Document Title:	**Evaluation of Sample Preparation Techniques for Matrix Effects and Absolute Recovery using UPLC-MS/MS using Opiates and Liver Tissue Specimens as the Model and Application to Post-Mortem Toxicology**
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Final Technical Report

Evaluation of sample preparation techniques for matrix effects and Absolute Recovery using UPLC-MS/MS using opiates and liver tissue specimens as the model and application to post-mortem toxicology

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Purpose:

With the "Opioid Crisis" which is currently occurring in the United States, being able to analyzed biological specimens for opiates (i.e. heroin and oxycodone) and opioids is extremely important. There has been a slow but steady increase in the number of deaths involving natural and semisynthetic opiates over the past 20 years. In the United States overdose deaths involving heroin increased by 20.6% from 2014-2015.[1] Determining manner and cause of death from an opioid overdose is usually associated with analyzing body fluids and tissues for the presence and concentration of an opioid. Blood is the body fluid analyzed for drug concentrations.[2,3] In forensic toxicology, blood is collected from a living person for human performance testing cases and analyzed as whole blood. However, in post-mortem cases, blood is no longer a true whole blood, since bodily processes that maintain homeostasis have stopped. Assumptions are commonly made that all bodily processes have stopped. Therefore, blood in the thoracic cavity can become contaminated as a result of post-mortem redistribution[4,5]. This involves drugs in the liver or gastrointestinal system leaking out and equilibrating with thoracic blood; thereby, potentially falsely elevating the drug concentration in the blood. This can affect the determination of manner and cause of death which may have dire criminal and/or civil consequences. Analysis of liver in conjunction with blood is a routine way to circumvent this issue. Advantages to using liver as an alternate matrix are: that it is large which provides ample sample size, drug concentrations do not fluctuate after death, and the liver is somewhat homogeneous. Disadvantages to using liver as an alternate specimen are the protein, fat, phospholipid matrix, and potential putrification products that make up the liver. These disadvantages necessitate effective clean-up or sample preparation before analysis of the liver.

With the increase in use of opioids to treat pain and subsequent increase in compliance testing of the individuals using these medications, newer sample preparation techniques have been developed and marketed. These techniques are primarily designed to increase the automatability of the analysis of blood or urine samples. The use of these techniques for difficult matrices such as liver has occurred with limited understanding of the effects that liver has on the analysis of the drug(s) of interest. These new techniques are fairly simple and easily automated, which makes them attractive for use in sample preparation. The newer sample preparation techniques are based on traditional techniques or hybrids with manufacturer's improvement(s) and the extracts are designed to be analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) which is more susceptible to matrix effects [6-13] than gas chromatography mass spectrometry (GC-MS). These traditional techniques involve solid-phase extraction (SPE), liquid-liquid extraction (LLE), and filtration. These techniques have limited published, peer-reviewed data and even manufacturer presented methods regarding use with tissue matrices such as liver. In order for these techniques to be effectively used for liver analysis, matrix effects and absolute recovery must be evaluated before method validation can be initiated in order to understand how effective these techniques are in cleaning up sample matrix, or at least a better understanding of the effect of the matrix on the analysis.

Project Design and Methods

This study was initially designed to validate a SPE method using SWGTOX[14] and FDA[15] Bioanalytical Guidelines (Aim 1) using opiates as the model drugs. Then nine techniques would be evaluated for matrix effects (ME), recovery (RE), and process efficiency (PE) (Aim 2) and the results compared to Aim 1 and the other techniques in Aim 2. The opiates analyzed were codeine, hydrocodone, hydromorphone, morphine, oxycodone, oxymorphone, and the heroin metabolite 6-acetyl morphine (6AM) The sample preparation techniques evaluated were performed following the manufacturer's guidelines[16-28], whenever possible or using a laboratory-validated liquid-liquid extraction technique. If the manufacture did not have a method specifically for liver tissue, the homogenate was processed as a urine or serum matrix. The liver extracts were analyzed using a previously validated ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for opiate analysis. The SPE techniques evaluated were the United Chemical Technologies (UCT) Clean Screen DAU and Xcel plates, and the Waters microElution MCX plate. The LLE techniques evaluated were previously validated LLE for GC-MS, and the Biotage Isolute Supported Liquid Extraction (SLE+) plate. The filtration techniques evaluated were the Biotage Isolute Phospholipid Depletion (PLD +) plate, the Thomson eXtreme Filter Vial, the UCT Clean Screen FASt plate, and Waters Oasis PRiME HLB plate. The LLE – SPE hybrid method evaluated was the UCT Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) process. The Phenomenex StrataXC SPE technique and the Waters Ostro pass through plate were later evaluated.

