before death, antemortem specimens are collected to determine if there was evidence of drug use before admission into the hospital[2]. Postmortem blood can sometimes be problematic during the investigation due to changes in drug concentrations after death. There are many factors that could cause this, such as decomposition and postmortem redistribution (PMR)[3]. PMR involves the redistribution of drugs into heart blood from solid organs such as the lungs and liver[3].

To establish if toxicants were present and capable of contributing to death samples undergo screening, identification, and quantification for a large range of over-the counter, prescription, and illicit drugs[5,6]. In forensic toxicology laboratories, these analyses are performed using

instrumental methods such as immunoassay, gas chromatography-mass spectrometry (GC/MS), and liquid chromatography-tandem mass spectrometry (LC/MS/MS).

A GC/MS method was developed for the separation, identification, and quantification of 31 alkaline compounds (bupropion, meperidine, fluoxetine, diphenhydramine, doxylamine, tramadol, N-desmethyltramadol, chlorpheniramine, EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), venlafaxine, brompheniramine, dextromethorphan, methadone, O-desmethylvenlafaxine, amitriptyline, nortriptyline, doxepin, cyclobenzaprine, desmethyldoxepin, mirtazapine, promethazine, sertraline, citalopram, clomipramine, desmethylcitalopram, paroxetine, olanzapine, zolpidem, diltiazem, verapamil, norverapamil) in whole blood. This group includes a wide range of substances including antidepressants, antihistamines, and hypnotics among others.

Reliable analytical data are required for the correct interpretation and evaluation of toxicological findings[7]. In an effort to ensure that reliable analytical data is being produced, methods and instruments must be validated. Validation is the process of performing a set of experiments that estimates the reliability and accuracy of an analytical method[7,8]. The goal of validation is to establish evidence that demonstrates a method's capability to perform and to identify any limitations[8]. Common examples of when it is necessary to verify that a method's performance parameters are fit for a particular analysis include new analytical methods, addition of new compounds to established analytical methods, and in demonstrating equivalence between an established method/instrument and a new method/instrument[8].

The Scientific Working Group for Forensic Toxicology (SWGTOX) created a set of guidelines to use for method validation in forensic toxicology. In these guidelines, SWGTOX lists required validation parameters based on the scope of the method being validated. For a

quantitative method, the following validation parameters need to be evaluated: interference, carryover, calibration model, bias, precision, limit of quantitation (LOQ), and limit of detection (LOD)[8].

The aim of this project was to validate a precise and sensitive quantification method for the analysis of alkaline drugs in whole blood, which would allow for better interpretation of toxicological findings through the generation of more reliable analytical data.

Experimental

Chemicals and material

Table 1 lists the 31 drug standards used. Sodium carbonate (Na_2CO_3), ammonium hydroxide (NH_4OH), and isoamyl alcohol were purchased from EMD Millipore® (Darmstadt, Germany). Heptane, methanol, and hydrochloric acid (HCl) were purchased from J.T.Baker® (Center Valley, PA). Acetonitrile (AcCN) was purchased from Thermo-Fisher Scientific (Pittsburgh, PA). Toluene and proadifen hydrochloride (SKF-525A) were purchased from Sigma-Aldrich (St. Louis, MO).

Table 1. Thirty one drug standards obtained from Cerilliant® or Alltech®.

Source	Analytes
Cerilliant®	Bupropion, Meperidine, Fluoxetine, Diphenhydramine, Doxylamine, Tramadol, N-desmethyltramadol, Chlorpheniramine, EDDP, Venlafaxine, Dextromethorphan, Methadone, O-desmethylvenlafaxine, Amitriptyline, Nortriptyline, Doxepin, Cyclobenzaprine, Desmethyldoxepin, Mirtazapine, Promethazine, Sertraline, Citalopram, Clomipramine, N-desmethylcitalopram, Paroxetine, Olanzapine, Zolpidem, Verapamil, Norverapamil
Alltech®	Brompheniramine and Diltiazem

Liquid-liquid extraction

The liquid-liquid extraction (LLE) was performed following the alkaline extraction scheme in place in the laboratory. Calibrators (10, 25, 50, 100, 250, 500, 1000, and 2000 ng/mL) and controls (65, 130, and 650 ng/mL) were prepared in whole blood using the drug standards listed above (Table 1). In a 15 mL screw-top centrifuge tube, 250 ng of internal standard (SKF-525A) and 100 µL of 20% Na_2CO_3 were added to 1 mL whole blood sample, calibrator, or control. The samples were briefly vortexed before 10 mL of heptane:isoamyl alcohol (95:5) was added. The centrifuge tubes were capped and mixed for 15 minutes on a test tube rocker. Tubes were centrifuged with a Thermo Scientific™ Heraeus™ Megafuge™ 16, for 10 minutes at 2500 rpm and the organic layers transferred to new centrifuge tubes. A back extraction was performed by adding 3 mL of 1 N HCl to each tube. Tubes were capped, rocked for 15 minutes, and centrifuged for 10 minutes at 2500 rpm. The organic layer was discarded and 650 µL of 20% Na_2CO_3, 8 drops of concentrated NH_4OH, and 150 µL toluene:acetonitrile (85:15) were added to each tube. Tubes were capped, rocked for 5 minutes, and centrifuged for 10 minutes at 2500 rpm. The aqueous layer was discarded and the organic phase was transferred to a GC autosampler vial with fixed insert.

Chromatographic conditions

Samples (2µL) were injected onto an Agilent 7890B GC with a 5977A MS detector and 7693 autosampler. The GC was equipped with a capillary column (Agilent HP-5MS, 30 m x 0.25 mm, 0.25 µm film thickness) and run in full-scan mode (scan range 40-570 *m/z*) with a solvent delay at 3.40 minutes. Helium was employed as the carrier gas. The injector temperature was 280 ºC and the initial oven temperature was 100 ºC, which was held for one minute. The oven was

ramped at 15 ºC/min to 325 ºC. The final temperature was held for five minutes for a total run time of 21.00 minutes. Three mass spectral libraries were used: an in-house library created using neat reference samples materials (OCME), the 2008 Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) library, and a combined Wiley and National Institute of Standards and Technology (NIST) library. Enhanced ChemStation (MSD ChemStation F.01.01.2317) was used to qualitatively assess the samples for the presence of each analyte using the selected *m/z* values listed in Table 2. MassHunter Workstation Software (Quantitative Analysis Version B.07.00/Build7.0.457.0 for GC/MS) was used to quantitatively analyze the samples. One quantifier ion and three qualifier ions were used to determine the presence and concentration of the analytes of interest. The confirmation ions are presented in Table 2 with the target ions that were used for quantification.

Table 2. Retention times (RT) and monitored *m/z* values.

Analyte	**Quantifier *m/z***	**Qualifier *m/z***	**RT (min)**
Bupropion	100.1	44.0, 139.0, 224.1	6.473
Meperidine	247.1	172.1, 71.1, 218.1	7.638
Fluoxetine	104.0	44.0, 91.0, 309.0	8.423
Diphenhydramine	165.0	58.1, 73.1, 152.0	8.499
Doxylamine	71.1	58.1,167.0, 180.0	8.843
Tramadol	263.2	58.1,135.0, 77.0	9.049
N-desmethyltramadol	188.1	135.0, 150.0, 249.1	9.250
Chlorpheniramine	203.0	28.1, 167.0, 72.1	9.435
EDDP	277.2	262.1, 220.1, 165.0	9.636
Venlafaxine	134.0	58.1, 179.1, 91.0	9.959
Brompheniramine	247.0	167.1, 72.1, 180.0	10.073
Dextromethorphan	271.1	150.1, 215.1, 171.0	10.285
Methadone	72.1	294.1, 223.1, 165.0	10.332
O-desmethylvenlafaxine	120.0	91.0, 58.1, 46.0	10.473
Amitriptyline	58.1	202.1, 215.0, 189.0	10.641
Nortriptyline	44.1	215.1, 202.1, 189.0	10.748
Doxepin	165.0	58.1, 178.1, 189.1	10.844
Cyclobenzaprine	215.1	58.0, 202.0, 189.0	10.914
Desmethyldoxepin	178.0	44.0, 165.1, 222.1	10.942
Mirtazapine	195.1	208.1, 221.1, 265.1	10.980
Promethazine	72.0	284.1, 180.0, 198.0	11.144
SKF-525A	86.0	99.0, 164.9	11.360
Sertraline	274.0	262.0, 159.0, 304.0	11.651
Citalopram	324.1	58.1, 238.0, 208.0	11.847
Clomipramine	269.1	58.1, 85.1, 227.0	11.892
Desmethylcitalopram	238.0	44.0, 138.0, 220.0	12.007
Paroxetine	192.1	138.0, 177.0, 109.0	12.774
Olanzapine	242.0	229.0, 213.0, 198.0	13.566
Zolpidem	235.1	219.1, 307.1, 92.0	13.939
Diltiazem	71.1	58.1, 121.0, 150.0	14.639
Verapamil	303.2	151.0, 58.0, 260.1	15.589
Norverapamil	289.2	151.0, 165.0, 260.1	15.834

Method Validation

The following studies were performed using the 'Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology'

revision 1.003 as a guide: matrix interference, drug interference, carryover, calibration model, bias, precision, LOQ, and LOD.

