

Challenges in Sample Preparation for Forensic Quantitative Screening of Over 120 Drugs of Abuse on a Triple Quadrupole Mass Spectrometer

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Overview

Purpose: To develop and analytically evaluate various sample preparation techniques along with an HPLC-MS/MS method that employs a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer for the quantitation of 122 pharmacologic agents in human urine for forensic toxicology.

Methods: Enzymatic hydrolysis followed by liquid-liquid extraction prior to HPLC-MS/MS analysis.

Results: Limits of quantitation defined as acceptable back-calculated calibration curves, passing ion ratio confirmation, and precise quality controls were met for 122 compounds.

Introduction

Rapid screening is a goal for many forensic toxicology laboratories. Newer, faster triple quadrupole mass spectrometers enable laboratories to include more compounds in one chromatographic run thereby saving analytical run time. The next challenge arises in finding a suitable sample processing technique that works for a variety of compounds across a wide chemical space with varying sensitivities and taking into account the different LOQ requirements. In this study, several liquid-liquid extraction (LLE) schemes were compared to see which method was a better fit for analyzing the wide range of compounds in human urine in a forensic toxicology setting.

Methods

Sample Preparation

- Enzymatic hydrolysis
- Liquid-liquid extraction (LLE)
 - Basic, Neutral, Acidic with EthylAcetate:Hexane (1:1 v/v),
 - Amtox A and B tubes (Ameritox Labs, Hilliard, OH)
- The organic layer was evaporated to dryness and reconstituted
- Calibrators and controls were prepared by spiking compounds into blank synthetic urine in the range of 0.5 to 500 ng/mL.

Liquid Chromatography

- Pump: Thermo Scientific™ Dionex™ UltiMate™ 3000RS with OAS autosampler.
- Mobile phases: 10 mM ammonium acetate in water(A) and methanol (B) (Fisher Scientific™ Optima™ grade)
- Column: Thermo Scientific™ Accucore™ PFP, 2.6 µm, 100 x 2.1 mm
- Gradient: initial 0.5-min hold at 2% mobile phase B followed by 10-min ramp to 100% B.
- Total run time was 15 minutes

Mass Spectrometry

- Mass Spectrometer: TSQ Endura triple quadrupole mass spectrometer with a heated electrospray ionization (HESI II) sprayer.
- Two selected reaction monitoring (SRM) transitions were monitored for each analyte to obtain ion ratio confirmation (IRC) and one SRM transition was monitored for each of the 84 stable-labeled internal standards used.
- Compounds are both positively and negatively ionized.

Data Analysis

Data was acquired and processed with Thermo Scientific™ TraceFinder™ software version 3.2. Calibration ranges, LODs, and LOQs were evaluated based on concentration accuracy; back-calculated concentrations had to be within 30%.

Method Evaluation

Limits of detection, precision and accuracy were evaluated by processing and analyzing calibrators and replicate controls. Matrix effects were determined by spiking 12 different lots of blank donor urine at 10 ng/mL and comparing results to that of a sample prepared in water.

The above methods were tested with over 100 compounds from a wide chemical space including amphetamines, antidepressants, barbiturates, benzodiazepines, drugs of abuse, and opioids, a space which includes polar and non-polar compounds as well as positively and negatively ionizing compounds.

TABLE 1. Extraction recoveries for basic, neutral and acidic LLE and extraction tubes A and B. While LLE under basic conditions gave higher recoveries for a greater number of compounds than other extraction techniques, the AmtoxA LLE tubes gave the best compromise on recovery over the entire compound list.

