Increasing Sample Throughput for Detecting Drugs of Abuse and Metabolites in Urine by UHPLC-MS/MS for Forensic Toxicology

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OVERVIEW

Purpose: To demonstrate ability to measure a comprehensive panel of drugs of abuse and their metabolites in non-hydrolyzed urine samples in approximately 2 minutes using UHPLC-MS/MS.

Methods: 101 drugs of abuse and metabolites were spiked into blank urine at multiple concentrations around their cutoff levels. These samples were diluted with an equal volume 20% methanol containing 36 isotopically-labeled standards prior to UHPLC-MS/MS. Separations were accomplished using the Thermo Scientific™ Vanquish™ UHPLC system by injection of 2 uL onto a sub-2um column at 1 mL/min. Compounds were detected with a Thermo Scientific™ TSQ Endura™ mass spectrometer utilizing heated electrospray ionization with polarity switching. Timed selected reaction monitoring (SRM) was employed to maximize detection efficiency for the large number of compounds analyzed.

Results: The Vanquish UHPLC/TSQ Endura system is able to measure ~100 drugs of abuse and metabolites in diluted urine samples at or below cutoff levels in under 1.4 minutes.

INTRODUCTION

Owing to its high analytical specificity and sensitivity, LC-MS/MS has become commonplace in reanalyzing urine samples after a positive immunoassay test to confirm the presence of drugs of abuse for forensic toxicology. Despite the drawbacks (e.g., cross-reactivity), immunoassay is still the default “first pass” for urine drug analysis owing to its speed and low cost versus LC-MS/MS. Advancements in UHPLC systems, sub-2 um LC columns and modern triple quadrupole detectors have greatly improved the separation efficiency and detection capability of large numbers of compounds with high sensitivity. This work investigates the feasibility of high-throughput measurements of approximately 100 drugs of abuse and metabolites by reducing time consuming sample preparation steps and employing two minute UHPLC-MS/MS analyses per sample.

MATERIALS AND METHODS

Sample Preparation

All standards were obtained from Cerilliant (Round Rock, TX) and used as received. Blank urine was obtained from a healthy male volunteer. After centrifugation of urine at 10,000 rpm for 10 min, urine supernatant was spiked with drugs of abuse and metabolites at concentrations equivalent to 0.1, 0.25, 0.5, 1, 2, 5 and 10 times the cutoff concentrations as listed in Table 2. Prepared urine samples were diluted with equal volume of a stock solution of isotopically-labeled standards in 20% methanol prior to LC-MS/MS analyses.

Liquid Chromatography

2 uL was injected onto a 2.1 x 50 mm, 1.9 um Thermo Scientific™ Hypersil GOLD™ aQ (Thermo Fisher Scientific), which was thermostatted at 40 C. Compound separation was accomplished with the Vanquish UHPLC system using a binary reverse-phase gradient as shown in Table 1. Mobile phases were (A) 0.1% formic acid in H_2O and (B) ACN. LC effluent was diverted to waste until after the column void to prevent salts from fouling the ion source.

Mass Spectrometry

The TSQ Endura MS with heated electrospray ionization was employed to detect all target drugs and internal standards. Most experiments used polarity switching to detect positively- and negatively-charged compounds in the same LC run. A total of 241 SRM transitions were monitored using a cycle time of 0.13 s, with most SRM time windows set to a width of 0.1 min (6 s).

Table 1: LC Gradient

Time (min)	%B	Flow Rate (mL/min)
0.0	0	1.0
0.4	22.5	1.0
1.0	80	1.0
1.29	80	1.0
1.3	0	1.0
1.4	0	1.2
2.1	0	1.2

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RESULTS

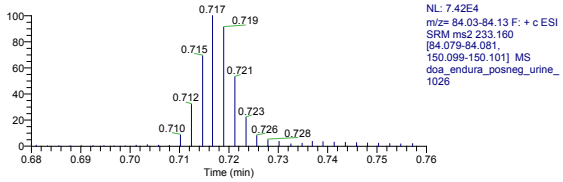
Table 2: Measured Drugs of Abuse in Urine