Matrix interference, interference from other commonly encountered analytes, and carryover studies were performed for all 31 of the drugs listed in Table 2. SKF-525A was used as the internal standard. Calibration model, bias, precision, LOQ, and LOD studies were performed on EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine.

Matrix interference

Matrix interferents are non-targeted compounds (i.e., matrix components, other drugs and metabolites, or impurities) present in the matrix, which may impact the ability to detect, identify, or quantitate a targeted analyte. Seventeen blank whole blood samples (Table 3) were extracted and evaluated without the addition of internal standard. Fourteen of these blood samples were procured at autopsy or during an external postmortem exam of the body by a County Medical Examiner and had negative EMIT immunoassay results. These 14 samples are listed with their case number in Table 3. The other three were purchased.

Table 3. List of the 17 blank whole blood samples that were extracted and analyzed to demonstrate the absence of interference from the matrix.

15-0002-SC	**NB1**
15-0008-heart	**NB2**
15-0011-SC	**NB3**
15-0019-CoME	**NB4**
15-0028-CoME	**NB5**
15-0033-CoME	**NB6**
15-0043-heart	**NB7**
15-0048-SC	**NB8**
15-0052-CoME	**NB9**
15-0057-heart	**NB10**
15-0066-CoME	**NB11**
15-0072-CoME	**NB12**
15-0081-CoME	**NB13**
15-0087-CoME	**NB14**
Bovine Whole Blood in EDTA	**NB15**
Whole Human Blood – Single Donor 10884	**NB16**
Blank Whole Blood (pooled)	**NB17**

SC = subclavian
CoME = procured by County Medical Examiner
15-00XX = Case number used at the WVOCME

Each sample was analyzed to demonstrate the absence of common interferences from the matrix by monitoring the quantifier and qualifier ions of the analytes of interest at their respective retention times.

Interferences from other commonly encountered analytes

Interferences from non-targeted analytes that are present in the sample may impact the ability to detect, identify or quantitate a targeted analyte during analysis. Analytes that are commonly encountered in routine casework were evaluated at high therapeutic or lethal concentrations to determine their potential to interfere with the analytes of interest. Six separate drug mix solutions containing commonly encountered analytes were prepared from neat

reference materials and injected one time each. Table 4 lists the components of each drug mix solution and their associated concentrations.

Table 4. Commonly encountered analytes prepared in methanol.

Analyte	Concentration (ng/mL)
Low Dose Opioids Mix: fentanyl, norfentanyl, buprenorphine, norbuprenorphine, acetylfentanyl, oxymorphone	500
Opiate Mix: morphine, codeine, hydrocodone, hydromorphone, naloxone, acetylcodeine, oxycodone, oxymorphone, dihydrocodeine, 6-monoacetylmorphine	5,000
Cocaine Mix: cocaine, benzoylecgonine, ecgonine methyl ester	5,000
Benzodiazepine Mix: diazepam, nordiazepam, 7-aminoclonazepam, temazepam, alprazolam, clonazepam, lorazepam, midazolam, chlordiazepoxide, demoxepam, oxazepam	5,000
Amphetamine Mix: phenethylamine, pseudoephedrine, amphetamine, MDA, MDMA, methamphetamine, phentermine, phenylpropanolamine	5,000
Acid/Neutral Mix: acetaminophen, ibuprofen, butalbital, phenytoin, barbital	50,000

Interference was determined based on whether or not ions of these analytes were found at similar retention times to the drugs of interest. Each sample was analyzed to demonstrate the absence of interference from commonly encountered analytes by monitoring the quantifier and qualifier ions of the analytes of interest at their respective retention times.

Carryover

Analyte carryover into a subsequent sample may lead to inaccurate qualitative or quantitative results when analyzing samples. The analytes of interest were evaluated at high

therapeutic or lethal concentrations to determine their potential for carryover. Over three different runs, an extracted negative matrix sample was analyzed immediately following a 5000 ng/mL sample of the extracted alkaline drugs of interest.

All three runs of the 5000 ng/mL sample were analyzed to ensure that all thirty-one of the alkaline compounds of interest, as well as the internal standard, were present Then each of the three extracted negative matrix samples was analyzed to demonstrate the absence of quantifier and qualifier ions of the analytes of interest.

Calibration Model

MassHunter creates a calibration curve by plotting response ratio (area of analyte/area of internal standard) vs. concentration. To determine the concentration of a sample, the response ratio is determined and the concentration can be calculated using the regression equation for the curve. A calibration model is the mathematical equation that demonstrates this relationship between the concentration of analyte and the corresponding instrument response and the use of an incorrect calibration model may lead to inaccurate quantitative results. SWGTOX guidelines state that the calibration model should use at least six non-zero calibrators[8]. Eight concentrations (10, 25, 50, 100, 250, 500, 1000, and 2000 ng/mL) of the analytes were prepared and analyzed in five different analytical runs. The presence of quantifier and qualifier ions at their respective retention times was required to deem a calibration point usable for the determination of the calibration model and subsequent studies. Following SWGTOX guidelines, the origin for each regression equation was not included as a calibration point[8].

The appropriateness of the chosen calibration model was confirmed using statistical tests for model fit by first determining if weighting needed to be applied to the data. SWGTOX

guidelines state that weighting is required if there is a statistical difference in the variance at the lowest and highest concentrations[8]. The variance at these concentrations was calculated as the square of the standard deviation. An F-test was performed to determine if a statistical difference existed between the two variances and the p-value was examined. If $p > 0.05$, then the difference between the two measurements of variance was not statistically significant and the data was homoscedastic. If $p < 0.05$, the difference in the variance between the two measurements was statistically significant and the data was heteroscedastic, therefore weighting was used. If an analyte required that weighting be used, the weighting factor was determined. In MassHunter the weighting choices are $1/x$ and $1/x^2$. The weighting factor was determined based upon a graph of the variance as a function of the concentration. A $1/x$ weighting factor was chosen if the variance varied linearly with the concentration. While a $1/x^2$ weighting factor was chosen if the variance varied in a parabolic fashion with the concentration.

Once the weighting factor was determined, the model order was determined using the regression equation for a linear model and a quadratic model (Equation 1):

$$Linear: y = mx + b$$

$$Quadratic: y = ax^2 + bx + c \quad (1)$$

SWGTOX guidelines state that the simplest calibration model that best fits the concentration-response relationship should be used[8]. To determine if the addition of the quadratic term to the regression equation was justified, a two-way ANOVA test was performed. If the use of a quadratic model lead to a significant increase in variance ($p < 0.05$); then a quadratic model was used. If the increase in variance was not significant ($p > 0.05$); a linear model was used. All statistical analyses were performed using an Excel spreadsheet[9].

Bias

Bias is the closeness of agreement between the mean value of a large series of measurements and the accepted value. The presence of bias leads to inaccurate quantitative results when using GC/MS and must be evaluated. Three pooled, fortified matrix samples were created by spiking bovine whole blood at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations. LLE was performed in triplicate for each concentration over five different days, for a total of 15 samples at each concentration. SWGTOX guidelines state that the maximum acceptable bias is ±20% at each concentration[8]. Bias was calculated using the following equation:

$$Bias\ (\%) at\ Concentration_x = \left[\frac{Grand\ Mean\ of\ Calculated\ Concentration_x - Nominal\ Concentration_x}{Nominal\ Concentration_x}\right] x100 \quad (2)$$

Precision

Precision is the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogenous sample. Imprecision leads to inaccurate quantitative results. The same data from the bias study was used to evaluate within-run and between-run precision. Precision is expressed as the coefficient of variation (%CV). SWGTOX guidelines state that the % CV shall not exceed 20% at each concentration[8]. Within- and between-run precision were calculated using the one-way ANOVA approach with the run number as the grouping variable. Using this approach, the within-run precision was calculated for each concentration using the following equation, where MS_{wg} is the mean square within groups obtained from the ANOVA table:

$$Within - Run\ CV(\%) = \left[\frac{\sqrt{MS_{wg}}}{grand\ mean\ for\ each\ concentration}\right] x100 \quad (3)$$

Likewise, the between-run precision was calculated for each concentration using the following equation, where MS_{bg} is the mean square between groups obtained from the ANOVA table and *n* is the number of observations in each group:

$$Between - Run\ CV(\%) = \left[\frac{\sqrt{\frac{MS_{bg}+(n-1)*MS_{wg}}{n}}}{grand\ mean\ for\ each\ concentration}\right] x100 \quad (4)$$

The ANOVA calculations were performed using an Excel spreadsheet.

