thermoscientific

Confident Quantitation with LC-MS/MS: Fast, Robust, Reliable, Reproducible, Sensitive Quantitation of Drugs of Abuse in Urine

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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

RESULTS

Table 2: Measured Drugs of Abuse in Urine

Compound	RT (min)	Cutoff (ng/mL)	LLOQ (ng/mL)
2-Hydroxyethylflurazepam	0.97	10	1
6B-Naltrexol	0.62	10	5
6-MAM	0.63	10	1
7-Aminoclonazepam	0.68	10	1
7-Aminoflunitrazepam	0.75	10	1
7-Aminonitrazepam	0.55	10	2.5
Acetaminophen	0.49	100	25
alpha-Hydroxyalprazolam	0.96	10	5
alpha-Hydroxymidazolam	0.90	10	2.5
alpha-Hydroxytriazolam	0.95	10	5
Alprazolam	1.02	10	1
Amobarbital	0.91	200	400
Amphetamine	0.58	50	5
Benzoylecgonine	0.71	20	1
Bromazepam	0.88	10	2.5
Buprenorphine	0.94	10	10
Buprenorphine-3B-Glucuronide	0.82	5	2.5
Butalbital	0.85	200	200
Carisoprodol	0.96	25	6.25
Chlordiazepoxide	0.84	10	1
cis-Tramadol	0.77	10	1
Clonazepam	0.96	10	5
Cocaethylene	0.87	20	2
Cocaine	0.82	20	2
Codeine	0.58	10	2.5
Codeine-6B-Glucuronide	0.55	10	10
Cotinine	0.30	10	1
Desalkylflurazepam	0.99	10	2.5
Diazepam	1.06	10	1
Dihydrocodeine	0.57	10	2.5
EDDP	0.97	10	2.5
Ephedrine	0.54	100	10
Fentanyl	0.91	1	0.25
Flunitrazepam	0.99	10	1
Flurazepam	0.93	10	1
Gabapentin	0.56	100	10
Hydrocodone	0.64	10	2.5
Hydromorphone	0.50	10	2.5
Hydromorphone-3B-Glucuronide	0.43	10	2.5
Ketamine	0.71	5	0.5
Lorazepam	0.96	10	5
Lorazepam Glucuronide	0.87	10	10
MDA	0.62	50	50
MDEA	0.69	50	5
MDMA	0.65	50	5
Meperidine	0.82	10	1
Methadone	1.02	10	1
Methamphetamine	0.63	50	12.5
Methylphenidate	0.77	25	2.5
Midazolam	0.92	10	2.5
Morphine	0.45	10	1
Morphine-3B-Glucuronide	0.40	10	2.5
Morphine-6B-Glucuronide	0.44	10	20
Naloxone	0.57	10	5
Naloxone-3B-Glucuronide	0.48	10	5
Naltrexone	0.62	10	5
N-Desmethyltramadol	0.77	10	2.5
N-Desmethylzopiclone	0.75	10	2.5
Nicotine	0.26	10	1
Nitrazepam	0.94	10	5
Norbuprenorphine	0.84	5	2.5
Norbuprenorphine Glucuronide	0.70	5	10
Norchlordiazepoxide	0.82	10	5
Norcodeine	0.56	10	10
Nordiazepam	0.98	10	2.5
Norephedrine	0.48	100	10
Norfentanyl	0.72	1	0.5
Norhydrocodone	0.63	10	10
Norketamine	0.70	5	0.5
Normeperidine	0.81	10	1
Noroxycodone	0.61	10	10
Noroxymorphone	0.45	10	5
Norpropoxyphene	0.99	25	2.5
O-Desmethyltramadol	0.63	10	1
Oxazepam	0.95	10	10
Oxazepam Glucuronide	0.85	10	20
Oxycodone	0.62	10	10
Oxymorphone	0.47	10	1
Oxymorphone-3B-Glucuronide	0.40	10	10
PCP	0.89	10	1
Pentazocine	0.86	20	2
Pentobarbital	0.91	200	400
Phenobarbital	0.81	200	200
Phentermine	0.66	50	5
Pregabalin	0.56	100	10
Propoxyphene	1.01	25	2.5
Pseudoephedrine	0.55	100	10
Ritalinic Acid	0.69	25	6.25
Secobarbital	0.94	200	400
Tapentadol	0.78	10	1
Tapentadol Glucuronide	0.67	10	1
Temazepam	1.01	10	1
Temazepam Glucuronide	0.89	10	10
THC	1.35	15	30
THC-COOH	1.21	15	3.75
THC-COOH glucuronide	1.10	15	3.75
THC-OH	1.20	15	150
Zolpidem	0.84	10	1
Zolpidem Phenyl-4-carboxylic acid	0.70	10	1
Zopiclone	0.76	10	1

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).

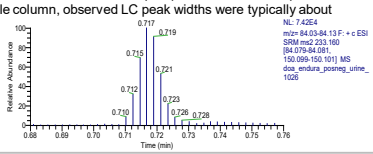

Figure 1: SRM acquisition points under LC peak – Norfentanyl at 1 ng/mL in urine

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%. 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.

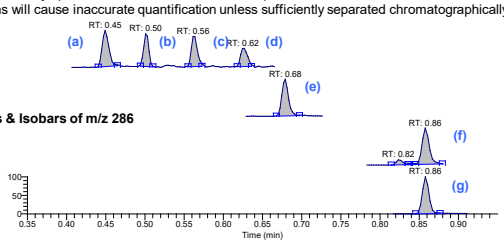

Figure 2: Isomers & Isobars of m/z 286

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.

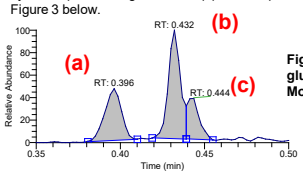

Figure 3: Glucuronide isomers in urine – (a) Morphine-3B-glucuronide, (b) Hydromorphone-3B-glucuronide, (c) Morphine-6B-glucuronide

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. Glass vials and dilution of urine samples with 20% MeOH were employed to help abate any issues.

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.

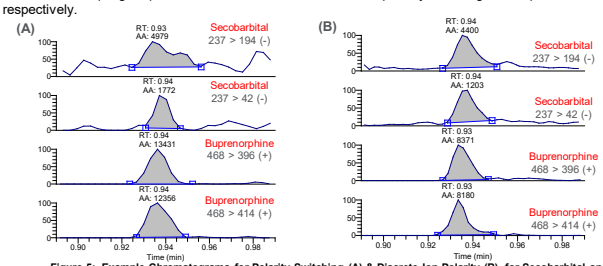

Figure 5: Example Chromatograms for Polarity Switching (A) & Discrete Ion Polarity (B) for Secobarbital and Buprenorphine at the 0.5 cutoff level

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, up to 8-fold, was observed for some negative ion compounds such as secobarbital when discrete ion polarity was used versus polarity switching. Compounds in positive ion mode did not show as significant a difference owing to a lesser increase in dwell time and duty cycle.