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Article

Simultaneous Quantitation of 78 Drugs and Metabolites in Urine with a Dilute-And-Shoot LC–MS-MS Assay

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A novel LC–MS-MS assay that simultaneously detects and quantitates 78 drugs and metabolites was developed and validated for chronic pain management. Urine specimen was diluted and mixed with internal standards (ISs) before injected into LC–MS-MS. Seventy-two analytes were detected with positive electrospray ionization mode and the remaining six analytes with negative mode. Two separate gradient elution chromatographic programs were established with the same mobile phases on the same bi-phenyl HPLC column. The assay was linear for all analytes with linear regression coefficient ranging 0.994–1.000. The intra-assay precision was between 1.7 and 8.8% and inter-assay precision between 1.9 and 12.2%, with bias <20% for all but six analytes. All analytes in urine specimens were stable for 7 days at 4°C, and no significant matrix effect or carryover was observed. A suboptimal recovery rate (60.0–156.8%) was observed for six analytes, potentially due to the lack of available deuterated ISs, requiring comparison to a chemically different IS. Method comparison using patient and proficiency testing samples demonstrated that this assay was sensitive and accurate. The assay improves on currently existing assays by including glucuronide conjugates, allowing direct detection of metabolites that might otherwise be missed by existing methods.

Introduction

Both prescribed drug misuse and illicit drug abuse risks exist in patients in chronic pain management programs (1). It was reported that 75% of patients in chronic pain management programs were found non-compliant with their prescription and 11% tested positive for illicit drug use (2). It is worth noting that abuse of prescription medications is increasing faster than illicit drugs. The incidence rate of prescription drug abuse has increased over 80% from 2000 to 2006, exceeding the incidence rate of abuse of the illicit drugs such as cocaine, heroin, marijuana and ecstasy combined (3). Drug compliance testing provides an objective measure of a patient's pattern of drug use. It may aid in identifying patients who are inappropriately taking prescribed medications or illicit drugs, which may interfere with treatment. Without drug testing, it could be challenging to identify drug abusers as obvious signs, behaviors and symptoms are not always present in those patients (4, 5).

Urine drug testing is a useful tool for pain management providers to assist in diagnostic and therapeutic decision-making. The main advantages of choosing urine as the specimen for drug testing include noninvasive sampling, drugs and metabolites present in high concentrations and relatively long detection windows (5). Adherence monitoring with urine drug testing has become a common practice in recent years. It has been shown that random urine drug testing increased compliant use of opioids with concomitant decreased illicit drug use in pain management practices (6, 7). Immunoassay drug tests are most commonly used in initial urine drug screens. They are based on interactions between antibodies and drugs of interest and are designed to classify substances as either present or absent in patient urine. However, because of the nature of immunoassays, they are prone to false positives caused by cross reactions and false negatives because of limited sensitivity (8). Therefore, results of immunoassay drug screens are presumptive and cannot be used solely to determine compliance status. Gas chromatography–mass spectrometry (GC–MS) methods are considered the gold standard for confirmatory testing. However, GC–MS methods are time consuming and in many cases require a derivatization step that contributes to sample loss (9). Compared with GC–MS, the liquid chromatography–tandem mass spectrometry (LC–MS-MS) assays generally require much simpler sample preparation with reasonably high sensitivity and specificity, are subjected to fewer interferences and have the potential to quantitate multiple analytes in a single method (10).

Many pain medications, such as opiates, opioids and benzodiazepines, are metabolized and conjugated in the liver. Parent drugs and metabolites are excreted in urine in both free and glucuronide conjugated forms (11). The urine concentration of conjugates can vary substantially depending on individual metabolism rates and sample collection time (8, 11). To improve assay sensitivity and reduce the number of monitored transitions, glucuronide hydrolysis may be included in sample preparation prior to LC–MS-MS. Chemical hydrolysis, such as acid hydrolysis, is fast and efficient, but its efficiency is subject to variables like acid concentration, temperature and pressure (12). Enzymatic hydrolysis (β-glucuronidase) is more specific, but can lead to incomplete hydrolysis if the enzyme concentration or incubation time is not properly optimized (12, 13). Solid-phase extraction (SPE) is frequently used for urine sample cleanup in recent LC–MS-MS method reports of urine drug testing (8, 9, 14, 15). However, SPE sample extraction typically involves laborious procedures and has inconsistent recoveries for all analytes, especially when many analytes were measured simultaneously (8, 15). Alternatively, direct injection of diluted urine samples into LC–MS-MS, or dilute-and-shoot methods, have been successfully employed in a several recent pain management urine drug testing assay (2, 16, 17). Although these reported dilute-and-shoot based assays were subject to various amounts of ion suppression due to matrix effects, it provides a simple but robust solution to develop an LC–MS-MS pain medication panel.

