Validation of dilute and shoot and evaluation of SPE method for barbiturates and THC carboxylic acid panel in urine

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Outline

Barbiturates and THCA panel is commonly tested in clinical laboratories by dilute and shoot approach which comes with high matrix interference at low concentration, and long term deterioration of the LC - MS system. The purpose of study reported here was to validate in lab developed dilute and shoot assay and evaluate solid phase extraction (SPE) approach as alternative.

Methods

Instrumentation:

LC -MS/MS analysis was done using an ExionLC UHPLC system coupled to an API 4500 mass spectrometer with a turbo-ionspray ESI source operated in negative ion mode. All automated extractions and plate processing were carried out using Orochem's Oroflex Personal Pipettor Robot and EZYPRESS HT 96-well plate positive pressure manifold. Evaporation was carried out by a nitrogen gas using the Evaporex EVX 192 plate evaporator.

Materials:

Xenobiotics and buffer chemicals were purchased from Sigma Aldrich, E. Coli beta-glucoronidase (BG) from Campbell, and HPLC -MS grade solvents from Pharmco -Aaper. Orpheus C18 SPE plates, Celerity Deluxe and Panthera Deluxe Polymeric SPE plates (Orochem Technologies Inc) were used for all extractions. Amobarbital, butabarbital, butalbital, phenobarbital secobarbital and THCA standards and ISs were purchased from Cerilliant. HPLC grade water and methanol were purchased from Pharmco -Aaper. Human urine was fortified with standard barbiturates and THCA .

Procedures:

I. Dilute and shoot

100 µL of urine was spiked with 50 µL of IS spiking solution in 90% methanol, vortexed with 50 µL of 200 mM phosphate pH 6.8 buffer, mixed with 20 µL of BG solution and incubated (55 deg C, 30 min). 150 µL of 20 mM ammonium formate buffer pH 3.7 in 40/60 water/methanol was added prior to vortexing and centrifuging. Assay was validated for accuracy, precision, linearity, carryover limit, matrix interference/ effect and xenobiotic interference and effect.

II. SPE screening

We screened Orpheus C18, and two types of polymeric DVB SPE plates (Celerity and Panthera Deluxe): each plate was conditioned with 1 ml of methanol and 1 ml of water, loaded with 100 µL of fortied urine (spiked at cut-off and ~30% of ULOQ concentration), mixed with 50 µL of IS spiking solution in 90% methanol, vortexed with 50 µL of 200 mM ammonium acetate pH 6.8 buffer, mixed with 20 µL of BG solution and incubated (55 deg C, 30 min). Washing procedures and volumes of methanol applied for

elutions were varied. Elutes were evaporated, reconstituted in 30% methanol and analyzed. Recovery and reproducibility were measured for different protocols using varying concentrations of methanol with or without 0.1-1% formic acid instead of water and varying elution volumes.

III. Optimized Panthera Deluxe SPE procedure

96 well plates packed with 30 mg were used for testing. 2 mL collection plate was used for collection.

add 100 uL spiked urine (samples)
add 100 uL of 200 mM ammonium acetate pH 6.8 mixed with enzyme sol 5/2 v/v
add 50 uL of IS spiking solution
mix gently and incubate for 30 min at 55 deg C. Cool for 5 min.
LOAD
WASH with 1 mL H2O followed by 1 mL 20% MeOH
ELUTE with 1.5 mL MeOH (2 × 0.75 mL)
evaporate to dryness
reconstitute in 500 uL of 30% MeOH

NOTE: Lower amount of packing allows for smaller elution volumes.

IV. LC -MS/MS analysis

Orochem Gazelle C18 column (1.7 µm, 50 × 2.1 mm) was used with a 30-95% methanol (0.1% FA) gradient mobile phase and Restek 0.2 µm pre-column filter. Analysis was performed in ESI negative ion mode. Total run time was 7 minutes.