Post-mortem liver tissue was obtained from the VCU Health Autopsy Suite and the specimen containers were deidentified and only marked with a number starting with 1, and

then the next container was not necessarily marked with the next number in sequence. Drug standards (codeine, hydrocodone, hydromorphone, morphine, oxycodone, oxymorphone, and 6-AM) and their respective deuterated internal standards (codeine-d6, hydrocodone-d6, hydromorphone-d6, morphine-d6, oxycodone-d6, oxymorphone-d3, and 6-AM-d6) were purchased from Cerilliant Corporation. The UPLC-MSMS instrument was a Waters AcQuity XEVO-TQ-S Micro UPLC-MS/MS system. The analytical column used was a Restek Ultra Biphenyl 3um, 100 x 2.1 mm column. Chemicals and solvents used were LC-MS grade or better when available, otherwise ACS grade or better were used. The mobile phase was A (20 mM ammonium formate in water) and B (20 mM ammonium formate in methanol), see Table 1 for gradient. Liver tissue was homogenized in a 1:4 ratio with saline using either a Biotage Bead Ruptor 24 or a Dremel Tissue Tearor depending on the amount of tissue needed. The Bead Ruptor 24 involved placing 0.4 g tissue plus 0.8 mL saline in a 7 mL tube with 2.8 mm ceramic beads. The vial was capped and run 1 cycle for 30 seconds at 5.3 m/sec. The manufacture notes that speed is more important than rpm on the Bead Ruptor.

ME, RE & PE were evaluated at two fortified concentrations (0.06 and 7.5 mg/kg) and were analyzed as six replicates. The ME, RE & PE were determined mathematically from drug fortified samples with no matrix (non-extracted external standard, NEET), samples containing drugs and matrix before extraction (Before), and samples where a blank matrix is extracted then fortified with drug (After) but before instrument analysis.

Method validation was performed using SWGTOX and FDA bioanalytical guidelines. Validation included determining the calibration model, bias and precision, limits of detection and quantitation (LOD & LOQ), interferences, carryover, dilution integrity, ion

enhancement/suppression, matrix effect, and absolute recovery of the opiates. Calibrators were analyzed in duplicate each day of method validation and were analyzed on five different days. Quality controls (LOQ, 3LOQ, M, H, and Dilution integrity) were prepared as a batch, aliquoted for specific testing and stored appropriately. The controls were analyzed in triplicate each day of analysis.

Data Analysis

The Waters MassLynx software v4.0 was used to calculate drug concentrations using the six calibrator concentrations analyzed. Statistical calculations involving determinations of mean, standard deviation(SD), coefficient of variation (%CV), bias, precision, matrix effect, recovery and process efficiency were determined using Microsoft Excel 2016 software.

%CV was calculated as: $$\%\mathrm{CV} = \frac{\text{standard deviation}}{\text{mean response}}$$

Bias was calculated as: $$\text{Bias } \% = \left[\frac{\text{Calculated Concentration - Nominal Concentration}}{\text{Nominal Concentation}}\right] x100$$

Matrix Effect was calculated as: $$\mathrm{ME} = \left(\frac{\bar{X}\ \text{Height After}}{\bar{X}\ \text{Height NEET}}\ 1\right) x100$$

Recovery was calculated as: $$\mathrm{RE} = \left(\frac{\bar{X}\ \text{Height Before}}{\bar{X}\ \text{Height After}}\right) x100$$

Process efficiency was calculated as: $$\mathrm{PE} = \left(\frac{\text{Height Before}}{\text{Height NEET}}\right) x100$$

Findings

Aim 1 was to initially validate the UCT Clean Screen DAU columns as the sample preparation technique. The UCT DAU method has been around for many years and is considered to be the “gold standard” of SPE sample preparation. Post-mortem liver samples