In this study, we developed and validated a simple and cost-effective LC–MS-MS assay which is able to simultaneously quantitate 78 drugs and metabolites that cover major categories of illicit drugs and drugs commonly prescribed in chronic pain patients. This dilute-and-shoot assay involves minimum sample preparation

by including glucuronide conjugates with commercially available standards, eliminating the need for an extra hydrolysis step. To maximize ionization efficiency, two separate LC–MS-MS methods, one employing positive and the other negative ionization, were built to measure 72 and 6 analytes, respectively. Isotope-labeled internal standards (ISs) were incorporated to facilitate reliable quantitation of the samples. As a major urinary metabolite of propoxyphene, norpropoxyphene has been reported to be unstable and become cyclized degradation products (8, 18, 19). To ensure accurate quantitation, both norpropoxyphene and its degradation products were quantified and summed before results reported.

Materials and methods

Chemicals and reagents

All certified reference standards and isotope-labeled IS solutions were purchased from Cerilliant (Round Rock, TX, USA). Methanol (Optima® LC/MS grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Trazodone (T-030, 1 mg/mL) and its metabolite *meta*-chlorophenylpiperazine (*m*-CPP, C-089, 1 mg/mL) methanol stocks were purchased from Cerilliant. Formic acid (LC/MS grade, 98%) was purchased from Sigma-Aldrich (St Louis, MO, USA). Liquichek™ Urine Toxicology Negative Control (blank human urine) was purchased from Bio-Rad (Hercules, CA, USA). Deionized water was generated with a Milli-Q water purification system from Millipore (Billerica, MA, USA).

Instrumentation and conditions

LC–MS-MS analysis was performed on an AB Sciex 5500 Q-trap mass spectrometer (Framingham, MA, USA) coupled with a Shimadzu Nexera X2 ultra-high pressure liquid chromatography (UHPLC) system (Kyoto, Japan). The temperature of the thermostatted column and the autosampler were set at 40 and 5°C, respectively. Of the 78 drugs and metabolites measured, 72 were detected in a positive ionization method and 6 in a negative ionization method (Table I). In both the methods, the chromatography separation was performed with a Raptor™ Bi-phenyl column, 3.0 × 50 mm, 2.7 μm (Restek, Bellefonte, PA, USA) and gradient elution comprising 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). A Raptor™ EXP® Guard Column Cartridge (2.7 μm, 3.0 × 5 mm) was installed preceding the bi-phenyl analytical column for the sake of sample cleanup. The gradient program for positive ionization method started from 5% mobile phase B at 0–0.2 min, increasing to 25% at 3 min. The mobile phase B content was further increased to 100% at 7.5 min, and held until 9.0 min, after which it was dropped to 5% at 9.5 min and held until 11.0 min. The total run time was 11.1 min with a flow rate of 0.6 mL/min. The gradient program for positive ionization method started from 10% mobile phase B at 0–0.2 min, increasing to 40% at 0.7 min. The mobile phase B content was further increased to 99% at 3.0 min, and held until 3.5 min, after which it was dropped to 10% at 3.6 min and held until 4.5 min. The total run time was 4.5 min with a flow rate of 0.7 mL/min.

