A Comparative Study of Common Urine Sample Preparation Techniques of a Comprehensive Panel of Pain Management Drugs by LC/MS Analysis for Forensic Toxicology

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MSACL 2012
Poster # 2

Agilent Technologies

Introduction

There are two common techniques to pre-treat urine samples for the LC/MS analysis of large panels in forensic toxicology: 'dilute and shoot' (D&S) and solid phase extraction (SPE.) Both are widely used, but each has its' own advantages and disadvantages. This research project compares and contrasts these sample preparation approaches using a comprehensive and rapid targeted LC/MS analysis method of a panel of over 65 compounds extracted from urine samples. The recovery results from several batches are reported. SPE clean-up/extraction methods are based upon the Plexa PCX phase cartridges and the methodology is reported. D&S techniques are based on a 1/10 dilution in de-ionized water.

Sample Preparation

Step 1: Glucuronide hydrolysis	
1ml urine + 10µl of ß-glucuronidase *Note: need 1,000 units per ml of urine; ß-glucuronidase was ≥100,000 units/ml, so using at least 1,000 units per ml*	
Step 2: SPE (Agilent Plexa PCX – 30mg)	
Sample	0.2ml urine
Pre-treatment	Dilute with KH_2PO_4
SPE Conditioning	0.5ml MeOH 0.5ml H_2O
SPE Wash	0.5ml 50% MeOH in H_2O Dry under vacuum for 5 minutes
Elution	0.5 ml EtAc:MeOH:NH_3OH
Step 3: Sample reconstitution	
Sample were evaporated to ~200µl, then 100µl of 0.2%HCl in MeOH was added. Samples were then evaporated to dryness and reconstituted in 100µl of 0.01% formic acid for injection.	

Table 1. Solid Phase Extraction (SPE) procedure.

Step 1: Glucuronide hydrolysis	
1ml urine + 10µl of ß-glucuronidase *Note: need 1,000 units per ml of urine; ß-glucuronidase was ≥100,000 units/ml, so using at least 1,000 units per ml*	
Step 2: 1:10 Dilution	
Sample	0.1ml urine
Dilution	Add 0.9ml H_2O
Filtering	2,500rpm for 20 minutes using 3K mass cut-off filter
Step 3: Sample transfer	
Transfer filtered and diluted urine to autosampler for injection.	

Table 2. Dilute and Shoot (D&S) procedure.

Instrument Parameters

Sample Information

65+ forensic analytes that respond in positive MS polarity were spiked at a concentrations of 100ng/ml into several clean urine matrix batches.

HPLC Parameters

Agilent 1260 HPLC binary pump, well plate sampler with thermostat, temperature-controlled column compartment

Parameter	Value
Column	Zorbax Poroshell EC-C18, 2.1 x 100mm, 2.7µm
Column Temp	55°C
Injection Volume	1µl (SPE), 10µl (D&S)
Autosampler	4°C
Needle Wash	Flushport, 5 seconds
Mobile Phase A	NH_4OH + Formic Acid in H_2O
Mobile Phase B	Formic Acid in Acetonitrile
Flow Rate	0.5 ml/min

Table 3. LC parameters

Mass Spectrometer Parameters

Agilent 6420 QqQ Mass Spectrometer

Ion Source Conditions	
Ion Mode	ESI +
Capillary Voltage	2000 V
Drying Gas (N_2)	12 L/min
Drying Gas Temp	350°C
Nebulizer Gas (N_2)	50 psi
Δ EMV	0 V
Dwell Time	dynamic

Table 4. Mass spectrometer parameters

Dynamic MRM

Dynamic MRM allows a mass spectrometer to acquire select MRM data during a specified retention time window, decreasing the number of ion transitions being monitored simultaneously. Cycle time is kept consistent to keep an even distribution of data points and ensure accurate quantitation.

Parameter	Value
Cycle Time	330 ms
Total MRMs	174
Max Concurrent MRMs	31
Retention Time Window	30 sec
Min/Max Dwell Time	7.5/161.5 ms
Q1/Q2 Resolution	0.7 amu

Table 5. Dynamic MRM parameters

SPE Recovery Data

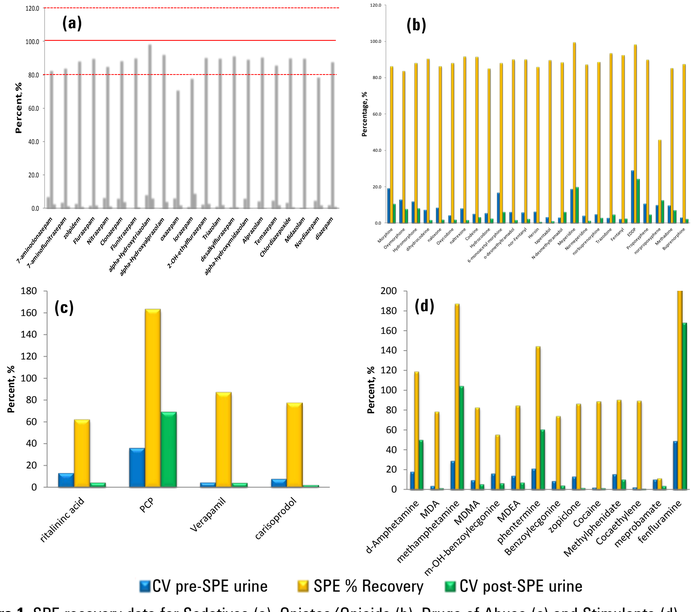

Figure 1. SPE recovery data for Sedatives (a), Opiates/Opioids (b), Drugs of Abuse (c) and Stimulants (d).