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were obtained from the VCU Health Autopsy Suite. Initially only ~25 g of deidentified opiate free liver samples were requested. A preliminary study using ten concentrations from 0.02 – 50 mg/kg, demonstrated that the assay was linear from 0.02 – 10 mg/kg for all seven opiates. The five quality control materials were prepared at and aliquoted for the required stability studies. On Day 1 of validation, the calibrators and controls were extracted and analyzed. No 6-AM was detected in any of the control materials but its deuterated internal standard which was added was present. The controls were prepared again with the assumption that 6-AM was not added. When the new controls were analyzed, the same results were obtained. Thoughts then turned to a stability issue. This was addressed by preparing a 1 mg/kg 6-AM liver homogenate and then splitting the homogenate between test tubes containing no additive and ones containing 12 mg sodium fluoride / 15 mg potassium oxalate and allowing them to stand for 1 hr. The results of these samples were the same. Various individuals were consulted about the phenomenon, and a research toxicologist informed us that the enzymes and co-factors were present, all that was needed was some oxygen which was incorporated in the homogenization process. After much research, it was determined that while the liver hepatocyte may die in a couple of days, the liver enzymes are still active for at least 4 months, when stored frozen.

The liver tissue obtained was quickly used up due to the 6-AM stability issue. The pathologists were questioned about obtaining larger pieces of tissue. It was determined that they were only collecting “opioid free” tissue not “opiate free” tissue. Larger pieces (~100 g) were requested from all autopsies, and the samples would be screened before use. Autopsy were being requested less frequently, so an attempt was made to use store purchased beef liver as the matrix and then look at species differences later. The beef liver was a poor

specimen, it was not very dense and did not hold together well, which made it difficult to cut and then homogenize. Upon reviewing the results from the initial analytical runs, it was observed that the UCT DAU had inconsistent recoveries for the opiates. A small amount of opiate free liver tissue was obtained, so it was decided that Aim 1 should be postponed until Aim 2 was completed and then Aim 1 would be performed on the most effective technique.

Aim 2 was performed on 12 techniques, see Table 2 for results. Acceptability for ME was set at ±25 % and for RE and PE at 75 – 125 %. These criteria were later reevaluated because extraction of drugs from liver tissue is not as simple as extraction of drugs from urine or serum matrix. Liver is a far more complex matrix and while acceptability criteria should be set before beginning testing, the criteria may need to be reevaluated based on the results obtained.

No technique met the initial acceptability criteria set. However, most techniques were useable for analyzing opiate concentrations in post-mortem liver tissue, provided the ME, RE, and PE were consistent across the concentration range evaluated, low 0.06 mg/kg, high 7.5 mg/kg. The UCT Clean Screen DAU had consistent RE and PE, but the ME was not consistent between the two concentrations. The Phenomenex Strata XC had consistent PE, but the ME and RE were not consistent due to the low abundance of the After extraction fortified samples. The UCT Xcel had RE that were slightly below the criteria, but the ME, RE and PE were consistent across the two concentrations. The Waters uElution MCX had inconsistent ME, RE and PE results across the two concentrations. The traditional LLE had inconsistent ME, RE and PE across the two concentrations. The Biotage Isolute SLE+ results were acceptable for ME, RE and PE across the two concentrations, except for morphine. The RE and PE for morphine was ~30%, but this was consistent across the two concentrations. A morphine recovery of 30% is expected

with the SLE+. Morphine is a zwitter-ion. Zwitter-ions have two or more functional groups which due to their chemical nature prevent the molecule from being able to reach an unionized state, no matter what the pH of the solution. Therefore, in an LLE method, 50% recovery is the highest expected recovery. The Biotage Isolute PLD+ had PE that was acceptable, but the ME and RE were not consistent across the two concentrations. The Thomson eXtreme filter vial was not consistent across the two concentrations. This was due to the wide variability in the peak height results for each concentration. The UCT FASt was not analyzed because the eluate was red in color, and the Co-PI stated that it was not going to be analyzed on the UPLC-MSMS. The Waters Oasis PRiME HLB had two ME and one PE that were slightly below the criteria, but the ME, RE and PE were consistent across the two concentrations. The Waters Ostro flow through plate had consistent RE and PE, but the ME were not consistent across the two concentrations. The UCT QuEChERS had consistent RE, but the ME and PE were not consistent across the two concentrations. This may be caused by the necessity of vigorously mixing in the LLE portion of the technique. It was observed that the more vigorous the mixing, the more consistent the results. A mechanical mixer was not available for use in this method evaluation, so the salt tubes were shaken by two or more individuals in the laboratory.