Results

I. Validation of dilute and shoot

Dilute and shoot assay conformed to industry standards: accuracy and precision were within 25% (20%) of the target while linearity test showed back - calculated concentrations of all calibrators (n=6) within 20% of target. AMR was determined to be 10 - 2500 ng/mL for all barbiturates except butabarbital (20 – 2500) and 4 - 500 ng/mL for THCA.

II. SPE recovery

We established that Panthera Deluxe (PNT) gives better reproducibility and recovery than Celerity Deluxe (CEL), (note especially THCA recoveries from PNT), while recoveries on C18 SPE were very poor (< 35% for all analytes). In all cases the optimized procedure was applied (see Procedures).

	RECOVERIES				CV %			
	LQC		HQC		LQC		HQC	
Analyte	PNT	CEL	PNT	CEL	PNT	CEL	PNT	CEL
AMO	100%	102%	101%	119%	7%	21%	5%	14%
BBAR	95%	100%	104%	115%	9%	21%	6%	10%
BTAL	98%	96%	102%	110%	9%	20%	1%	8%
PHE	104%	101%	105%	111%	9%	19%	3%	8%
SEC	100%	102%	103%	113%	11%	18%	3%	12%
THCA	87%	86%	78%	62%	12%	10%	4%	35%

III . Comparison of dilute and shoot (D&S) and SPE on Panthera (PNT)

Accuracy and reproducibility was assessed by comparison of QC samples and calibrators across three batches. PNT procedure gives overall better results than D&S. It also gives greater AMR and lower LLOQ namely for butabarbital and THCA. Results for xenobiotic interference/effect testing and matrix ion suppression/enhancement were comparable between procedures while PNT showed superior results in terms of matrix interference/ effect tested at cut-off concentration. Response factors for barbs are clearly improved with SPE (Figure 1.)

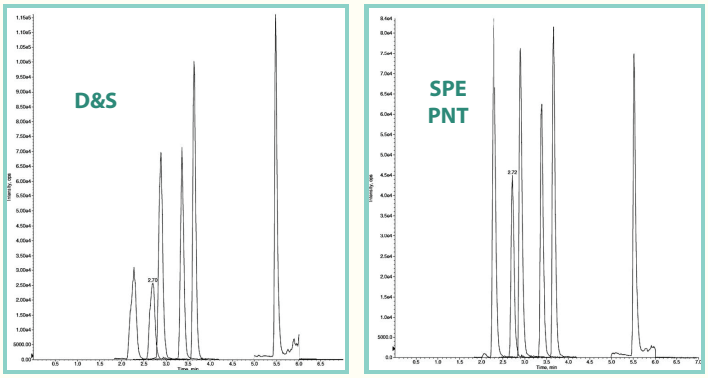

Figure 1. UHPLC -MS/MS chromatogram of fortied urine (at cut-off) sample processed by dilute and shoot (left) and Panthera SPE process (right). Peaks in eluting order (r.t. min): phenobarbital (2.34), butabarbital (2.76), butabital (2.94), amobarbital (3.42), secobarbital (3.69), THCA (5.50)

Matrix effect, LLOQ & AMR	Absolute mean bias across 10 spiked samples (at cut-off)		LLOQ and AMR	
Analyte	SPE PNT	D&S	SPE PNT LLOQ*	D&S AMR
AMO	10%	15%	0.9 ng/mL	10-2500 ng/mL
BBAR	8%	18%	1.5 ng/mL	20-2500 ng/mL
BTAL	9%	11%	0.4 ng/mL	10-2500 ng/mL
PHE	9%	7%	0.7 ng/mL	10-2500 ng/mL
SEC	8%	24%	0.5 ng/mL	10-2500 ng/mL
THCA	16%	14%	1.8 ng/mL	4-500 ng/mL

* as 10 × (stdev of baseline//slope of the linear curve)

Conclusion

We developed a fast and reliable SPE method for analysis of barbiturates and THCA in urine toxicology setting. This improved method demonstrated reduced matrix effect and it expanded a lower end of AMR for almost an order of magnitude. The sample processing time per plate is about 15 minutes, while maintaining the total cost of sample preparation at a very competitive level.

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