Compound	Basic	Neutral	Acidic	TxA	TxB	Compound	Basic	Neutral	Acidic	TxA	TxB
6-MAM	Green	Green	Green	Green	Green	MDA	Green	Green	Green	Green	Green
7-Aminoclonazepam	Green	Green	Green	Green	Green	MDMA	Green	Green	Green	Green	Green
7-Aminoflunitrazepam	Green	Green	Green	Green	Green	Meperidine	Green	Green	Green	Green	Green
Acetaminophen	Green	Green	Green	Green	Green	Meprobamate	Green	Green	Green	Green	Green
α-Hydroxyalprazolam	Green	Green	Green	Green	Green	Methadone	Green	Green	Green	Green	Green
Alprazolam	Green	Green	Green	Green	Green	Methamphetamine	Green	Green	Green	Green	Green
Amitriptyline	Green	Green	Green	Green	Green	Methotrimeprazine	Green	Green	Green	Green	Green
Amphetamine	Green	Green	Green	Green	Green	Methylphenidate	Green	Green	Green	Green	Green
Atenolol	Green	Green	Green	Green	Green	Metoprolol	Green	Green	Green	Green	Green
Atropine	Green	Green	Green	Green	Green	Mirtazapine	Green	Green	Green	Green	Green
Benzoylcegonine	Green	Green	Green	Green	Green	Morphine	Green	Green	Green	Green	Green
Brompheniramine	Green	Green	Green	Green	Green	Nicotine	Green	Green	Green	Green	Green
Buprenorphine	Green	Green	Green	Green	Green	Norbuprenorphine	Green	Green	Green	Green	Green
Bupropion	Green	Green	Green	Green	Green	Norchlordiazepoxide	Green	Green	Green	Green	Green
Carbamazepine	Green	Green	Green	Green	Green	Norcodeine	Green	Green	Green	Green	Green
Carbamazepine-epoxide	Green	Green	Green	Green	Green	Norcyclobenzaprine	Green	Green	Green	Green	Green
Carisprodol	Green	Green	Green	Green	Green	Nordiazepam	Green	Green	Green	Green	Green
Chlordiazepoxide	Green	Green	Green	Green	Green	Nordoxepin	Green	Green	Green	Green	Green
Chlorpheniramine	Green	Green	Green	Green	Green	Norfentanyl	Green	Green	Green	Green	Green
Chlorpromazine	Green	Green	Green	Green	Green	Norketamine	Green	Green	Green	Green	Green
Cimetidine	Green	Green	Green	Green	Green	Normeperidine	Green	Green	Green	Green	Green
Citalopram	Green	Green	Green	Green	Green	Norpropoxyphene	Green	Green	Green	Green	Green
Clomipramine	Green	Green	Green	Green	Green	Norsertaline	Green	Green	Green	Green	Green
Clonazepam	Green	Green	Green	Green	Green	Nortrimipramine	Green	Green	Green	Green	Green
Clozapine	Green	Green	Green	Green	Green	Nortriptyline	Green	Green	Green	Green	Green
Cocaeethylene	Green	Green	Green	Green	Green	Norverapamil	Green	Green	Green	Green	Green
Cocaine	Green	Green	Green	Green	Green	O-Desmethyltramadol	Green	Green	Green	Green	Green
Codeine	Green	Green	Green	Green	Green	Olanzapine	Green	Green	Green	Green	Green
Cotinine	Green	Green	Green	Green	Green	Oxazepam	Green	Green	Green	Green	Green
Cyclobenzaprine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Desalkylflurazepam	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Desipramine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Desmethyl-clomipramine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Dextromethorphan	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Diazepam	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Dihydrocodeine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Diltiazem	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Diphenhydramine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Doxepin	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Doxylamine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Duloxetine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Ecgonine ethyl ester	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Ecgonine methylester	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Ephedrine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Fentanyl	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Flunitrazepam	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Flurazepam	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Hydrocodone	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Hydromorphone	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Hydroxyzine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Imipramine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Ketamine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Lamotrigine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Lidocaine	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
Lorazepam	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
LSD	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
maprotiline	Green	Green	Green	Green	Green	Oxycodone	Green	Green	Green	Green	Green
RECOVERY KEY	greater				less						

Results

Extraction Recoveries: While LLE under basic conditions gave higher recoveries for a greater number of compounds than other extraction techniques, the AmtoxA LLE tubes gave the best compromise on recovery over the entire compound list, taking into account required LOQs for all compounds (TABLE 1). LOQs met forensic toxicology requirements for 98% of the compounds tested (TABLE 2).

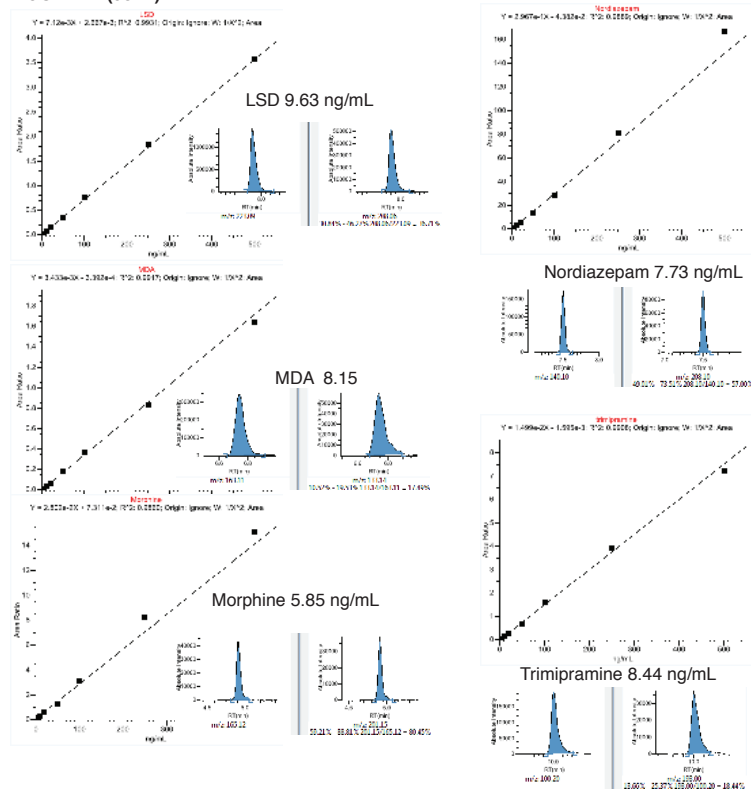
TABLE 2. Limits of Quantitation and QC Precision for Compounds Tested.