Analytes were detected by mass spectrometry using scheduled multiple reaction monitoring (MRM) in either positive or negative electrospray ionization (ESI) modes. All analytes were monitored within a ±0.5 min retention time window. The dwell time was automatically calculated by the software Analyst® under the dynamic MRM mode with a total cycle time of 0.3 and 0.4 ms, respectively, for positive and negative ionization modes. For the positive ionization method, the source parameters were: curtain gas, 35 L/min; collisional activated dissociation (CAD), medium; heated nebulizer temperature, 550°C; nebulizing gas (GS1), 50 L/min and heater gas (GS2), 60 L/min. For the negative ionization method, the source parameters were: curtain gas, 35 L/min; CAD, medium; heated nebulizer temperature, 600°C; GS1, 50 L/min and GS2, 50 L/min. Two characteristic MRM transitions were monitored for each analyte, with the exception of amphetamine, buprenorphine-3β-D-glucuronide, norbuprenorphine and norbuprenorphine-3β-D-glucuronide for which only one transition was available. The MRM ratios, which are defined as the peak area ratios between primary and secondary ion transitions, were only acceptable within ±30% or better for all analytes. All data were collected using the AB Sciex Analyst® software and quantified with the MultiQuant® 2.1 software.

Preparation of calibrators and quality control materials

All calibrators and quality controls (QCs) were prepared separately for positive and negative ionization methods, comprising 72 and 6 compounds, respectively. First, the 40× positive or negative working solution was prepared by mixing each individual compound in 50% methanol in water. The concentration of each compound in the above working solutions was 40 times of their individual cutoff value. Different predetermined cutoffs were used for different analytes, based on their distinct clinical significance. The 40× working solution was then diluted with 50% methanol in water, producing five calibrator solutions: calibrator 5 (8×), calibrator 4 (4× cutoff), calibrator 3 (1× cutoff), calibrator 2 (0.4× cutoff) and calibrator 1 (0.2× cutoff). High-level (QC-H, 3× cutoff) and low-level (QC-L, 0.5× cutoff) QCs were diluted from separately prepared 40× working solutions with 50% methanol in water. The calibrator and QC bulk solutions were then aliquoted into microcentrifuge tubes (2.0 mL) and stored at −20°C.

Similarly, IS mix was also prepared separately for positive and negative ionization methods, by adding individual deuterated IS in 100% methanol. IS mix was aliquoted and stored at −20°C.

Sample preparation

One hundred microliter urine specimens, calibrators or controls were centrifuged for 3 min at 10,000 rpm (Eppendorf centrifuge model 5430). After centrifugation, 10 μL of urine specimen supernatant (or calibrator, QC), and 10 μL of IS mix, was diluted in 480 μL of sample diluent (95% mobile phase A + 5% mobile phase B, for patient samples) or 470 μL of sample diluent plus 10 μL of Bio-Rad blank urine (for calibrators and QCs) before injection. The purpose of adding 10 μL of Bio-Rad blank urine for calibrators and QCs is to ensure the matrix resemblance to patient samples in the final preparation.

Assay validation

The method was validated for linearity, limit of detection (LOD), lowest limit of quantitation (LLOQ), precision, accuracy,