Limit of quantitation

The LOQ is an estimate of the lowest concentration of an analyte in a sample that can be reliably measured. SWGTOX guidelines state that the LOQ may be defined as the lowest acceptable non-zero calibrator if all detection and identification criteria are met[8]. The LOQ was administratively set to be equal to the lowest non-zero calibrator for each analyte.

Limit of detection

The LOD is an estimate of the lowest concentration of analyte in a sample that is reliably differentiated from the signal due to the blank matrix and identified by the analytical method used. SWGTOX guidelines state that for analytes following a linear calibration model, the LOD may be estimated from a minimum of three linear calibration curves constructed over the working concentration range over different runs[8]. The LOD was estimated from the standard deviation of the *y* intercept (s_y) and the average slope (Avg_m) using the following equation:

$$LOD = \frac{3.3s_y}{Avg_m} \quad (5)$$

The LOD for each analyte was estimated using Equation 5 if a linear calibration model was established Following SWGTOX guidelines for analytes that did not follow a linear calibration model, the LOD was defined as the lowest non-zero calibrator[8].

Results and Discussion

Matrix interference

Interference from the matrix was not observed in any of the whole blood samples analyzed. A representative GC/MS total ion chromatogram (TIC) for an extracted negative matrix can be seen in Figure 1.

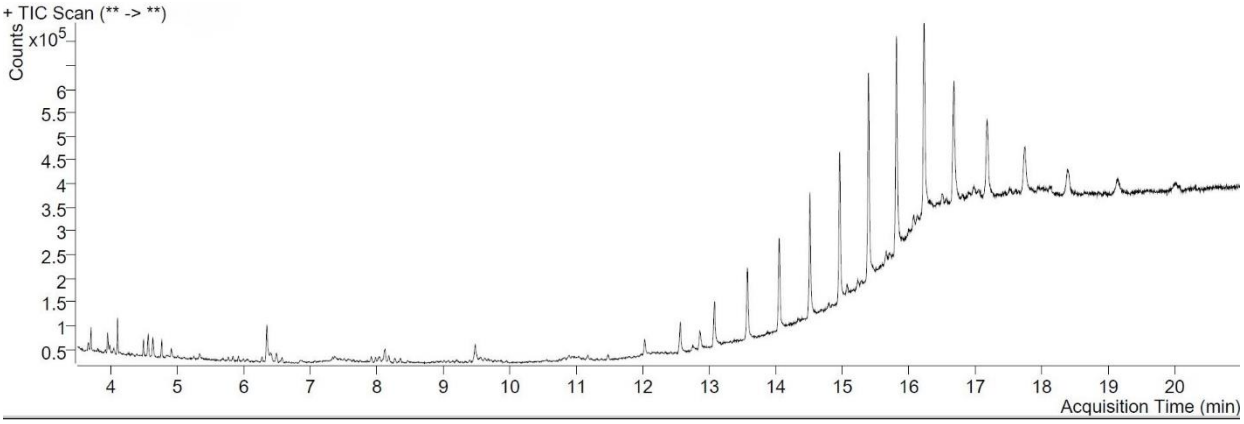

Figure 1. Representative GC/MS TIC for one extracted negative whole blood sample, showing no matrix interferences.

Interferences from other commonly encountered analytes

No interferences were observed from any of the commonly encountered drugs. The TIC for each mix can be found in Appendix A (Figures 1-6).

Carryover

Each negative matrix sample was analyzed with both ChemStation and MassHunter software and there was no response observed for any of the quantifier and qualifier ions at the retention times of the 31 analytes of interest.

Calibration Model

No quantifier and qualifier ions were identified in the 10 ng/mL calibrator, deeming it unacceptable for use. The lowest calibrator deemed to be acceptable for EDDP, methadone, amitriptyline, nortriptyline, and sertraline was 25 ng/mL and the response ratios for the calibrators used can be found in Appendix B (Tables 1-5). For paroxetine, the lowest calibrator was determined to be 50 ng/mL and the response ratios for the calibrators used can be found in Appendix B (Table 6).

The heteroscedasticity of the data was tested by comparing the variance of the measurements at the lowest concentration with the variance of the measurements at the highest concentration to determine the p-value (Table 5). The p-value calculated for each analyte was less than 0.05, indicating a statistically significant difference between the variance of the two measurements; therefore, a weighting factor was applied to the data.

Table 5. Heteroscedasticity testing for each analyte.

Analyte	**p-value**
EDDP	7.74E-8
Methadone	1.31E-8
Amitriptyline	2.90E-8
Nortriptyline	8.34E-6
Sertraline	2.26E-10
Paroxetine	2.54E-5

A graph of the variance vs. concentration for each calibrator determined what weighting factor should be used for the regression model. The variance of each analyte varied in a parabolic fashion with the concentration so a $1/x^2$ weighting factor was chosen. A representative graph of the variance at each concentration can be seen in Figure 2.

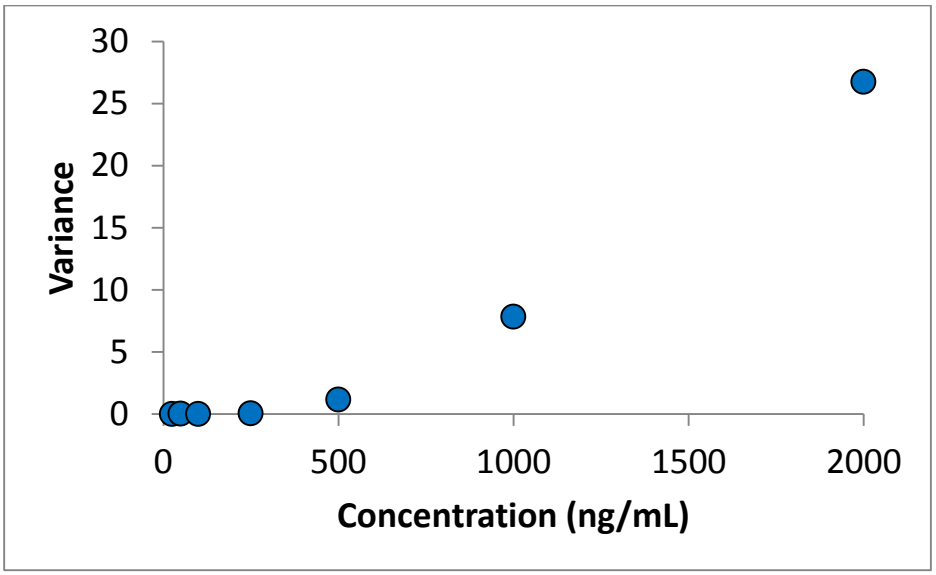

Figure 2. Representative graph of the variance at each concentration.

The regression equation for a linear and quadratic model, using $1/x^2$ weighting, was determined using MassHunter for all five days of the calibration model study, (Appendix B: Tables 7-12). Using an Excel spreadsheet, a two-way ANOVA test was performed to determine if the increase in variance, or explainable error, was statistically significant upon addition of the quadratic term. The increase in variance was not significant for EDDP, methadone, and amitriptyline and a linear model was chosen (Table 6). The p-value for sertraline was not calculated in the Excel spreadsheet used, because there was a decrease in the explained variance; therefore, the simplest model (linear) was chosen[9]. The increase in variance was significant, $p < 0.05$, for nortriptyline and paroxetine and a quadratic model was chosen (Table 6).

Table 6. The calculated p-values for each analyte using a two-way ANOVA test.

