LC-MS/MS Analysis of Large Drug Panels in Urine Using Three Sample Preparation Techniques

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Overview/Introduction

Urine drug testing to support pain management is a mainstay of the clinical toxicology laboratory. Reduced reimbursement has continued to put increasing pressure on laboratories. Many toxicology labs are moving to larger drug panels to increase throughput and efficiency, while reducing turn-around-time and cost. Methods using LC-MS with 50 or more drugs and metabolites are increasingly common. These panels increase throughout and improve laboratory efficiency, but create many challenges. While "dilute and shoot" methods are easy and affordable, they can result in shortened LC column lifetimes and increased MS instrumentation downtime. Matrix effects can also effect sensitivity and overall method performance. Supported Liquid extraction (SLE) and Solid Phase Extraction (SPE) provide cleaner samples for LC-MS/MS and more robust clinical assays. Successful sample preparation method development requires understanding the retention mechanism of the analytes in the assay. The octanol-water partition coefficient (logP) and acid-dissociation constant (pKa) of the compounds of interest are important properties that can guide sample preparation strategy and optimization of method conditions.

Methods

Three sample preparation methodologies were evaluated for 52 drug analytes:

Supported Liquid Extraction ISOLUTE® SLE+

- Works for acidic, basic and neutral drugs
- EtOAc, CH_2Cl_2 or MTBE for elution
- Addition of IPA can increase partitioning of more hydrophilic compounds

Polymeric Reversed Phase SPE EVOLUTE® EXPRESS ABN

- More hydrophilic compounds will exhibit poor retention
- Organic wash must be carefully controlled

Mixed Mode Polymeric SPE EVOLUTE® EXPRESS CX

- Reversed phase and cation exchange mechanisms
- Compounds can be retained by ion exchange and reversed phase
- pH sample pretreatment controls ionization and retention
- Wash steps must be carefully selected based upon retention mechanism
- Must know retention mechanism of compounds!

Hydrolyzed urine specimens fortified with 52 drugs and metabolites from multiple drug classes were treated with acid or base and extracted using three sample preparation methodologies. Wash and elution solvents were optimized for each extraction protocol. Samples were analyzed on a Sciex 5500 triple quadrupole MS coupled to a Shimadzu Nexera LC and autosampler. Two transitions were monitored for each analyte. Recovery, matrix effect, and process efficiency were evaluated at 25, 50 and 100 ng/mL (Matuszewski BK et al, *Anal. Chem.* 75, 3019-3030, 2003). Average recovery and matrix effect for the quantitative MS/MS transition for each compound are reported in Table 3.

Extraction Parameters

Table 1: Extraction Methods

	ISOLUTE SLE+	EVOLUTE EXPRESS ABN	EVOLUTE EXPRESS CX
Specimen volume	200 µL		
Ammoniun acetate pH 4	200 µL		
enzyme	13 µL		
hydrolyze	55 °C for 30 minutes		
Sample Pretreatment	200 µL 0.1% NH4OH	200 µL 0.1% NH4OH	200 µL 4% H3PO4
Condition	-	1 mL MeOH	1 mL MeOH
Equilibrate	-	1 mL 0.1% NH4OH	1 mL 4% H3PO4
Load (µL)	400 µL	entire pretreated sample	
Wash 1	-	1 mL 0.1% NH4OH	1 mL 4% H3PO4
Wash 2	-	1 mL 10% MeOH in water	1 mL of 50% MeOH in water
Dry	-	dried 5 min under nitrogen 40 psi	
Elute option 1	2 x 0.75 mL 90:10 CH2Cl2:2-propanol		2 x 0.75 mL 78:20:2 CH2Cl2:2-propanol:NH4OH
OR Elute option 2	-	-	**OR** 2 x 0.75 mL 78:20:2 CH2Cl2:MeOH:NH4OH
Dry	-	dried under nitrogen at 40°C	dried under nitrogen 40°C
Reconstitute	150 µL 90:10 0.1% FA in water:0.1% FA in MeOH		