Table I
MRM Transitions, Retention Time and Compound Tuning Parameters

Compound	Precursor ion (*m*/*z*)	Primary ion (*m*/*z*)	Secondary ion (*m*/*z*)	Retention time (min)	DP	CE	Ionization mode
2-Hydroxyethylflurazepam	333.2	211.2	109.0	7.1	110	55	Positive
6-Acetylmorphine (6-MAM)	328.1	165.0	191.0	4.2	100	51	Positive
6β-Naltrexol	344.1	308.3	326.0	4.6	100	40	Positive
7-Aminoclonazepam	286.2	121.1	222.2	5.5	100	41	Positive
7-Aminoflunitrazepam	284.2	135.1	227.2	6.1	100	40	Positive
Alprazolam	309.2	281.2	205.2	7.5	160	50	Positive
Amphetamine	136.0	119.0	–	2.9	20	13	Positive
Benzoylecgonine	290.1	168.1	105.0	5.4	100	37	Positive
Buprenorphine	468.3	396.3	55.0	6.0	70	55	Positive
Buprenorphine-3β-D-glucuronide	644.3	468.4	–	5.5	20	55	Positive
Carisoprodol	261.3	55.1	97.1	6.4	80	40	Positive
Chlordiazepoxide	300.1	227.0	165.1	6.2	81	37	Positive
Clonazepam	316.1	270.2	214.1	7.0	150	35	Positive
Codeine	300.1	115.1	165.0	4.1	100	100	Positive
Codeine-6-glucuronide	476.3	300.3	215.1	4.2	80	44	Positive
Diazepam	285.2	193.1	154.1	7.7	160	44	Positive
EDDP	278.2	234.2	219.1	6.3	100	50	Positive
Fentanyl	337.2	188.1	105.1	6.0	100	45	Positive
Flunitrazepam	314.2	268.2	239.1	7.4	150	36	Positive
Flurazepam	388.2	315.0	288.2	6.1	80	48	Positive
Gabapentin	172.2	137.1	55.0	3.1	98	30	Positive
Hydrocodone	300.2	199.1	171.1	4.5	100	40	Positive
Hydromorphone	286.2	185.0	157.0	3.0	120	40	Positive
Hydromorphone-3-glucuronide	462.3	286.2	185.1	2.4	50	41	Positive
Lorazepam	321.1	275.1	229.2	6.9	150	30	Positive
Lorazepam-glucuronide	497.0	275.1	303.0	6.5	111	53	Positive
MDA	180.2	133.0	135.1	3.6	60	23	Positive
MDEA	208.1	105.2	163.2	4.6	40	26	Positive
MDMA	194.1	163.1	135.1	4.2	80	29	Positive
Meperidine	248.1	91.0	70.1	5.3	100	72	Positive
Meprobamate	219.2	158.2	97.2	5.5	101	25	Positive
Methadone	310.2	265.2	105.0	6.5	80	30	Positive
Methamphetamine	150.0	91.2	119.0	3.6	20	16	Positive
Midazolam	326.1	291.1	249.1	6.3	100	48	Positive
Morphine	286.2	152.0	128.0	2.5	100	78	Positive
Morphine-3-glucuronide	462.2	286.2	201.2	2.0	80	44	Positive
Morphine-6-glucuronide	462.2	286.2	201.2	2.8	20	47	Positive
Naloxone	328.1	212.2	253.2	3.9	80	51	Positive
Naltrexone	342.1	267.2	55.0	4.4	100	40	Positive
Norbuprenorphine	414.3	152.0	–	5.6	100	129	Positive
Norbuprenorphine-3β-D-glucuronide	590.4	414.4	–	4.9	30	52	Positive
Nordiazepam	271.2	140.1	165.1	7.2	160	39	Positive
Norfentanyl	233.1	84.0	55.0	4.9	80	32	Positive
Norhydrocodone	286.1	199.1	241.1	4.1	120	30	Positive
Normeperidine	234.3	42.1	56.0	5.2	80	42	Positive
Noroxycodone	302.1	227.1	284.1	3.9	120	41	Positive
Norpropoxyphene	326.3	44.1	252.3	6.2	50	45	Positive
NPD (norbuprenorphine degradent)	308.3	100.0	143.0	6.3	80	26	Positive
O-Demethyl Tramadol	250.2	58.1	42.0	4.1	50	14	Positive
Oxazepam	287.2	241.1	104.0	7.0	150	30	Positive
Oxazepam-glucuronide	463.1	287.2	241.0	6.6	100	24	Positive
Oxycodone	316.2	212.1	241.1	4.3	160	58	Positive
Oxymorphone	302.1	227.0	198.1	2.7	100	38	Positive
Oxymorphone-3-glucuronide	478.3	284.2	227.1	1.8	80	40	Positive
PCP (Phencyclidine)	244.3	91.0	159.0	6.1	40	24	Positive
Pregabalin	160.1	97.1	83.1	2.4	70	20	Positive
Propoxyphene	340.3	266.3	57.7	6.2	80	13	Positive
Sufentanil	387.2	238.2	111.2	6.3	100	39	Positive
Temazepam	301.2	255.2	177.2	7.4	150	55	Positive
Temazepam-glucuronide	477.1	301.2	283.2	7.0	90	23	Positive
Tramadol	264.2	42.1	58.0	5.1	50	100	Positive
Triazolam	343.2	239.1	308.2	7.4	100	56	Positive
α-Hydroxyalprazolam	325.2	297.2	216.2	7.1	160	35	Positive
α-Hydroxymidazolam	342.2	203.1	168.1	6.6	100	35	Positive
α-Hydroxytriazolam	359.2	331.1	176.1	7.1	100	37	Positive
Zaleplon	306.1	236.1	264.2	7.2	107	39	Positive
Zopiclone	389.1	245.0	217.2	5.4	85	25	Positive
Zolpidem	308.1	235.2	263.2	5.8	75	69	Positive
Zolpidem-Phenyl Carboxylic acid	338.1	265.2	293.2	5.1	126	51	Positive
Amitriptyline	278.2	191.1	91.1	6.4	95	33	Positive
Desipramine	267.2	44.1	72.0	6.2	100	62	Positive
Imipramine	281.2	58.0	86.1	6.3	90	30	Positive
Nortriptyline	264.2	105.0	233.1	6.3	95	29	Positive
Butalbital	223.0	42.0	180.0	2.5	−95	−48	Negative
Phenobarbital	231.1	42.1	188.0	2.4	−90	−54	Negative