Analyte	p-value
EDDP	0.698
Methadone	0.249
Amitriptyline	0.132
Nortriptyline	1.85E-7
Sertraline	>0.05
Paroxetine	3.80E-5

A linear calibration model with inverse weight by concentration squared ($1/x^2$) was established from 25 ng/mL to 2000 ng/mL for EDDP, methadone, amitriptyline, and sertraline. A quadratic calibration model with inverse weight by concentration squared ($1/x^2$) was established from 25 ng/mL to 2000 ng/mL for nortriptyline. A quadratic calibration model with inverse weight by concentration squared ($1/x^2$) was established from 50 ng/mL to 2000 ng/mL for paroxetine.

Bias

Using the regression model determined in the calibration model study, the calculated concentrations at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) levels were determined as shown in Appendix C (Tables 1-6). Using Equation 2, bias was calculated at low, medium, and high concentrations for EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine (Table 7).

Table 7. Percent bias at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations.

	Bias (%)		
Analyte	**Low**	**Medium**	**High**
EDDP	-0.53	4.57	7.68
Methadone	-3.20	10.47	8.65
Amitriptyline	-3.64	10.76	8.82
Nortriptyline	-4.89	17.94	2.69
Sertraline	-2.93	12.20	12.58
Paroxetine	-3.64	16.11	2.38

The bias for EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine falls below the maximum acceptable bias at each concentration (75, 750, and 1500 ng/mL).

Precision

Using the same data that was used to the bias study, a one-way ANOVA was performed at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations for EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine. The MS_{wg} and the MS_{bg} for each analyte at all three concentrations can be found in Appendix C (Table 7). Equation 3 was used to calculate the within-run precision at low, medium, and high concentrations for EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine (Table 8). Equation 4 was used to calculate the between-run precision (Table 9).

Table 8. Within-run precision at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations.

	Precision (% CV)		
Analyte	**Low**	**Medium**	**High**
EDDP	9.28	12.02	3.99
Methadone	5.66	6.04	3.37
Amitriptyline	6.62	5.86	3.16
Nortriptyline	12.86	5.43	2.73
Sertraline	9.38	5.70	3.39
Paroxetine	12.22	7.56	6.49

Table 9. Between-run precision at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations.

	Precision (% CV)		
Analyte	**Low**	**Medium**	**High**
EDDP	11.59	10.46	5.51
Methadone	7.29	7.83	5.24
Amitriptyline	7.49	7.90	4.69
Nortriptyline	17.32	7.43	5.05
Sertraline	8.37	8.53	5.71
Paroxetine	18.88	8.48	8.34

The % CV for EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine falls below the maximum acceptable precision at each concentration (75, 750, and 1500 ng/mL).

Limit of quantitation

The LOQ was set at 25 ng/mL for EDDP, methadone, amitriptyline, nortriptyline, and sertraline. The LOQ was set at 50 ng/mL for paroxetine. The extracted ion chromatograms (EIC) for the quantifier ions for EDDP (277.2 *m/z*), methadone (72.1 *m/z*), amitriptyline (58.1 *m/z*), nortriptyline (44.1 *m/z*), sertraline (274.0 *m/z*), and paroxetine (192.1 *m/z*) at the lowest calibrator can be seen in Figure 3.

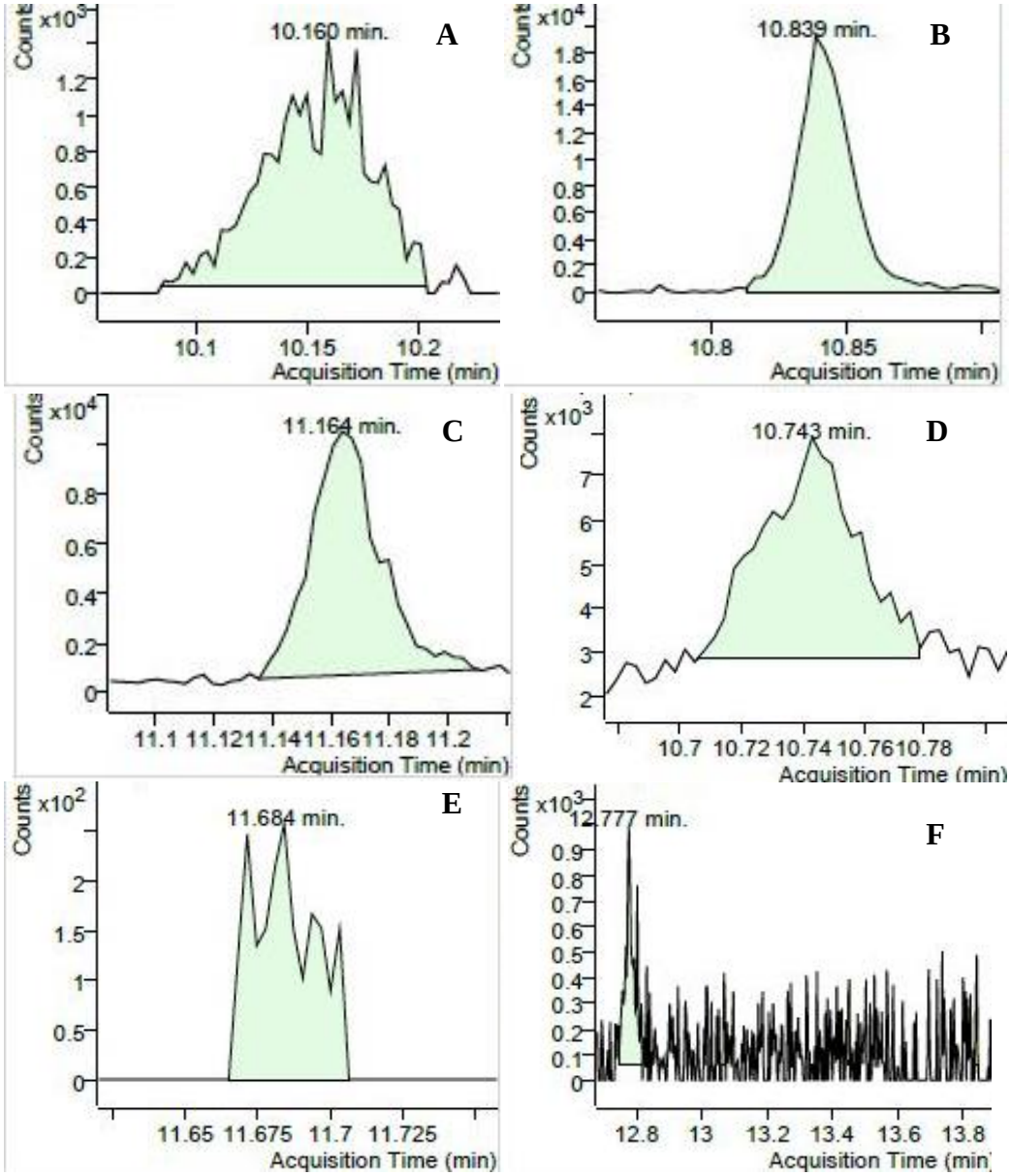

Figure 3. The EIC for the quantifier ions for EDDP (A), methadone (B), amitriptyline (C), nortriptyline (D), and sertraline (E) at 25 ng/mL and for paroxetine (F) at 50 ng/mL.

The EIC for the quantifier and qualifier ions for EDDP (277.2, 262.1, 220.1, 165.0 *m/z*), methadone (72.1, 262.1, 220.1, 165.0 *m/z*), amitriptyline (58.1, 202.1, 189.0, 215.0 *m/z*), nortriptyline (44.1, 202.1, 189.0, 215.0 *m/z*), sertraline (274.0, 262.1, 220.1, 165.0 *m/z*), and paroxetine (192.1, 138.0, 177.0, 109.0 *m/z*) at the lowest calibrator can be seen in Figure 4.