Column Stability for 3000x D&S Urine Samples

Zorbax Poroshell 120 EC-C18 column stability results for various analytes picked out Randomly throughout the course of 6min analysis . Stability is expressed in Retention Time %RSD.

Analyte	%RSD	Analyte	%RSD	Analyte	%RSD
Morphine	0.7	Mepreridine	0.4	Triazolam	0.0
Codeine	0.4	Zolpidem	0.3	Naltrexone	0.1
Hydrocodone	0.4	Fentanyl	0.1	Chlordiazepoxide	0.1
MDMA	0.3	EDDP	0.1	Dexmethyldiazepam	0.1
Nor-Fentanyl	0.2	Nitrazepam	0.1	Cocaethylene	0.2
Heroin	0.2	Propoxephine	0.1	11-nor-9-carboxy-Δ9-THC	0.0
Methylphenidate	0.2	Buprenorphine	0.3		

Table 6. Retention time stability for 3000 D&S injections on a single Poroshell Column

6420 Sensitivity Results: D&S vs SPE Sample Preparation

Compound	LLOQ (ng/ml) D&S	LLOQ (ng/ml) SPE	ULOQ (ng/ml)
6-monoacetyl morphine	10	<1	1000
buprenorphine	10	1	1000
codeine	25	<1	1000
dihydrododeine	25	<1	1000
EDDP	10	<1	1000
fentanyl	1	<1	1000
heroin	10	<1	1000
hydrocodone	10	<1	1000
hydromorphone	5	<1	1000
meperidine	5	<1	1000
methadone	10	<1	1000
morphine	5	<1	1000
naloxone	5	<1	1000
naltrexone	10	<1	1000
N-desmethyltramadol	10	1	1000
norbuprenorphine	25	3	1000
norfentanyl	1	<1	1000
normeperidine	5	<1	1000
norpropoxyphene	5	<1	1000
o-desmethyltramadol	5	<1	1000
oxycodone	10	<1	1000
oxymorphone	5	<1	1000
propoxyphene	5	<1	1000
tapentadol	5	<1	1000
tramadol	1	<1	1000
Trazodone	1	<1	1000

Table 7. D&S vs. SPE LOQ for Opiates/Opioids

Compound	LLOQ (ng/ml) D&S	LLOQ (ng/ml) SPE	ULOQ (ng/ml)
amphetamine	5	<1	1000
benzoylecgonine	5	<1	1000
cocaethylene	5	<1	1000
cocaine	5	<1	1000
fenfluramine	1	<1	1000
MDA	5	<1	1000
MDEA	1	<1	1000
MDMA	5	<1	1000
meprobamate	10	5	1000
methamphetamine	1	<1	1000
methylphenidate	5	<1	1000
m-OH-benzoylecgonine	10	<1	1000
phentermine	1	<1	1000
zopiclone	5	<1	1000

Table 8. D&S vs. SPE LOQ for Stimulants

Compound	LLOQ (ng/ml) D&S	LLOQ (ng/ml) SPE	ULOQ (ng/ml)
2-OH-ethylflurazepam	200	5	1000
7-aminoclonazepam	10	<1	1000
7-aminoflunitrazepam	5	<1	1000
alpha-OH-midazolam	10	<1	1000
alprazolam	10	<1	1000
a-OH-alprazolam	20	<1	1000
a-OH-triazolam	50	<1	1000
chlordiazepoxide	10	<1	1000
clonazepam	25 to 50	<1	1000
desalkylflurazepam	20	1	1000
diazepam	10	<1	1000
flunitrazepam	10	1	500
flurazepam	5	1	1000
lorazepam	50	20	1000
midazolam	10	<1	1000
nitrazepam	25	5	1000
nordiazepam	25	<1	1000
oxazepam	50	25	1000
temazepam	25	<1	1000
triazolam	5	<1	1000
zolpidem	5	<1	1000

Table 9. D&S vs. SPE LOQ for Sedatives

Compound	LLOQ (ng/ml) D&S	LLOQ (ng/ml) SPE	ULOQ (ng/ml)
11-nor-9-carboxy-THC	-	25	-
carisoprodol	5	1	1000
PCP	1	<1	1000
ritalinic acid	5	1	1000
verapamil	2	<1	1000

Table 10. D&S vs. SPE LOQ for Drugs of Abuse

Conclusions

SPE recoveries for 90% of all analytes in the comprehensive panel were within ±20% of full recovery. In general, SPE sample preparation yielded more sensitive results for LLOQ than Dilute & Shoot approaches. Zorbax Poroshell 120 EC-C18 Column lifetime was outstanding, with little degradation in performance after 3000x Dilute & Shoot urine samples injected. Both sample preparation approaches are appropriate for Agilent 6420/30/60 QqQ systems to achieve typical linear ranges and sensitivity requirements.