(*continued*)

Table I Continued

Compound	Precursor ion (*m*/*z*)	Primary ion (*m*/*z*)	Secondary ion (*m*/*z*)	Retention time (min)	DP	CE	Ionization mode
Pentobarbital	225.0	42.0	182.1	2.6	−95	−56	Negative
Secobarbital	237.1	42.0	194.1	2.7	−95	−54	Negative
THC-COOH	343.1	245.2	107.1	3.6	−115	−40	Negative
THC-COOH glucuronide	519.2	343.1	299.2	3.5	−55	−34	Negative

DP, declustering potential; CE, collision energy.

interference and matrix effect, sample stability, carryover and correlation studies.

Linearity

Standard curve linearity was measured using the ratio of the analyte peak area to the IS area versus nominal concentration of standards by weighted linear regression ($1/X^2$). The acceptance criterion for a calibration curve was a correlation coefficient R of 0.990 or better.

LOD, LLOQ and dilution validation

LODs were evaluated by analyzing 15 Bio-Rad blank urine specimens fortified with IS over 3 days. An LOD was defined as the average concentration of the negative specimens plus three times the standard deviations (SDs) of the negative specimens. LLOQs were evaluated by analyzing serially diluted standards in 50% methanol urine specimen that were repeated 15 times over 5 days. The LLOQs were defined as the concentration at which the percent coefficient variation (% CV) was $\leq$20%. The accuracy acceptance criterion in LLOQ determination was $\pm$20%. The dilution validation was evaluated by 10-fold dilution of 40× working solution with Bio-Rad blank urine, which was repeated nine times over 3 days. The acceptance criterion for dilution validation was that the inter-assay percent CV was $\leq$20% and the % bias was within $\pm$20%.

Sample stability

The stability of the analytes in urine was assessed by storing urine samples (from three different negative patients) that have been fortified at their individual cutoff concentrations at 4°C for up to 7 days.

Carryover

Carryover was assessed by running four calibrator 1 samples (L_1-L_4) immediately after injecting three calibrator 5 (H_1-H_3) samples to verify the minimal sample carryover. Carryover was calculated as $100 * ((L_1 - (L_3 + L_4)/2)/((H_2 + H_3)/2 - (L_3 + L_4)/2)$ and must be <1% to be acceptable.

Interference and matrix effect

To evaluate interference, urine samples from 10 drug-negative patients (determined by in-house qualitative drug abuse screening on a Roche Cobas® 8000 platform) were analyzed separately to ensure that no visible interferences were present at the retention time of all analytes. The matrix effect was assessed by simultaneous post-column infusion (or 'tee-infusion') of standard compounds into the MS-MS detector during the chromatographic analysis of 10 separate patient negative urine samples (20). All analytes (0.1 μg/mL) were mixed in 50% methanol with 0.1% formic acid and infused at a flow rate of 10 μL/min in the course of the chromatographic analysis of the negative urine sample. The chromatographic signals of each selected MS-MS transition are examined to check for any signal perturbation (or ion suppression) of the MS-MS signal at the analytes' retention times.