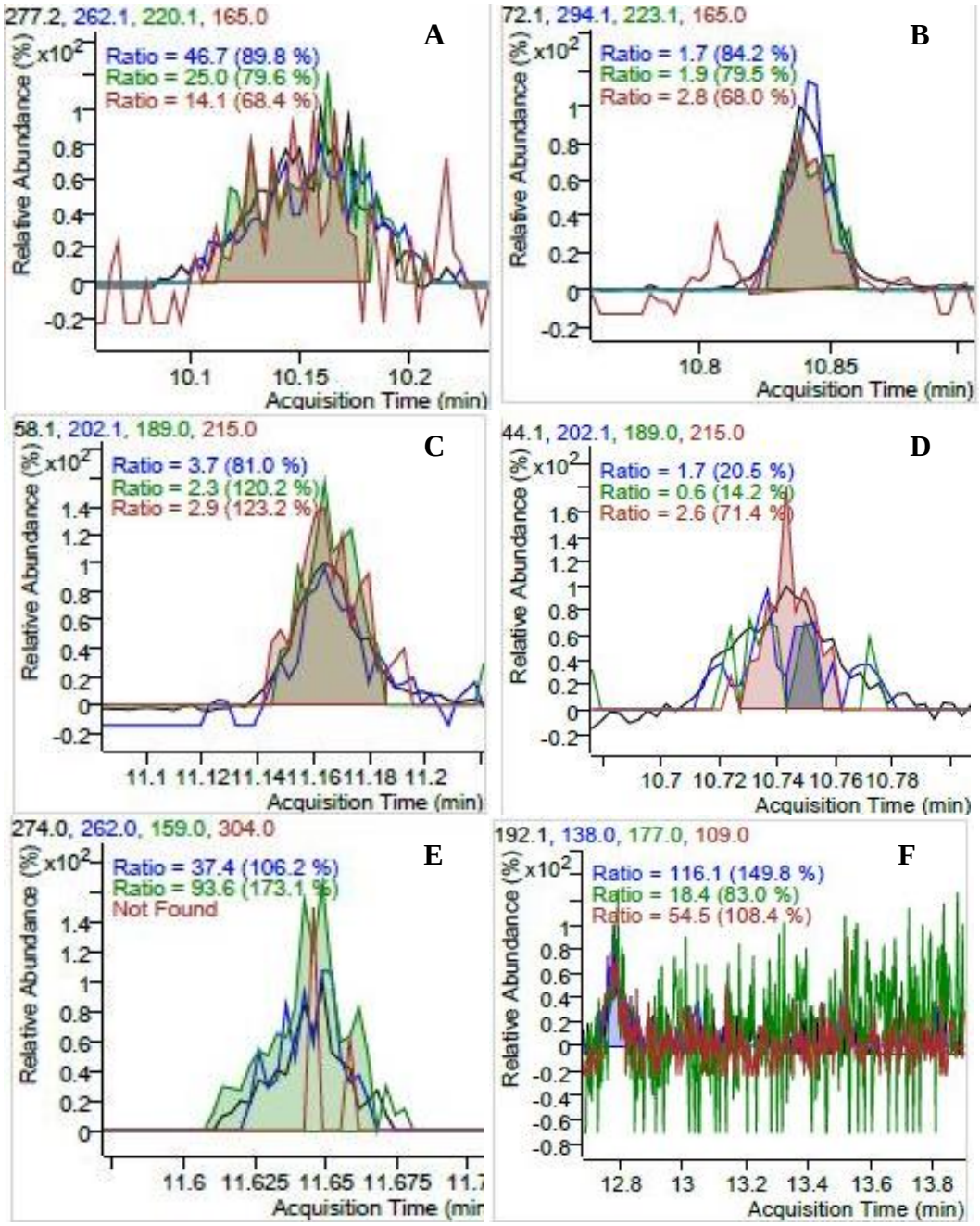

Figure 4. The EIC for the quantifier and qualifier ions for EDDP (A), methadone (B), amitriptyline (C), nortriptyline (D), and sertraline (E) at 25 ng/mL and for paroxetine (F) at 50 ng/mL.

Limit of detection

The data used in the calibration model study was used to determine the LOD for EDDP, methadone, amitriptyline, and sertraline (Appendix D: Table 1). Equation 5 was used to calculate the estimated LOD. The LOD for EDDP was estimated to be 18 ng/mL, methadone was estimated to be 6 ng/mL, amitriptyline was estimated to be 6 ng/mL, and sertraline was estimated to be 10 ng/mL.

Since a quadratic model was established for nortriptyline and paroxetine, Equation 5 could not be used to estimate the LOD. The LOD was administratively set to be equal to the LOQ for both analytes; nortriptyline at 25 ng/mL and paroxetine at 50 ng/mL.

Conclusions

No matrix interference or interference from other commonly encountered analytes was observed. All 31 alkaline compounds were analyzed to ensure that no carryover was observed for samples at high therapeutic or lethal concentrations. A regression model that was linear with inverse weight by concentration squared ($1/x^2$) was established with acceptable bias and precision for EDDP, methadone, amitriptyline, and sertraline. A quadratic calibration model with inverse weight by concentration squared ($1/x^2$) was established with acceptable bias and precision for nortriptyline and paroxetine. The LOQ was administratively set as the lowest acceptable calibrator for EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine. The LOD was estimated for EDDP (18 ng/mL), methadone (6 ng/mL), amitriptyline (6 ng/mL), and sertraline (10 ng/mL). The LOD was administratively set as the LOQ for nortriptyline (25 ng/mL) and paroxetine (50 ng/mL). The GC/MS method developed at the West

Virginia Office of the Chief Medical Examiner Toxicology Laboratory has been shown to work reproducibly and accurately.

For future studies, calibration model, bias, precision, LOQ, and LOD for the alkaline compounds not included in this project would be beneficial.

References

1. M. Tominaga, T. Michiue, O. Inamori-Kawamoto, A.M. Hishmat, S. Oritani, M. Takama, T. Ishikawa, H. Maeda. Efficacy of drug screening in forensic autopsy: Retrospective investigation of routine toxicological findings. *Leg. Med.* **2015**, *17(3)*, 172-176
2. R.J. Dinis-Oliveira, F. Carvalho, J.A. Duarte, F. Remião, A. Marques, A. Santos, T. Magalhães. Collection of biological samples in forensic toxicology. *Toxicol. Mech. Methods* **2010**, *20(7)*, 363-414.
3. E. Han, E. Kim, H. Hong, S. Jeong, J. Kim, S. In, H. Chung, S. Lee. Evaluation of postmortem redistribution phenomena for commonly encountered drugs. *Forensic Sci. Int.* **2012**, *219(1-3)*, 265-271.
4. F.T. Peters, O.H. Drummer, F. Musshoff. Validation of new methods. *Forensic Sci. Int.* **2007**, *165(2-3)*, 216-224.
5. O.H. Drummer. Postmortem toxicology of drugs of abuse. *Forensic Sci. Int.* **2004**, *142(2-3)*, 101-113.
6. I.I. Papoutsis, S.A. Athanaselis, P.D. Nikolaou, C.M. Pistos, C.A. Spiliopoulou, C.P. Maravelias. Development and validation of an EI-GC-MS method for the determination of benzodiazepine drugs and their metabolites in blood: Applications in clinical and forensic toxicology. *J. Pharm. Biomed. Anal.* **2010**, *52(4)*, 609-614.

7. C. Miller, O. Pleitez, D. Anderson, D. Mertens-Maxham, N. Wade. Asenapine (Saphris®): GC-MS Method Validation and the Postmortem Distribution of a New Atypical Antipsychotic Medication. *J. Anal. Toxicol*. **2013**, *37(8)*, 559-564.
8. Scientific Working Group for Forensic Toxicology. Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. **2013**, *Revision 1*.
9. B. Desharnais, F. Camirand-Lemyre, P. Mireault, C.D. Skinner. "Validation of calibration models: Development and testing of a practical procedure." Presentation at 2014 Annual Meeting of the Society of Forensic Toxicologists, Grand Rapids, MI, October 22, 2014.

Acknowledgements

This project benefitted from the input of Kristen Bailey, Myron Gebhardt, Dr. James Kraner, and Dr. Lauren Richards-Waugh, all of whom provided assistance and critiques of the presentation and these studies. The author also thanks the analysts and employees of the West Virginia Office of the Chief Medical Examiner Toxicology Laboratory, who gave valuable advice and encouragement and made the experience within their laboratory worthwhile.