Compound	RT (min)	Polarity	Cutoff (ng/mL)	LLOQ (ng/mL)	Int. Std.
2-Hydroxyethylflurazepam	0.97	Positive	10	1	EDDP_D3
6B-Naltrexol	0.62	Positive	10	5	Oxycodone_D6
6-MAM	0.63	Positive	10	1	6-MAM_D3
7-Aminoclonazepam	0.68	Positive	10	1	7-Aminoclonazepam_D4
7-Aminoflunitrazepam	0.75	Positive	10	1	7-Aminoflunitrazepam_D7
7-Aminonitrazpeam	0.55	Positive	10	2.5	Ephedrine_D3
Acetaminophen	0.49	Positive	100	25	Hydromorphone_D6
alpha-Hydroxyalprazolam	0.96	Positive	10	5	Oxazepam_D5
alpha-Hydroxymidazolam	0.90	Positive	10	2.5	Fentanyl_D5
alpha-Hydroxytriazolam	0.95	Positive	10	5	Oxazepam_D5
Alprazolam	1.02	Positive	10	1	Alprazolam_D5
Amobarbital	0.91	Negative	200	400	Amobarbital_D5
Amphetamine	0.58	Positive	50	5	Amphetamine_D6
Benzoylecgonine	0.71	Positive	20	1	Benzoylecgonine_D3
Bromazepam	0.88	Positive	10	2.5	PCP_D5
Buprenorphine	0.94	Positive	10	10	Buprenorphine_D4
Buprenorphine-3B-Glucuronide	0.82	Positive	5	2.5	Meperidine_D4
Butalbital	0.85	Negative	200	200	Butalbital_D5
Carisoprodol	0.96	Positive	25	6.25	Carisoprodol_D7
Chlordiazepoxide	0.84	Positive	10	1	Meperidine_D4
cis-Tramadol	0.77	Positive	10	1	Methylphenidate_D9
Clonazepam	0.96	Positive	10	5	Carisoprodol_D7
Cocaethylene	0.87	Positive	20	2	PCP_D5
Cocaine	0.82	Positive	20	2	Meperidine_D4
Codeine	0.58	Positive	10	2.5	Codeine_D3
Codeine-6B-Glucuronide	0.55	Positive	10	10	Ephedrine_D3
Cotinine	0.30	Positive	10	1	Cotinine_D3
Desalkylflurazepam	0.99	Positive	10	2.5	Nordiazepam_D5
Diazepam	1.06	Positive	10	1	Diazepam_D5
Dihydrocodeine	0.57	Positive	10	2.5	Codeine_D3
EDDP	0.97	Positive	10	2.5	EDDP_D3
Ephedrine	0.54	Positive	100	10	Ephedrine_D3
Fentanyl	0.91	Positive	1	0.25	Fentanyl_D5
Flunitrazepam	0.99	Positive	10	1	Nordiazepam_D5
Flurazepam	0.93	Positive	10	1	Buprenorphine_D4
Gabapentin	0.56	Positive	100	10	Gabapentin_D10
Hydrocodone	0.64	Positive	10	2.5	Hydrocodone_D6
Hydromorphone	0.50	Positive	10	2.5	Hydromorphone_D6
Hydromorphone-3B-Glucuronide	0.43	Positive	10	2.5	Morphine_D6
Ketamine	0.71	Positive	5	0.5	Benzoylecgonine_D3
Lorazepam	0.96	Positive	10	5	Carisoprodol_D7
Lorazepam Glucuronide	0.87	Positive	10	10	PCP_D5
MDA	0.62	Positive	50	50	Oxycodone_D6
MDEA	0.69	Positive	50	5	MDEA_D5
MDMA	0.65	Positive	50	5	Phentermine_D5
Meperidine	0.82	Positive	10	1	Meperidine_D4
Meprobamate	0.81	Positive	25	12.5	Tapentadol_D3
Methadone	1.02	Positive	10	1	Alprazolam_D5
Methamphetamine	0.63	Positive	50	12.5	6-MAM_D3
Methylphenidate	0.77	Positive	25	2.5	Methylphenidate_D9
Midazolam	0.92	Positive	10	2.5	Fentanyl_D5