Table II
IS MRM Transitions, Retention Time and Compound Tuning Parameters

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Retention time (min)	DP	CE	Ionization mode
2-Hydroxyethylflurazepam-D4	337.2	113.0	7.1	110	37	Positive
6-Acetylmorphine (6-MAM)-D3	331.1	165.1	4.2	100	51	Positive
7-Aminoclonazepam-D4	290.1	121.1	5.5	100	41	Positive
7-Aminoflunitrazepam-D7	291.2	138.1	6.1	100	40	Positive
Alprazolam-D5	314.2	286.1	7.5	160	39	Positive
Amphetamine-D5	141.1	124.0	2.9	20	13	Positive
Benzoylecgonine-D8	298.2	171.1	5.4	100	27	Positive
Buprenorphine-D4	472.3	59.1	6.0	70	97	Positive
Codeine-D3	303.3	115.1	4.1	100	100	Positive
Diazepam-D5	290.2	198.2	7.7	160	44	Positive
EDDP-D3	281.2	233.7	6.3	100	42	Positive
Fentanyl-D5	342.3	188.3	6.0	100	45	Positive
Gabepentin-D10	182.3	55.0	3.1	80	33	Positive
Hydrocodone-D6	306.2	202.1	4.5	100	40	Positive
Hydromorphone-D6	292.3	185.0	3.0	120	40	Positive
MDA-D5	185.2	110.0	3.6	60	30	Positive
MDEA-D5	213.1	105.1	4.6	40	26	Positive
MDMA-D5	199.2	164.8	4.2	80	25	Positive
Meperidine-D4	252.1	93.0	5.3	100	72	Positive
Methadone-D3	313.4	268.0	6.5	80	26	Positive
Methamphetamine-D5	155.2	121.2	3.6	20	15	Positive
Morphine-6-glucuronide-D3	465.2	289.2	2.8	20	47	Positive
Morphine-D3	289.2	152.2	2.5	100	78	Positive
Nordiazepam-D5	276.1	140.0	7.2	160	39	Positive
Norfentanyl-D5	238.2	84.0	4.9	80	32	Positive
Normeperidine-D4	238.2	42.0	5.2	80	42	Positive
O-Demethyl Tramadol-D6	256.3	64.2	4.1	50	14	Positive
Oxazepam-D5	292.3	246.2	7.0	150	30	Positive
Oxycodone-D6	322.1	218.2	4.3	160	58	Positive
Oxymorphone-D3	305.2	230.3	2.7	100	38	Positive
PCP (Phencyclidine)-D5	249.4	96.0	6.1	40	24	Positive
Propoxyphene-D11	351.3	277.3	6.3	80	13	Positive
Tramadol-13C, D3	268.0	58.0	5.1	50	35	Positive
Zolpidem-D7	315.1	242.1	5.9	100	45	Positive
Zopiclone-D4	393.1	245.0	5.4	85	25	Positive
α-Hydroxyalprazolam-D5	330.3	302.3	7.1	160	35	Positive
α-Hydroxytriazolam-D4	363.1	335.1	7.1	100	37	Positive
Imipramine-D3	284.2	61.0	6.3	90	63	Positive
Butalbital-D5	228.0	185.2	2.5	−95	−18	Negative
Phenobarbital-D5	236.0	42.0	2.4	−70	−54	Negative
Pentobarbital-D5	230.0	42.1	2.6	−110	−56	Negative
Secobarbital D5-IS	242.0	199.0	2.7	−115	−18	Negative
THC-COOH-D3	346.1	248.1	3.6	−115	−40	Negative
THC-COOH glucuronide-D3	522.0	193.0	3.5	−60	−28	Negative

DP, declustering potential; CE, collision energy.

Accuracy

To assess accuracy, analytes standard mix (each at their cutoff concentrations) was fortified in three sets of drug-negative