Appendix A

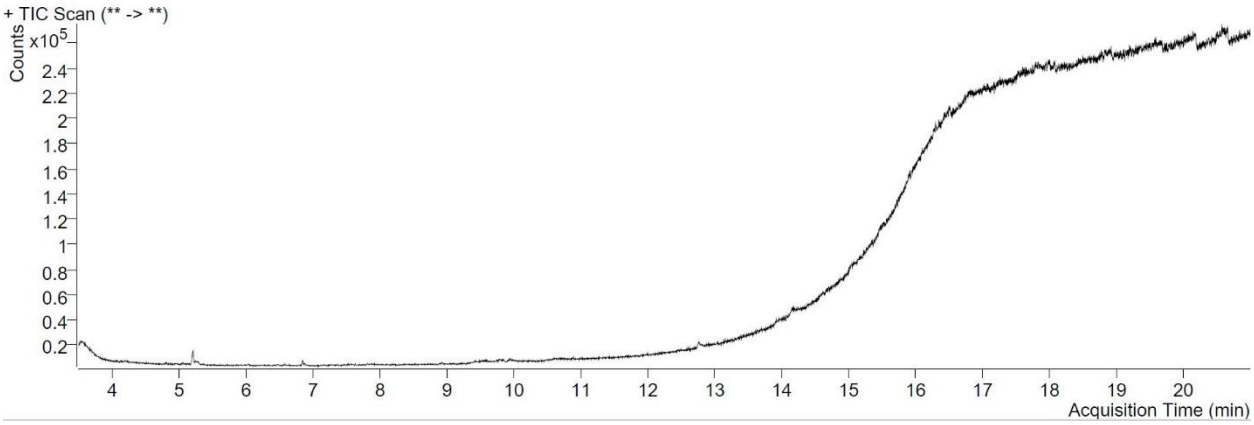

Figure 1. The TIC for the Low Dose Opioids mix.

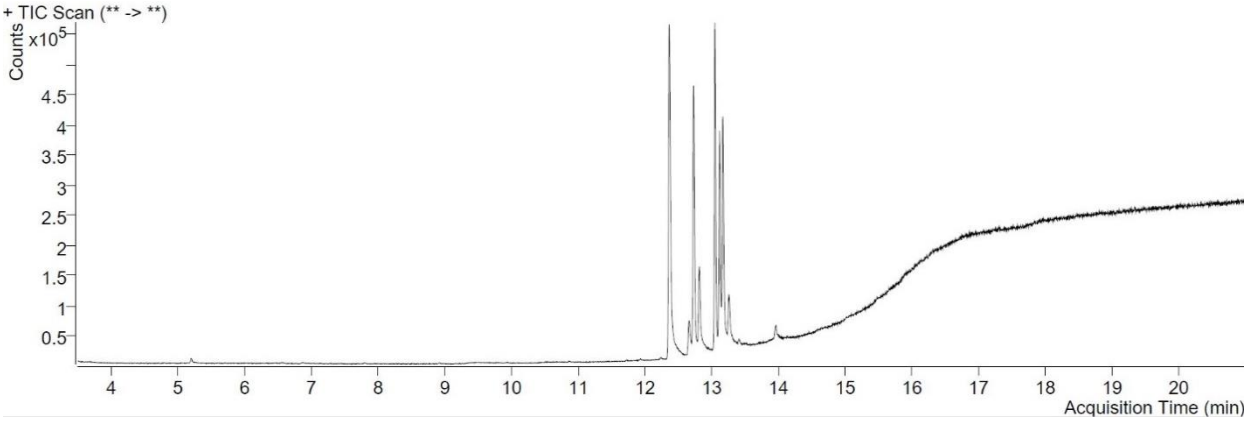

Figure 2. The TIC for the Opiate mix.

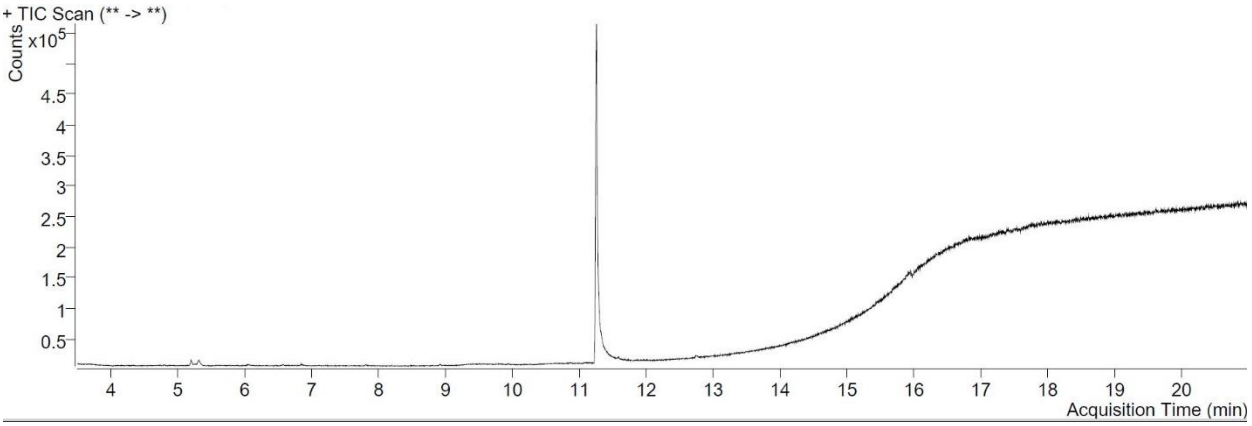

Figure 3. The TIC for the Cocaine mix.

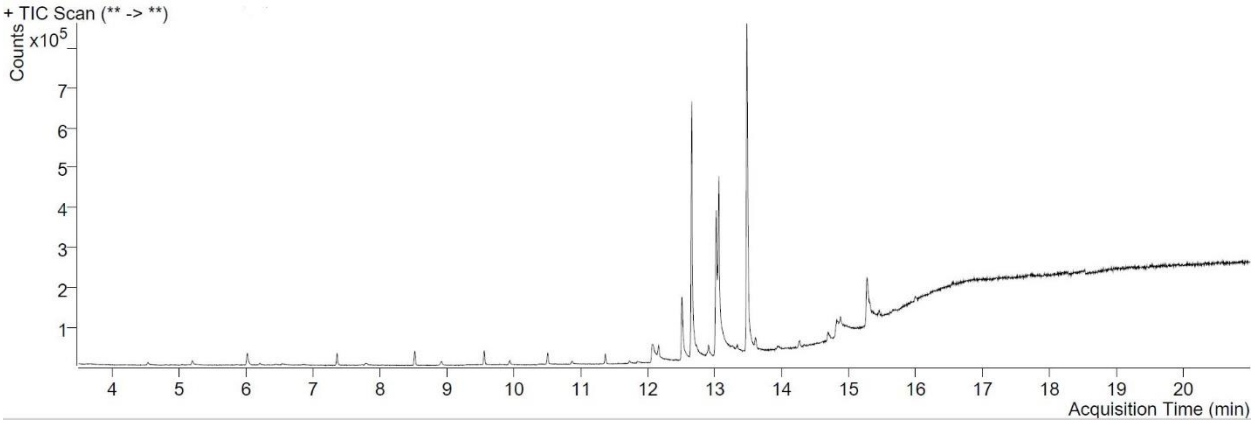

Figure 4. The TIC for the Benzodiazepine mix.

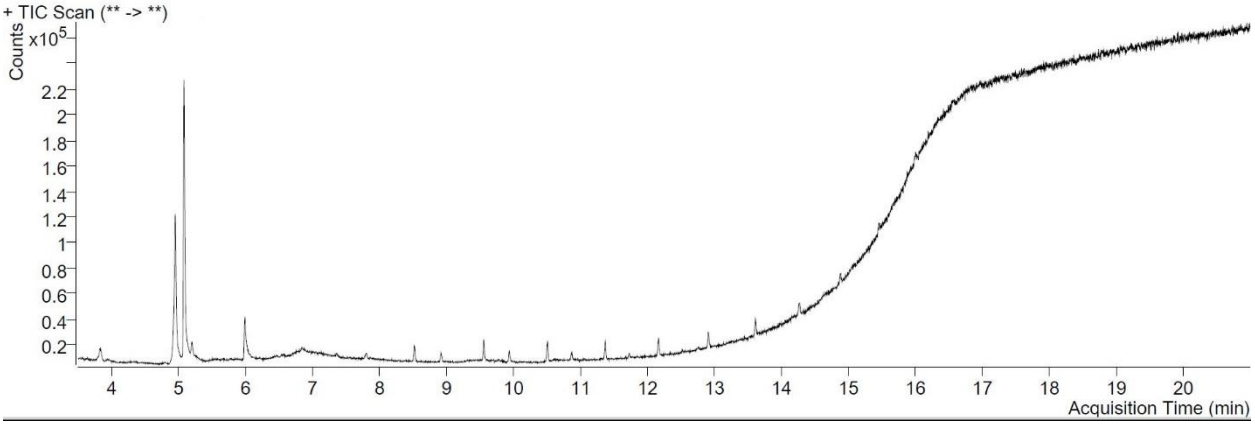

Figure 5. The TIC for the Amphetamine mix.