Morphine	0.45	Positive	10	1	Morphine_D6
Morphine-3B-Glucuronide	0.40	Positive	10	2.5	Morphine_3B-Glucuronide_D3
Morphine-6B-Glucuronide	0.44	Positive	10	20	Morphine_D6
Naloxone	0.57	Positive	10	5	Codeine_D3
Naloxone-3B-Glucuronide	0.48	Positive	10	5	Oxymorphone_D3
Naltrexone	0.62	Positive	10	5	Oxycodone_D6
N-Desmethyltramadol	0.77	Positive	10	2.5	Methylphenidate_D9
N-Desmethylzopiclone	0.75	Positive	10	2.5	7-Aminoflunitrazepam_D7
Nicotine	0.26	Positive	10	1	Nicotine_D4
Nitrazepam	0.94	Positive	10	5	Buprenorphine_D4
Norbuprenorphine	0.84	Positive	5	2.5	Norbuprenorphine_D3
Norbuprenorphine Glucuronide	0.70	Positive	5	10	MDEA_D5
Norchlordiazepoxide	0.82	Positive	10	5	Meperidine_D4
Norcodeine	0.56	Positive	10	10	Codeine_D3
Nordiazepam	0.98	Positive	10	2.5	Nordiazepam_D5
Norephedrine	0.48	Positive	100	10	Oxymorphone_D3
Norfentanyl	0.72	Positive	1	0.5	Norfentanyl_D5
Norhydrocodone	0.63	Positive	10	10	Hydrocodone_D6
Norketamine	0.70	Positive	5	0.5	Benzoylecgonine_D3
Normeperidine	0.81	Positive	10	1	Meperidine_D4
Noroxycodone	0.61	Positive	10	10	Oxycodone_D6
Noroxymorphone	0.45	Positive	10	5	Morphine_D6
Norpropoxyphene	0.99	Positive	25	2.5	Nordiazepam_D5
O-Desmethyltramadol	0.63	Positive	10	1	Hydrocodone_D6
Oxazepam	0.95	Positive	10	10	Oxazepam_D5
Oxazepam Glucuronide	0.85	Positive	10	20	Norbuprenorphine_D3
Oxycodone	0.62	Positive	10	10	Oxycodone_D6
Oxymorphone	0.47	Positive	10	1	Oxymorphone_D3
Oxymorphone-3B-Glucuronide	0.40	Positive	10	10	Morphine_3B-Glucuronide_D3
PCP	0.89	Positive	10	1	PCP_D5
Pentazocine	0.86	Positive	20	2	Norbuprenorphine_D3
Pentobarbital	0.91	Negative	200	400	Amobarbital_D5
Phenobarbital	0.81	Negative	200	200	Phenobarbital_D5
Phentermine	0.66	Positive	50	5	Phentermine_D5
Pregabalin	0.56	Positive	100	10	Gabapentin_D10
Propoxyphene	1.01	Positive	25	2.5	Alprazolam_D5
Pseudoephedrine	0.55	Positive	100	10	Ephedrine_D3
Ritalinic Acid	0.69	Positive	25	6.25	MDEA_D5
Secobarbital	0.94	Negative	200	400	Secobarbital_D5
Tapentadol	0.78	Positive	10	1	Tapentadol_D3
Tapentadol Glucuronide	0.67	Positive	10	1	7-Aminoclonazepam_D4
Temazepam	1.01	Positive	10	1	Alprazolam_D5
Temazepam Glucuronide	0.89	Positive	10	10	PCP_D5
THC	1.35	Positive	15	30	THC_D3
THC-COOH	1.21	Negative	15	3.75	THC-COOH_D3
THC-COOH glucuronide	1.10	Negative	15	3.75	THC-COOH-Glucuronide_D3
THC-OH	1.20	Positive	15	150	THC-COOH_D3
Zolpidem	0.84	Positive	10	1	Norbuprenorphine_D3
Zolpidem Phenyl-4-carboxylic acid	0.70	Positive	10	1	Benzoylecgonine_D3
Zopiclone	0.76	Positive	10	1	7-Aminoflunitrazepam_D7