The stability of 6-AM was readdressed. Carboxylesterases in blood metabolize heroin to 6-AM, and carboxylesterases in the liver metabolize 6-AM to morphine. The rate of metabolism of 6-AM in liver was evaluated since there are no reported cases of 6-AM being detected in liver tissue. Three liver samples that were collected near the same time were homogenized. The homogenate was aliquoted into test tubes and place in a 37 ^{o}C bath to simulate body temperature. Two samples were analyzed on one day and the third was analyzed on a separate

day. The aliquots were fortified with 6-AM at 1 mg/kg, briefly vortexed and then placed back in the 37 °C bath. At 15 min, 30 min, 1 hr., 2 hr., and 4 hr., aliquots were removed from the bath and placed in a dry ice:acetone bath to stop the enzymes. The aliquots were then analyzed using the UCT Xcel validated method. In two of the livers, the 6-AM concentration was < 0.02 mg/kg in 1 hour. In the other sample, the 6-AM concentration was 0.28 mg/kg at 4 hours, and using the metabolism curve to extrapolate the concentration, 6-AM should have been non-detected by 8 hr.

Aim 1 was revisited using the UCT Xcel technique, see Table 4. The calibration was assessed in duplicate over 5 days along with a blank and negative sample. The technique was linear from 0.02 – 10.0 mg/kg, and the r^2 values were > 0.992. Precision at the LOQ (0.02 mg/kg) are questionable for some of the opiates, which may indicate the need to narrow the linearity range and increase the LOQ. Prepared homogenate stability was assessed at three storage temperatures. The homogenates were not stable at room temperature for three days. They were beginning to putrefy by the time they were analyzed. Refrigerated homogenates were only stable for < 21 days, after which the oxycodone and oxymorphone concentrations trended lower over time. Homogenates that were stored frozen were stable for up to 2 freezing and thawing cycles. No common drugs of abuse interfered with the sample preparation or analytical methods. No carryover was observed in the blank or negative controls analyzed after the high calibrator (10 mg/kg).

Implications

Time was the most important point that was observed when completing this study. Liver tissue homogenates were only stable for < 3 weeks when stored in the refrigerator. Liver

homogenates were stable for two freeze-thaw cycles, but not for a third cycle. A recommendation for preparing quality control materials is to prepare the materials, aliquot into single use containers and store frozen until needed for analysis. Liver homogenates were not stable when left on the bench top for the weekend. Prepared sample extracts stored on the instrument at 10 °C were only stable for 48 hours post-preparation. Heroin and 6-AM are metabolized too rapidly in the body to be detected in liver samples; even individuals with poor liver function would metabolize them before the liver sample was collected and analyzed.

Liver is a difficult matrix to analyze. Sample preparation is not as simple for liver tissue as it is for blood or urine. Sample preparation techniques with similar principle techniques do not work similarly, and chemically-similar compounds do not always extract similarly using the same method. Not all sample preparation techniques are effective or reliable for the extraction of opiates from liver tissue. Before attempting to validate a sample preparation method for analyzing drugs in a matrix; the matrix effect, recovery and process efficiency of the method should be evaluated. Criteria should be established before the validation begins, but one must also be able to evaluate the results obtained.

Communication between the laboratory and the pathologist is important. Common assumptions on both parties can lead to misunderstandings, not only in specimen type, but also quantity and terminology.

Tables:

1. LC Gradient for analysis of opiates using Waters AcQuity system.

Time (min)	Flow Rate (mL/min)	%A	% B
Initial	0.6	95	5
1.50	0.6	60	40
3.00	0.6	0	100
3.50	0.6	0	100
3.60	0.6	95	5

2. Matrix Effect, Recovery and Process Efficiency for Aim 2 (n=6)

<u>UCT Clean Screen DAU</u>

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-29	-19	-27	-22	-34	-32
RE (%)	31	39	36	51	62	57
PE (%)	22	31	26	39	40	38

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	4	1	6	5	5	7
RE (%)	33	48	37	45	57	60
PE (%)	35	49	40	47	60	64

<u>Phenomenex StrataXC</u>

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-98	-99	-98	-99	-98	-98
RE (%)	972	1370	2470	3156	1598	1065
PE (%)	24	25	23	28	24	18

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-100	-100	-100	-100	-100	-100
RE (%)	>1000	>1000	>1000	>1000	>1000	>1000
PE (%)	22	25	24	28	24	20

<u>UCT Xcel</u>

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	16	7	11	7	19	19

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RE (%)	75	82	67	69	81	78
PE (%)	63	76	60	64	65	64

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	12	11	9	13	13	10
RE (%)	78	87	80	83	85	86
PE (%)	69	77	72	72	74	78