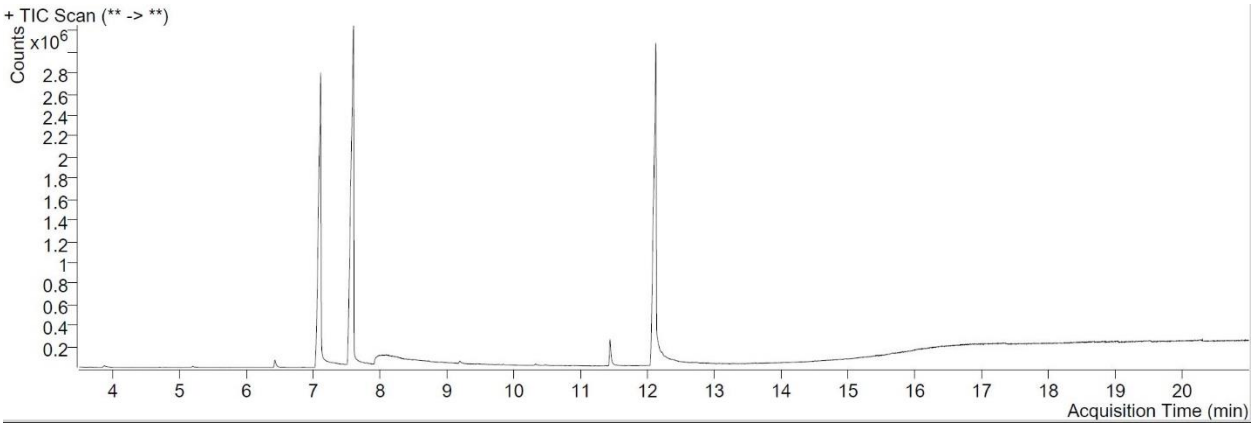

Figure 6. The TIC for the Acid/Neutral mix.

Appendix B

Table 1. Raw data for EDDP used in the calibration model study.

	Response Ratio				
Concentration (ng/mL)	**6/04/15**	**6/24/15**	**7/09/15**	**7/10/15**	**7/13/15**
25	0.052	0.017	0.011	0.012	0.021
50	0.108	0.050	0.039	0.036	0.038
100	0.253	0.101	0.082	0.073	0.081
250	0.631	0.239	0.214	0.204	0.217
500	1.427	0.734	0.556	0.474	0.511
1000	2.386	1.412	1.152	0.990	0.932
2000	5.103	2.807	2.251	1.787	2.072

Table 2. Raw data for methadone used in the calibration model study.

	Response Ratio				
Concentration (ng/mL)	**6/04/15**	**6/24/15**	**7/09/15**	**7/10/15**	**7/13/15**
25	0.350	0.358	0.331	0.320	0.262
50	0.664	0.680	0.656	0.650	0.559
100	1.638	1.327	1.321	1.238	1.206
250	3.850	3.133	3.485	3.247	3.068
500	8.348	9.640	9.078	7.885	7.245
1000	13.561	18.111	17.911	15.196	13.434
2000	29.958	38.139	33.232	26.345	28.277

Table 3. Raw data for amitriptyline used in the calibration model study.

	Response Ratio				
Concentration (ng/mL)	**6/04/15**	**6/24/15**	**7/09/15**	**7/10/15**	**7/13/15**
25	0.215	0.348	0.306	0.310	0.259
50	0.444	0.868	0.637	0.600	0.522
100	1.106	1.261	1.246	1.171	1.117
250	2.813	2.815	3.354	3.122	2.903
500	6.278	8.698	8.608	7.597	6.784
1000	10.562	16.955	16.990	14.712	12.606
2000	24.784	36.964	31.506	25.676	26.095

Table 4. Raw data for nortriptyline used in the calibration model study.

Concentration (ng/mL)	Response Ratio				
	AM 7/17/2015	PM 7/17/15	AM 7/20/15	PM 7/20/15	7/21/15
25	0.178	0.474	0.179	0.307	0.171
50	0.358	0.681	0.440	0.567	0.353
100	1.544	1.735	1.031	1.247	1.092
250	3.579	4.780	3.890	4.308	3.096
500	10.052	10.324	7.689	9.589	8.723
1000	21.066	27.671	22.788	21.350	21.125
2000	52.526	57.701	51.689	52.790	48.788

Table 5. Raw data for sertraline used in the calibration model study.

Concentration (ng/mL)	Response Ratio				
	6/04/15	6/24/15	7/09/15	7/10/15	7/13/15
25	0.002	0.004	0.002	0.003	0.004
50	0.009	0.011	0.008	0.009	0.006
100	0.028	0.019	0.018	0.017	0.018
250	0.100	0.053	0.043	0.045	0.045
500	0.213	0.146	0.120	0.115	0.106
1000	0.396	0.309	0.227	0.236	0.209
2000	1.161	0.659	0.468	0.415	0.438

Table 6. Raw data for paroxetine used in the calibration model study.

Concentration (ng/mL)	Response Ratio				
	AM 7/17/2015	PM 7/17/15	AM 7/20/15	PM 7/20/15	7/21/15
50	0.047	0.031	0.024	0.024	0.016
100	0.079	0.100	0.046	0.046	0.059
250	0.179	0.299	0.235	0.235	0.214
500	0.603	0.610	0.414	0.414	0.520
1000	1.298	1.715	1.458	1.458	1.198
2000	3.009	3.227	2.987	2.987	2.674

Table 7. Regression equations for EDDP determined using MassHunter, for all five days of the calibration model study, for a linear and quadratic model using $1/x^2$ weighting.

		6/04/15	6/24/15	7/09/15	7/10/15	7/13/15	Average
	Intercept	-0.0143	-0.0172	-0.0166	-0.0115	-0.0049	-0.0129
Linear	**1st order slope**	0.0026	0.0013	0.0011	0.0009	0.0010	0.0014
	Intercept	-0.0162	-0.0132	-0.0141	-0.0109	-0.0022	-0.0114
Quadratic	**1st order slope**	0.0027	0.0012	0.0010	0.0009	0.0009	0.0013
	2nd order slope	-6.72E-8	1.38E-7	8.65E-8	1.92E-8	9.25E-8	5.39E-8

Table 8. Regression equations for methadone determined using MassHunter, for all five days of the calibration model study, for a linear and quadratic model using $1/x^2$ weighting.

		6/04/15	6/24/15	7/09/15	7/10/15	7/13/15	Average
	Intercept	-0.0391	-0.1002	-0.1055	-0.0426	-0.0893	-0.0754
Linear	**1st order slope**	0.0153	0.0168	0.0163	0.0141	0.0136	0.0152
	Intercept	-0.0604	-0.0172	-0.0690	-0.0442	-0.0694	-0.0521
Quadratic	**1st order slope**	0.0160	0.0143	0.0152	0.0141	0.0129	0.0145
	2nd order slope	-7.39E-7	2.88E-6	1.26E-6	-5.47E-8	6.87E-7	8.07E-7

Table 9. Regression equations for amitriptyline determined using MassHunter, for all five days of the calibration model study, for a linear and quadratic model using $1/x^2$ weighting.

		6/04/15	6/24/15	7/09/15	7/10/15	7/13/15	Average
	Intercept	-0.0906	-0.0761	-0.106	-0.0452	-0.0708	-0.0778
Linear	**1st order slope**	0.0118	0.0157	0.0156	0.0135	0.0126	0.0138
	Intercept	-0.0812	0.0168	-0.0748	-0.0407	-0.0529	-0.0465
Quadratic	**1st order slope**	0.0115	0.0129	0.0146	0.0134	0.0121	0.0129
	2nd order slope	3.26E-7	3.22E-6	1.09E-6	1.58E-7	6.19E-7	1.08E-6

Table 10. Regression equations for nortriptyline determined using MassHunter, for all five days of the calibration model study, for a linear and quadratic model using $1/x^2$ weighting.

		AM 7/17/15	PM 7/17/15	AM 7/20/15	PM 7/20/15	7/21/15	Average
Linear	**Intercept**	-0.4119	-0.2016	-0.3777	-0.2748	-0.3699	-0.3272
	1st order slope	0.0205	0.0231	0.0193	0.0202	0.0184	0.0203
Quadratic	**Intercept**	-0.2477	-0.0063	-0.1747	-0.0977	-0.1792	-0.1411
	1st order slope	0.0155	0.0172	0.0131	0.0148	0.0126	0.0146
	2nd order slope	5.69E-6	6.77E-6	7.03E-6	6.14E-6	6.61E-6	6.45E-6

Table 11. Regression equations for sertraline determined using MassHunter, for all five days of the calibration model study, for a linear and quadratic model using $1/x^2$ weighting.