Compound	LOQ (ng/mL)	%RSD 1 ng/mL	%RSD 10 ng/mL	%RSD 100 ng/mL
6-MAM	1	7.22%	5.71%	6.68%
7-Aminoclonazepam	0.5	6.31%	4.62%	1.80%
7-Aminoflunitrazepam	0.5	3.49%	1.20%	1.82%
Acetaminophen	50	BLQ	BLQ	3.89%
α-Hydroxyalprazolam	1	1.57%	2.31%	2.61%
Alprazolam	0.5	2.71%	1.38%	9.56%
Amitriptyline	5	BLQ	9.10%	7.71%
Amphetamine	50	BLQ	BLQ	2.71%
Atenolol	1	5.58%	2.70%	9.70%
Atropine	0.5	3.44%	2.91%	4.23%
Benzoylcegonine	2	BLQ	5.00%	0.99%
Brompheniramine	2	BLQ	5.36%	7.81%
Buprenorphine	1	12.5%	11.68%	5.04%
Bupropion	2	BLQ	2.85%	1.30%
Butalbital	10	BLQ	BLQ	10.14%
Carbamazepine	2	BLQ	2.39%	2.71%
Carbamazepine-epoxide	0.5	1.93%	3.87%	5.03%
Carisprodol	0.5	3.66%	1.54%	4.04%
Chlordiazepoxide	0.5	10.3%	4.19%	3.53%
Chlorpheniramine	0.5	2.30%	2.40%	5.50%
Chlorpromazine	5	BLQ	13.75%	5.47%
Cimetidine	2	BLQ	5.83%	5.09%
Citalopram	5	BLQ	2.26%	9.39%
Clomipramine	2	BLQ	4.76%	4.94%
Clonazepam	1	4.78%	2.98%	5.75%
Clozapine	0.5	7.73%	2.76%	4.48%
Cocaeethylene	1	13.64%	3.55%	4.24%
Cocaine	50	BLQ	BLQ	2.22%
Codeine	5	BLQ	5.15%	9.83%
Cotinine	0.5	5.09%	2.17%	2.75%
Cyclobenzaprine	2	BLQ	8.90%	4.27%
Desalkylflurazepam	0.5	11.71%	2.72%	4.89%
Desipramine	5	BLQ	3.11%	6.33%
Desmethylclomipramine	10	BLQ	7.63%	5.38%
Dextromethorphan	1	7.53%	11.46%	9.13%
Diazepam	5	BLQ	2.08%	5.05%
Digoxin	2	BLQ	10.10%	8.03%
Dihydrocodeine	1	11.61%	2.30%	4.34%
Diltiazem	1	8.00%	1.94%	3.04%
Diphenhydramine	0.5	3.09%	2.24%	3.29%
Doxepin	10	BLQ	2.70%	5.98%
Doxylamine	5	BLQ	3.40%	1.03%
Duloxetine	5	BLQ	5.79%	3.71%
Ecgonine ethyl ester	5	BLQ	3.40%	9.21%
Ecgonine methyl ester	2	BLQ	1.40%	1.97%
EDDP	2	BLQ	3.41%	10.30%
Ephedrine	0.5	7.26%	8.72%	8.34%
Fentanyl	0.5	4.66%	6.76%	3.25%
Flunitrazepam	1	6.98%	1.15%	2.16%
Fluoxetine	2	BLQ	3.34%	3.47%
Flurazepam	0.5	2.24%	2.05%	2.39%
Hydrocodone	2	BLQ	1.68%	3.27%
Hydromorphone	0.5	4.42%	11.27%	3.05%
Hydroxyzine	0.5	3.43%	2.75%	5.18%
Imipramine	1	11.19%	5.07%	3.31%
Ketamine	0.5	8.02%	4.11%	1.45%
Lamotrigine	1	3.47%	5.80%	1.80%
Lidocaine	0.5	3.26%	1.45%	4.68%
Lorazepam	0.5	7.34%	1.81%	2.46%
LSD	0.5	4.48%	4.25%	1.06%
Maprotiline	10	BLQ	2.86%	7.27%
MDA	0.5	4.52%	7.40%	3.23%
MDMA	0.5	6.47%	2.07%	4.76%
Meperidine	2	BLQ	7.00%	4.20%
Meprobamate	0.5	2.69%	7.52%	2.37%
Methadone	0.5	8.08%	6.75%	4.44%
Methamphetamine	50	BLQ	BLQ	12.90%
Methotrimeprazine	10	BLQ	3.82%	4.40%
Methylphenidate	2	BLQ	2.04%	5.05%