Waters uElution MCX

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-66	3	-4	19	-3	1
RE (%)	146	71	75	79	151	137
PE (%)	50	73	72	94	146	138

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-6	22	14	21	18	18
RE (%)	11	46	37	46	45	37
PE (%)	10	56	43	55	54	43

Liquid-Liquid Extraction

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-36	-43	-41	-47	-55	-41
RE (%)	52	63	42	41	40	56
PE (%)	33	36	25	22	18	33

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-44	-46	-49	-57	-58	-44
RE (%)	81	87	80	75	73	82
PE (%)	45	47	41	32	31	46

Biotage Isolute SLE+

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-8	-17	-4	-6	-15	-8
RE (%)	32	109	86	94	102	96
PE (%)	29	90	82	88	86	87

High 7.5 mg/kg

	M	C	HM	HC	OC	OM

ME (%)	0	0	0	0	-1	0
RE (%)	30	9	87	90	93	94
PE (%)	30	97	87	90	82	95

Biotage Isolute PLD+

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	43	-15	31	45	3	7
RE (%)	62	95	59	61	92	75
PE (%)	89	80	78	88	94	87

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	51	47	44	45	49	50
RE (%)	55	58	56	55	59	59
PE (%)	83	86	81	80	87	88

Thomson eXtreme Filter Vial

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-2	7	-86	1	0	62
RE (%)	91	98	172	115	107	82
PE (%)	90	106	24	117	107	133

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-12	-13	-16	-17	-12	-11
RE (%)	81	82	80	80	80	82
PE (%)	71	71	67	66	71	72

Waters Oasis PRiME HLB

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-15	5	-24	-4	-23	-33
RE (%)	105	100	108	105	106	122
PE (%)	89	106	82	100	81	82

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	-34	-20	-31	-11	-15	-24
RE (%)	117	108	108	107	108	110
PE (%)	77	85	4	94	90	82

Waters Ostro Plate

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	35	49	3	32	49	11
RE (%)	41	32	33	30	30	32
PE (%)	52	47	34	39	45	35

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	30	-14	11	-15	-1	24
RE (%)	40	40	39	33	39	41
PE (%)	52	34	43	28	38	51

UCT QuEChERS

Low 0.06 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	159	206	129	120	41	80
RE (%)	114	180	129	132	126	126
PE (%)	294	550	295	289	178	225

High 7.5 mg/kg

	M	C	HM	HC	OC	OM
ME (%)	111	101	112	117	104	117
RE (%)	106	122	115	120	118	113
PE (%)	224	246	243	260	241	245

Table 3:

Method Validation UCT Xcel

Bias (%) n=15

	M	C	HM	HC	OC	OM
LOQ (0.02 mg/kg)	14	8	3	6	22	12
Low (0.06 mg/kg)	3	-9	-8	-11	-18	-19
Mid (0.60 mg/kg)	2	-8	-4	-7	-13	-10
High (7.50 mg/kg)	-15	-20	-13	-18	-27	-26
Dil (50.0 mg/kg)	-25	-11	-19	-8	-14	-21

Inter-run Precision (%) n=15

	M	C	HM	HC	OC	OM
LOQ (0.02 mg/kg)	11	37	8	11	20	19
Low (0.06 mg/kg)	5	18	7	7	6	11
Mid (0.60 mg/kg)	8	11	9	7	6	8
High (7.50 mg/kg)	5	10	13	8	11	11
Dil (50.0 mg/kg)	32	12	22	5	7	15

Intra-run Precision (%) n=3

	M	C	HM	HC	OC	OM
LOQ (0.02 mg/kg)	7	48	11	11	38	28
Low (0.06 mg/kg)	9	18	8	4	8	8
Mid (0.60 mg/kg)	12	14	11	9	4	10
High (7.50 mg/kg)	4	15	7	7	7	5
Dil (50.0 mg/kg)	7	13	9	6	5	9

Table 4:

Interferences

Amobarbital	EME	Norfentanyl
Amphetamine	Fentanyl	Norketamine
Amitriptyline	Heroin	Norpropoxyphene
Benzoylecgonine	Imipramine	Nortriptyline
Butalbital	Ketamine	Pentobarbital
Cocaethylene	MDA	Phenobarbital
Cocaine	MDMA	Propoxyphene
Desipramine	Meperidine	Secobarbital
Doxepin	Methadone	THC
EDDP	Methamphetamine	THCA
EMDP	Norcodeine	Tramadol

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