		6/04/15	6/24/15	7/09/15	7/10/15	7/13/15	Average
Linear	**Intercept**	-0.0100	-0.0031	-0.0037	-0.0026	-0.0020	-0.0043
	1st order slope	0.0005	0.0003	0.0002	0.0002	0.0002	0.0003
Quadratic	**Intercept**	-0.0070	-0.0015	-0.0032	-0.0025	-0.0014	-0.0031
	1st order slope	0.0004	0.0002	0.0002	0.0002	0.0002	0.0002
	2nd order slope	1.05E-7	5.72E-8	1.58E-8	5.92E-9	2.35E-8	4.14E-8

Table 12. Regression equations for paroxetine determined using MassHunter, for all five days of the calibration model study, for a linear and quadratic model using $1/x^2$ weighting.

		AM 7/17/15	PM 7/17/15	AM 7/20/15	PM 7/20/15	7/21/15	Average
Linear	**Intercept**	-0.0221	-0.0484	-0.0477	-0.0406	-0.0474	-0.0412
	1st order slope	0.00121	0.0015	0.0012	0.0013	0.0012	0.0013
Quadratic	**Intercept**	0.0023	-0.0362	-0.0232	-0.0209	-0.0341	-0.0224
	1st order slope	0.0008	0.0013	0.0008	0.0010	0.0010	0.0010
	2nd order slope	3.18E-7	1.89E-7	3.82E-7	3.07E-7	2.07E-7	2.81E-7

Appendix C

Table 1. The calculated concentration for EDDP at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations, using the regression model determined in the calibration study.

		Concentration (ng/mL)				
		AM 7/17/15	**PM 7/17/15**	**AM 7/20/15**	**PM 7/20/15**	**7/21/15**
Low (75 ng/mL)	**Rep1**	91	76	91	67	71
	Rep 2	67	75	80	75	67
	Rep 3	74	76	79	69	61
Medium (750 ng/mL)	**Rep1**	770	847	603	761	804
	Rep 2	729	765	904	682	767
	Rep 3	881	817	918	792	724
High (1500 ng/mL)	**Rep1**	1640	1654	1715	1532	1534
	Rep 2	1781	1499	1714	1581	1587
	Rep 3	1660	1539	1631	1495	1665

Table 2. The calculated concentration for methadone at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations, using the regression model determined in the calibration study.

		Concentration (ng/mL)				
		AM 7/17/15	**PM 7/17/15**	**AM 7/20/15**	**PM 7/20/15**	**7/21/15**
Low (75 ng/mL)	**Rep1**	81	70	87	70	69
	Rep 2	72	69	74	71	71
	Rep 3	75	66	75	67	72
Medium (750 ng/mL)	**Rep1**	830	909	848	785	799
	Rep 2	812	797	867	717	781
	Rep 3	948	861	912	809	753
High (1500 ng/mL)	**Rep1**	1749	1698	1647	1563	1544
	Rep 2	1787	1567	1685	1581	1532
	Rep 3	1697	1609	1596	1523	1668

Table 3. The calculated concentration for amitriptyline at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations, using the regression model determined in the calibration study.

		Concentration (ng/mL)				
		AM 7/17/15	**PM 7/17/15**	**AM 7/20/15**	**PM 7/20/15**	**7/21/15**
Low (75 ng/mL)	**Rep1**	82	72	86	71	68
	Rep 2	70	71	72	70	70
	Rep 3	75	66	73	67	71
Medium (750 ng/mL)	**Rep1**	825	909	858	784	805
	Rep 2	800	803	878	719	781
	Rep 3	934	870	930	805	760
High (1500 ng/mL)	**Rep1**	1715	1702	1665	1562	1549
	Rep 2	1775	1571	1687	1601	1548
	Rep 3	1700	1616	1596	1538	1659

Table 4. The calculated concentration for nortriptyline at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations, using the regression model determined in the calibration study.

		Concentration (ng/mL)				
		AM 7/17/15	**PM 7/17/15**	**AM 7/20/15**	**PM 7/20/15**	**7/21/15**
Low (75 ng/mL)	**Rep1**	85	65	86	77	72
	Rep 2	78	60	77	55	66
	Rep 3	86	54	66	56	87
Medium (750 ng/mL)	**Rep1**	899	952	907	848	869
	Rep 2	873	854	930	780	808
	Rep 3	994	921	971	871	791
High (1500 ng/mL)	**Rep1**	1638	1628	1531	1508	1441
	Rep 2	1682	1496	1568	1527	1426
	Rep 3	1622	1542	1491	1501	1504

Table 5. The calculated concentration for sertraline at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations, using the regression model determined in the calibration study.

		Concentration (ng/mL)				
		AM 7/17/15	**PM 7/17/15**	**AM 7/20/15**	**PM 7/20/15**	**7/21/15**
Low (75 ng/mL)	**Rep1**	82	75	86	74	66
	Rep 2	64	75	73	73	67
	Rep 3	71	64	70	75	77
Medium (750 ng/mL)	**Rep1**	827	918	890	794	773
	Rep 2	812	816	905	733	775
	Rep 3	945	873	956	830	775
High (1500 ng/mL)	**Rep1**	1784	1739	1760	1618	1581
	Rep 2	1848	1552	1799	1595	1583
	Rep 3	1759	1667	1721	1645	1680

Table 6. The calculated concentration for paroxetine at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations, using the regression model determined in the calibration study.

		Concentration (ng/mL)				
		AM 7/17/15	**PM 7/17/15**	**AM 7/20/15**	**PM 7/20/15**	**7/21/15**
Low (75 ng/mL)	**Rep1**	65	72	94	62	99
	Rep 2	48	71	77	65	80
	Rep 3	63	70	66	67	85
Medium (750 ng/mL)	**Rep1**	919	902	893	798	860
	Rep 2	809	844	915	852	721
	Rep 3	986	913	915	964	771
High (1500 ng/mL)	**Rep1**	1706	1709	1408	1431	1537
	Rep 2	1663	1339	1590	1474	1436
	Rep 3	1760	1517	1486	1519	1461

Table 7. The mean square within groups (MS_{wg}) and the mean square between groups (MS_{bg}) obtained from a one-way ANOVA for EDDP, methadone, amitriptyline, nortriptyline, sertraline, and paroxetine at low (75 ng/mL), medium (750 ng/mL), and high (1500 ng/mL) concentrations for the five runs used in the bias and precision studies.

	Low (75 ng/mL)		**Medium (750 ng/mL)**		**High (1500 ng/mL)**	
Analyte	**MS_{wg}**	**MS_{bg}**	**MS_{wg}**	**MS_{bg}**	**MS_{wg}**	**MS_{bg}**
EDDP	48	129	8879	2415	4164	15414
Methadone	17	61	2502	7608	3012	15866
Amitriptyline	23	42	2370	8170	2668	12234
Nortriptyline	84	289	2308	8351	1766	14646
Sertraline	47	18	2304	10859	3271	21303
Paroxetine	78	403	4330	7711	9929	29399

Appendix D

Table 1. The slope, average slope (Avg_m), *y* intercept, standard deviation of the *y* intercept, and LOD for EDDP, methadone, amitriptyline, and sertraline using a linear, non-forced, inverse with inverse weight by concentration squared ($1/x^2$) calibration model.

Analyte	**Date**	**Slope**	**Avg_m**	***y* intercept**	**s_y**	**LOD**
EDDP	6/24/15	0.001323		-0.01724		
	7/09/15	0.001081		-0.01661		
	7/10/15	0.000921	0.001068	-0.01151	0.00573	18
	7/13/15	0.000946		-0.00488		
Methadone	6/24/15	0.016847		-0.10024		
	7/09/15	0.016339		-0.10551		
	7/10/15	0.014087	0.015210	-0.04261	0.02867	6
	7/13/15	0.013568		-0.08925		
Amitriptyline	6/24/15	0.015684		-0.07610		
	7/09/15	0.015577		-0.10615		
	7/10/15	0.013548	0.014355	-0.04524	0.02499	6
	7/13/15	0.012611		-0.07077		
Sertraline	6/24/15	0.000282		-0.00313		
	7/09/15	0.000224	0.000230	-0.00367	0.00069	10
	7/10/15	0.000215		-0.00263		
	7/13/15	0.000201		-0.00204		