Table 2. (continued)

Compound	LOQ	%RSD	%RSD	%RSD
	(ng/mL)			
Metoprolol	5	BLQ	4.30%	4.51%
Mirtazapine	1	4.38%	1.90%	8.47%
Morphine	2	BLQ	7.78%	8.36%
Naproxen	2	BLQ	4.97%	2.80%
Nicotine	2	BLQ	1.92%	4.41%
Norbuprenorphine	1	11.81%	8.32%	9.04%
Norchloridazepoxide	1	9.12%	4.06%	0.84%
Norcodeine	2	BLQ	7.97%	6.99%
Norcyclobenzaprine	2	BLQ	3.60%	7.79%
Nordiazepam	1	5.28%	4.17%	6.35%
Nordoxepin	0.5	6.35%	2.34%	1.78%
Norfentanyl	0.5	4.88%	1.64%	2.04%
Norfluoxetine	20	BLQ	BLQ	6.12%
Norketamine	0.5	4.38%	1.52%	1.75%
Normeperidine	0.5	5.28%	4.85%	1.91%
Norpropoxyphene	20	BLQ	BLQ	9.28%
Norsertaline	10	BLQ	6.23%	6.11%
Nortrimipramine	10	BLQ	4.40%	5.19%
Nortriptyline	0.5	8.80%	6.23%	5.76%
Norverapamil	0.5	1.60%	8.22%	8.18%
O-Desmethyltramadol	1	1.43%	3.34%	6.99%
Olanzapine ¹	20	BLQ	BLQ	6.04%
Oxazepam	0.5	12.94%	3.60%	2.19%
Oxycodone	0.5	5.91%	NA	5.29%
Oxymorphone	0.5	15.76%	9.21%	5.41%
Paroxetine	1	13.75%	2.89%	3.37%
Phencyclidine	2	BLQ	11.76%	2.46%
Phenethylamine	2	BLQ	3.17%	6.56%
Pheniramine	0.5	4.87%	5.07%	4.74%
Phenobarbital	20	BLQ	BLQ	14.32%
Phentermine	10	BLQ	11.70%	7.51%
Phenylephrine	10	BLQ	2.04%	0.79%
Phenylpropranolamine	0.5	8.87%	2.66%	6.32%
Phenytoin	20	BLQ	BLQ	6.13%
Propoxyphene	50	BLQ	BLQ	2.19%
Propranolol	1	8.49%	2.13%	4.18%
Pseudoephedrine	10	BLQ	6.28%	3.15%
Quetiapine	0.5	4.36%	1.97%	8.07%
Quinidine	2	BLQ	5.51%	3.29%
Quinine	2	BLQ	6.29%	8.30%
Ranitidine	10	BLQ	5.46%	10.50%
Sertraline	5	BLQ	4.61%	4.80%
Strychnine	5	BLQ	3.51%	6.49%
Temazepam	0.5	2.79%	0.61%	5.20%
THC	2	BLQ	14.09%	14.16%
THC-COOH (neg)	1	10.55%	2.20%	2.19%
THC-COOH (pos)	1	8.04%	2.29%	2.28%
Theophylline	0.5	0.23%	0.67%	1.34%
Thioridazine ¹	100	1.36%	8.41%	5.30%
Tramadol	0.5	2.87%	3.68%	2.76%
Trazodone	0.5	3.49%	1.06%	1.18%
Trimipramine	0.5	3.75%	2.05%	4.10%
Verapamil	2	BLQ	4.99%	5.19%
Zolpidem	0.5	2.12%	1.70%	4.86%

FIGURE 1. (cont.)



Matrix effects were determined by comparing concentration of analyte in spiked donor urine to a sample prepared in water. Calculated concentration within $\pm 50\%$ was considered passing. 8.3% of the individual analyte/donor results were outside of this range. However, less than 2% of those compounds that had a stable-labeled analog internal standard were out of range whereas 21% of those without an analog were out of range.

Conclusion

- A single analytical HPLC-MS/MS method was developed for 122 chemically diverse compounds.
- The method includes both polar and non-polar as well as positively and negatively ionizing compounds.
- Stable-labeled analog internal standards are crucial to minimize matrix effects.
- The fast scanning speed and polarity switching of the TSQ Endura mass spectrometer enable the analysis of all 122 compounds plus 84 stable-labeled internal standards without loss of signal intensity.
- A single sample processing scheme was used for all compounds, making the method efficient.
- Forensic toxicological limits of quantitation were met or exceeded.

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