Separation & Detection Efficiency

Fast LC-MS/MS for large numbers of compounds requires an efficient UHPLC pump, LC column and triple quadrupole detector. At 1 mL/min with a 1.9 um particle column, observed LC peak widths were typically about 1.1 s at the base of the peak (see Figure 1).

Figure 1: SRM acquisition points under LC peak – Norfentanyl at 1 ng/mL in urine



Setting the SRM cycle time to 0.13 s allowed 8-10 acquisition points under each LC peak, as seen for Norfentanyl in Figure 1. Previous reports indicate measurement of 9 points under a Gaussian peak integrated at 0.1% relative abundance will yield measurement errors of less than 3%.[1] Acquisition speed and detection efficiency of the TSQ Endura is critical for such narrow LC peaks. For example, at 0.665 min in the LC run, the TSQ Endura was measuring the method maximum of 56 SRM transitions at an approximate dwell time of 1.3 ms (431 Hz acquisition rate). LC retention times were very consistent, varying less than 0.01 min (0.6 s) over approximately 300 injections. This allowed narrow Timed SRM windows of 0.1 min (6 s) for most compounds to maximize detection efficiency without compromising LC peak measurements.

Separation of Isomers/Isobars

Another critical aspect during method development was the separation of isomeric and isobaric compounds. Since the triple quad is generally operated as a unit-resolution mass spectrometer, isomers and isobars that do not have unique product ions will cause inaccurate quantification unless sufficiently separated chromatographically.

Figure 2: Isomers & Isobars of m/z 286

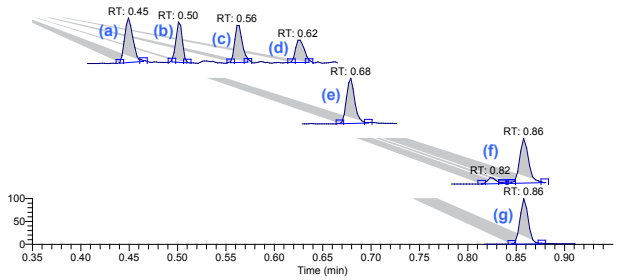


Figure 2 shows an example of the separation of isomers and isobars with the precursor ion at m/z 286. Compounds a-d, which have the common SRM transition of 286 > 152, are isomers morphine, hydromorphone, norcodeine and norhydrocodone, respectively. Peaks e & f are isomers 7-aminoclonazepam and norchlordiazepoxide, respectively. Peak at 0.86 min. having the same 286 > 227 transition as norchlordiazepoxide (f), is an interference also observed in the urine blank. Peak g is Pentazocine (286 > 218).

While most isomers and isobars (color coded) in Table 2 were baseline separated, not all isomers were well resolved with this LC method. For example, isomers amobarbital and pentobarbital showed no separation; ephedrine and pseudoephedrine were only partially separated (data not shown). Opiate conjugates hydromorphone-3B-glucuronide (b) and morphine-6B-glucuronide (c) were also partially separated as shown in Figure 3 below.

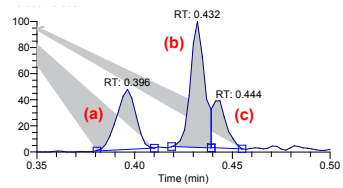


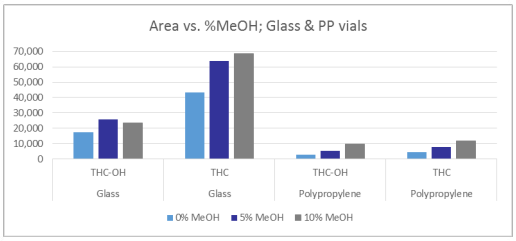
Figure 3: Glucuronide isomers in urine
(a) Morphine-3B-glucuronide,
(b) Hydromorphone-3B-glucuronide,
(c) Morphine-6B-glucuronide

Figures of Merit

Table 2 provides an overview of the drugs of abuse and metabolites measured in urine using polarity switching on the Vanquish UHPLC/TSQ Endura system. Retention times, ion polarity, internal standards, cutoff levels and the lower limits of quantitation (LLOQs) are also listed. LLOQs were determined by N=5 replicate injections, where the acceptance criteria were %CV < 20%, Mean %Difference < 20% and ion ratio confirmations (IRCs) pass for 4 of 5 injections.

All compounds were fit to linear regression curves with 1/x weighting using internal calibration based on area ratios. R^2 > 0.990 was observed for all compounds except morphine-6B-glucuronide, the cannabinoids and the barbiturates. Morphine-6B-glucuronide regression was affected below the cutoff concentration owing to the closely eluting hydromorphone-3B-glucuronide (see Figure 3). Poor regression for the barbiturates was due to low ionization efficiency in negative mode as a result of using 0.1% formic acid in the mobile phase. The cannabinoids were likely affected due to sample solubility and adsorption losses.[2,3] As shown in Figure 4, these issues were also observed for THC and 11-OH THC during method development, especially with polypropylene autosampler vials. Glass vials and dilution of urine samples with 20% MeOH were employed to help abate these issues.