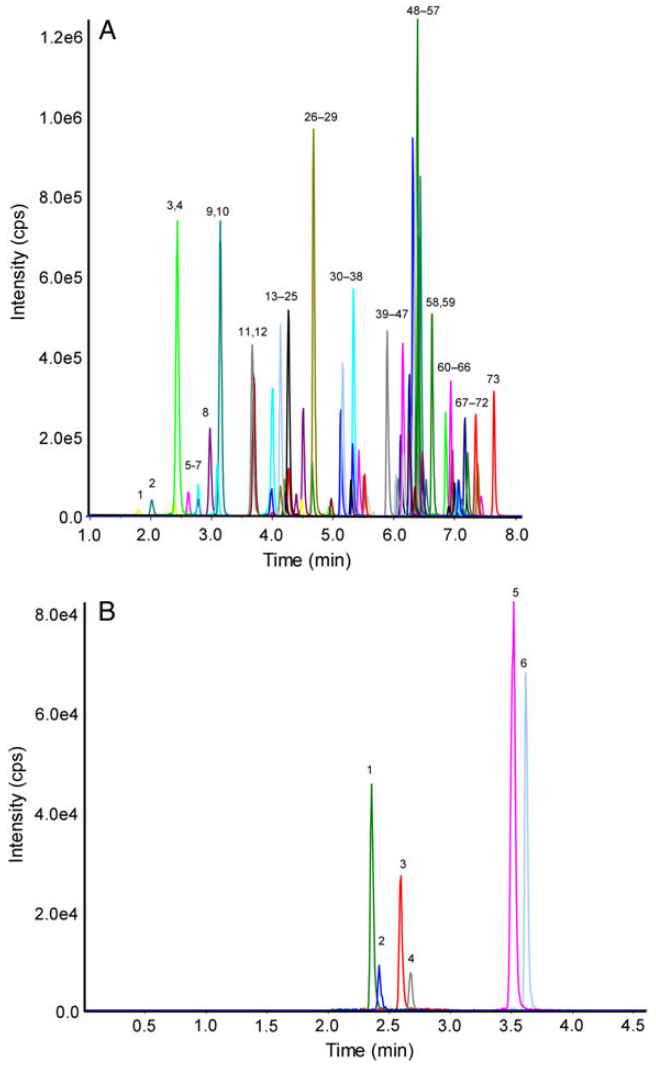

Figure 1. Complete MRM chromatograms of 73 analytes in a positive ESI mode and 6 in a negative ESI mode. (A) Analytes detected in a positive mode: 1. Oxymorphone-3-glucuronide, 2. Morphine-3-glucuronide, 3. Hydromorphone-3-glucuronide, 4. Pregabalin, 5. Morphine, 6. Oxymorphone, 7. Morphine-6-glucuronide, 8. Amphetamine, 9. Hydromorphone, 10. Gabapentin, 11. Methamphetamine, 12. MDA, 13. Noroxycodone, 14. Naloxone, 15. Norhydrocodone, 16. *O*-Demethyl Tramadol, 17. Codeine, 18. MDMA, 19. 6-Acetylmorphine (6-MAM), 20. Codeine-6-glucuronide, 21. Oxycodone, 22. Naltrexone, 23. Hydrocodone, 24. 6β-Naltrexol, 25. MDEA, 26. Norbuprenorphine-3β-D-glucuronide, 27. Norfentanyl, 28. Tramadol, 29. Zolpidem-phenyl carboxylic acid, 30. Normeperidine, 31. Meperidine, 32. Benzoylecgonine, 33. Zopiclone, 34. 7-Aminoclonazepam, 35. Buprenorphine-3β-D-glucuronide, 36. Meprobamate, 37. Norbuprenorphine, 38. Zolpidem, 39. Fentanyl, 40. Buprenorphine, 41. 7-Aminoflunitrazepam, 42. Flurazepam, 43. PCP (Phencyclidine), 44. Chlordiazepoxide, 45. Norpropoxyphene, 46. Propoxyphene, 47. Desipramine, 48. Sufentanil, 49. Midazolam, 50. Imipramine, 51. EDDP, 52. NPD (norbuprenorphine degradent), 53. Nortriptyline, 54. Amitriptyline, 55. Carisoprodol, 56. Lorazepam-glucuronide, 57. Methadone, 58. Oxazepam-glucuronide, 59. α-Hydroxymidazolam, 60. Lorazepam, 61. Oxazepam, 62. Clonazepam, 63. Temazepam-glucuronide, 64. α-Hydroxytriazolam, 65. 2-Hydroxyethylflurazepam, 66. α-Hydroxyalprazolam, 67. Nordiazepam, 68. Zaleplon, 69. Flunitrazepam, 70. Triazolam, 71. Temazepam, 72. Alprazolam, 73. Diazepam (B) Analytes detected in a negative mode: 1. Phenobarbital, 2. Butalbital, 3. Pentobarbital, 4. Secobarbital, 5. THC-COOH glucuronide, 6. THC-COOH.

urine specimens from six different patients. Accuracy was determined by the ratio of recovered analyte concentration to nominal concentration.