Methods (continued)

LC Parameters

Mobile phase A (MPA): 0.1% formic acid (FA) in water
Mobile phase B (MPB): 0.1% FA in MeOH, gradient separation
Flow Rate: 0.6 mL/min
Column: Restek RAPTOR biphenyl 2.7 µm, 50 x 3.0 mm
LC: Shimadzu UPLC
Injection volume: 2 µL

Results and Discussion

Supported Liquid Extraction ISOLUTE® SLE+

- 80 to >90% recovery for most compounds except:
- 40-80% recovery for norhydrocodone, morphine and amphetamine
- 36% recovery for zolpidem-phenyl-4-COOH
- <20% recovery pregabalin and gabapentin

Polymeric Reverse Phase SPE EVOLUTE® EXPRESS ABN

- 80 to >90% recovery for many compounds
- Poor recovery for opiates and some opioids:
- 30-70% for 6-AM, hydrocodone, norhydrocodone, codeine, dihydrocodeine, oxycodone, norbuprenorphine, norfentanyl, tapentadol and n-desmethyltapentadol
- ≤10% morphine, hydromorphone, oxymorphone
- 27% recovery for naloxone
- 56% recovery 7-aminoclonazepam
- Lower recoveries for 7-aminoclonazepam, amphetamine and methamphetamine and ritalinic acid
- 70-80% recovery 9-carboxy-THC, amitriptyline and nortriptyline
- <20% recovery pregabalin and gabapentin

Mixed Mode Polymeric Reverse Phase Cation Exchange SPE EVOLUTE® EXPRESS CX

- 80 to >90% recovery for most compounds using CH_2Cl_2:2-propanol:NH_4OH except:
- Poor recovery for barbiturates
- 30% recovery for ritalinic acid
- ≤20% recovery pregabalin, gabapentin, meprobamate and carisoprodol
- Lowering organic wash to 10% improved recovery of carisoprodol and barbiturates
- Recovery improved for pregabalin (100%), meprobamate (30%), gabapentin and ritalinic acid (80%) and using CH_2Cl_2:MeOH:NH_4OH, but samples were not as clean

Conclusions

- Supported liquid extraction and mixed-mode SPE worked best for the most compounds
- Compromises in recovery and sample cleanliness are necessary with large drug panels in urine that have analytes with very different properties
- Knowing the logP and pKa of your compounds is essential for successful method development

Table 2. Summary of Sample Preparation Results

Drug Class	CX	SLE+	ABN	Comment
Opiates	+++	++	+	low recoveries for most drugs on ABN
Opioids	+++	+++	++	low recoveries for a few drugs on ABN
Benzodiazepines	+++	+++	++	most variability, 7-AC, zolpidem metabolite lower
Barbiturates	+	+++	+++	recovery on CX requires 10% organic wash
Stimulants	+++	++	++	lower recovery ritalinic acid for most options
Illicit Drugs	+++	+++	++	
Pregabalin and Gabapentin	++			must add MeOH to elution solvent on CX - samples not as clean
Carisoprodol and Meprobamate	++	+++	++	lower organic wash on CX for meprobabmate