Figure 4: Effect of solution organic % and vial composition on Cannabinoids' response



Polarity Switching vs. Discrete Ion Polarity

To the authors' knowledge, this is the first report of measuring several dozen drugs of abuse and metabolites in urine by LC-MS/MS with polarity switching in approximately 2 minutes per sample. A recent report[4] presented a similar dilute-and-shoot method for 78 drugs and metabolites in urine. However, this method uses separate positive and negative ion LC-MS/MS runs totaling 11.1 and 4.5 minutes, respectively.

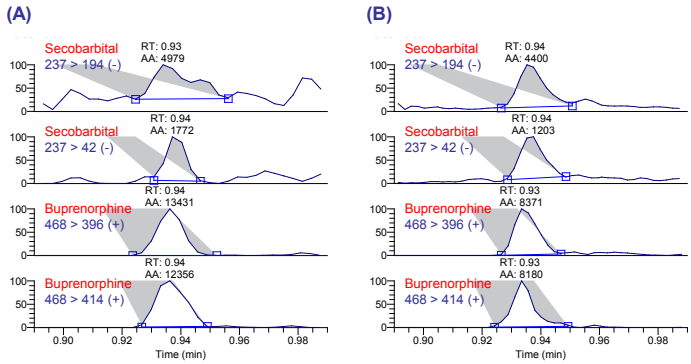
For comparison purposes, the same urine samples were analyzed concurrently with polarity switching, positive ion only and negative ion only acquisitions using the same LC method. As Table 2 indicates, most barbiturates (e.g., secobarbital) did not perform adequately using polarity switching to achieve LLOQs at the designated cutoff levels.

Figure 5 shows example chromatograms at 0.5 times the cutoff for secobarbital and buprenorphine by polarity switching (A) and by discrete ion polarity acquisitions (B). Note the improvement in S/N for the quantifier SRM transition of secobarbital (237 > 194) when data were acquired in negative mode only. The improvement is further reflected by the %CVs, which were 20.2% and 5.4% for secobarbital for polarity switching versus negative ion only, respectively. Also, the IRCs only passed in 2 of 5 injections at this concentration with polarity switching; all 5 injections passed with negative ion only. In fact, the LLOQ for secobaribtal was 0.25 times the cutoff (50 ng/mL) with discrete negative ion acquisition versus 2 times the cutoff (400 ng/mL) for polarity switching.

In contrast, the differences in performance were not as significant with buprenorphine. For example, at 0.5 times the cutoff (5 ng/mL), the %CVs were 17.0% and 15.6% for polarity switching versus positive ion only, respectively.

The reasons for these differences result from the absolute changes in SRM dwell times and in the triple quad duty cycle for the compounds during these modes of acquisition. As a result of the polarity switching time (50 ms per cycle), the actual TSQ measurement time is reduced from 130 ms to 80 ms. At the retention time for secobarbital and buprenorphine, a total of 46 SRM transitions were measured: 40 positive and 6 negative. During polarity switching experiments, the dwell time is ~0.7 ms for all SRM transitions. When acquiring in positive mode only, the 40 SRM transitions have a dwell time of ~2.3 ms. However, in negative mode only, the 6 SRM transitions have a dwell time of ~21 ms. The substantial increase in dwell time *and* duty cycle (2.2% versus 16.7%) for secobarbital during negative mode only acquisition account for the significant improvements in S/N and %CVs observed. Conversely, the duty cycle for buprenorphine only increases from 2.2% to 2.5%.

Figure 5: Example Chromatograms for Polarity Switching (A) & Discrete Ion Polarity (B) for Secobarbital and Buprenorphine at the 0.5 cutoff level



CONCLUSIONS

- The reproducible chromatographic performance of the Vanquish UPHLC system along with the speed and sensitivity of the TSQ Endura mass spectrometer showed herein supports the feasibility to measure ~100 drugs of abuse and metabolites in diluted urine for forensic toxicology samples in about 2 minutes per sample using fast UHPLC-MS/MS.
- Diligent LC method development allowed for the baseline separation of most isomeric and isobaric compounds measured by UHPLC-MS/MS in under 1.4 minutes.
- Most target compounds had LLOQs at or below the designated cutoff levels in diluted urine. Some problematic compounds, such as THC, could be improved by refining the sample preparation to prevent adsorption losses.
- Improved performance in LLOQ was observed for some negative ion compounds when discrete ion polarity was used versus polarity switching. This was due to the significant increase in compound dwell time and duty cycle. Compounds in positive ion mode did not show as significant a difference owing to a lesser increase in dwell time and duty cycle.

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