Table 3: Compound Properties, Percent Average Recovery and Matrix Effect

Compound	Properties			Average Recovery/Matrix Effect					
				SLE+		ABN		CX	
	logP	pKa 1	pKa 2	REC	ME	REC	ME	REC	ME
11-Nor-delta-9-carboxy-THC	5.1	4.2	9.3	90%	62%	78%	161%	87%	63%
6-monoacetylmorphine	0.6	8.7	9.7	82%	84%	35%	91%	102%	92%
7-aminoclonazepam	0.5	3.0	5.0	68%	62%	56%	54%	82%	78%
alpha-hydroxy-alprazolam	1.5	5.0	13.7	100%	108%	92%	142%	93%	106%
alprazolam	3.0	5.1	18.3	102%	107%	89%	147%	93%	107%
amitriptyline	4.8	9.8		94%	76%	80%	86%	92%	81%
amphetamine	1.8	10.0		66%	74%	<20%	86%	132%	42%
benzoylecgonine	-0.6	3.2	9.5	103%	115%	130%	107%	105%	101%
buprenorphine	3.6	9.6		90%	93%	76%	153%	89%	97%
butalbital	1.6	8.5		105%	117%	103%	71%	<20%	87%
carisoprodol	1.9	15.0		108%	134%	97%	144%	21%	106%
chlordiazepoxide (Librium)	3.1	6.5		92%	86%	111%	91%	119%	51%
clonazepam	3.2	1.9	11.7	145%	160%	116%	79%	120%	574%
cocaine	2.3	8.9		87%	85%	75%	99%	105%	80%
codeine	1.3	9.2		100%	99%	29%	92%	109%	89%
diazepam	3.1	2.9		100%	105%	86%	201%	103%	94%
dihydrododeine	1.6	9.3		93%	90%	29%	79%	110%	82%
EDDP	4.6	9.6		116%	85%	115%	100%	134%	63%
fentanyl	3.8	8.8		101%	83%	94%	111%	107%	79%
gabapentin	-1.3	4.6	9.9	<20%	79%	<20%	107%	<20%	96%
hydrocodone	2.0	8.6		100%	100%	60%	94%	110%	87%
hydromorphone	1.6	8.6	10.1	94%	97%	10%	96%	112%	77%
ketamine	3.4	7.5		109%	118%	77%	86%	113%	48%
lorazepam	3.5	10.6	12.5	97%	102%	85%	160%	93%	96%
MDMA	1.9	10.1		93%	90%	55%	85%	100%	89%
meperidine	2.5	8.2		111%	111%	96%	109%	109%	66%
meprobamate	0.9	>12.0		99%	131%	112%	90%	<20%	105%
methadone	5.0	9.1		125%	80%	98%	109%	102%	86%
methamphetamine	2.2	10.2		102%	82%	32%	83%	141%	35%
morphine	0.9	9.1	10.3	47%	97%	<10%	104%	112%	61%
naloxone	1.6	7.8	10.7	105%	88%	27%	88%	127%	80%
n-desmethyltapentadol	2.3	10.6		78%	101%	44%	101%	93%	92%
norbuprenorphine	2.3	10.5		113%	94%	60%	172%	97%	100%
nordiazepam	3.2	2.9	12.3	106%	129%	84%	222%	98%	88%
norfentanyl	1.4	10.0		82%	103%	56%	89%	109%	89%
norhydrocodone	1.6	10.0		76%	89%	43%	95%	102%	88%
norketamine	2.9	7.5		105%	102%	50%	92%	108%	54%
normeperidine	2.1	9.3		104%	98%	77%	107%	103%	91%
nortriptyline	4.4	10.5		100%	71%	71%	87%	90%	80%
oxazepam	2.9	10.6	12.5	114%	104%	84%	189%	106%	105%
oxycodone	1.0	8.1		104%	79%	45%	77%	113%	79%
oxymorphone	0.8	8.2	10.0	96%	88%	<10%	89%	122%	71%
phencyclidine	4.5	10.6		118%	88%	96%	118%	106%	51%
phenobarbital	1.4	8.1		103%	111%	94%	72%	<20%	103%
pregabalin	-1.4	4.8	10.2	<20%	96%	<20%	103%	<20%	94%
ritalinic acid	-0.4	3.7	10.1	<20%	116%	47%	106%	30%	106%
secobarbital	2.0	8.5		101%	110%	101%	89%	30%	105%
tapentadol	3.0	10.2		108%	91%	65%	80%	106%	85%
temazepam	2.8	4.7		112%	126%	90%	237%	101%	109%
tramadol	2.5	9.3		128%	83%	98%	79%	110%	79%
zolpidem	3.0	5.7		121%	99%	100%	126%	108%	99%
zolpidem-4-phenyl-COOH	0.6	3.4	5.7	36%	97%	90%	122%	64%	98%

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