Precision

Intra-assay imprecision was estimated by analyzing 10 control specimens at two levels (QC-H and QC-L) on the same day. Inter-assay imprecision was estimated by analyzing 20 control specimens at two levels (QC-H and QC-L) over 20 days with a positive ionization method or 5 days with a negative ionization method.

Correlation studies

To ensure accuracy of the results, correlation studies were performed in two studies. One study involved splitting 20 patient urine samples and had them tested by our method and by the national reference laboratory ARUP (Salt Lake City, UT, USA). The ARUP assay included a screening test by high-resolution time-of flight or immunoassay and confirmation/quantitation by either GC–MS or LC–MS-MS for positive samples. A second study was performed testing two urine proficiency testing (PT) samples from the 2014 Drug Monitoring for Pain Management (DMPM)—a program offered by the College of American Pathologists (CAP). These test results were compared against the peer group averages using LC–MS-MS-based methods.

Trazodone and m-CPP interference study

To investigate a potential false-positive amphetamine result from the comparison method, the methanol stocks of trazodone or *m*-CPP (1 mg/mL) were separately diluted to five different concentrations using blank urine: 1, 5, 10, 20 and 50 μg/mL, followed by Amphetamine II immunoassay on Roche Cobas® 8000 module c502 (positive cutoff 1 μg/mL).

Results and discussion

Assay optimization

THC-COOH, its glucuronide, and barbiturates were reported to have better ionization efficiency when analyzed with a negative ESI mode (8, 9, 21–23). The same ionization approach was employed in this study. Recent improvements in sensitivity and selectivity with negative ESI have contributed to its widespread use in pharmacokinetic, drug metabolism and pesticide residue studies (24–26). Weak organic acids, such as acetic or formic acid, are often added to mobile phases in positive ESI methods. It is commonly accepted that the acidic environment promotes protonation of analytes in a positive ionization mode. And it is a reasonable assumption that adding base to mobile phase solutions would help deprotonation in a negative-ion mode. However, previous studies with a negative ESI mode showed that adding volatile bases resulted in limited sensitivity and poor solution stability (24). On the contrary, addition of 0.1–0.2% acetic or formic acid has been shown to increase a signal-to-noise ratio (27). Although acetic acid was demonstrated to have better ionization efficiency than formic acid in negative ESI (24), 0.1% formic acid was chosen for mobile phases in our negative ESI method to eliminate the need of switching mobile phases between positive and negative ionization methods. The negative method sensitivity was acceptable with 0.1% formic acid added in mobile phases (see detailed discussion below about LOD and LLOQ).

MRM transitions were optimized by a direct infusion of each analyte at 10–100 ng/mL prepared in 50% methanol in water fortified with 0.1% formic acid. The ion transition

Table III
IS Paring Table For All Analytes

Compound	IS applied	Concentration (ng/mL)
2-Hydroxyethylflurazepam	2-Hydroxyethylflurazepam-D4	50
6-Acetylmorphine (6-MAM)	6-Acetylmorphine (6-MAM)-D3	50
6β-Naltrexol	Oxycodone-D6	50
7-Aminoclonazepam	7-Aminoclonazepam-D4	30
7-Aminoflunitrazepam	7-Aminoflunitrazepam-D7	10
Alprazolam	Alprazolam-D5	30
Amphetamine	Amphetamine-D5	50
Benzoylecgonine	Benzoylecgonine-D8	50
Buprenorphine	Buprenorphine-D4	10
Buprenorphine-3β-D-glucuronide	Buprenorphine-D4	10
Carisoprodol	Gabepentin-D10	200
Chlordiazepoxide	Nordiazepam-D5	50
Clonazepam	Oxazepam-D5	50
Codeine	Codeine-D3	50
Codeine-6-glucuronide	Morphine-6G-D3	50
Diazepam	Diazepam-D5	50
EDDP	EDDP-D3	30
Fentanyl	Fentanyl-D5	5
Flunitrazepam	Alprazolam-D5	30
Flurazepam	α-Hydroxyalprazolam-D5	30
Gabapentin	Gabepentin-D10	200
Hydrocodone	Hydrocodone-D6	50
Hydromorphone	Hydromorphone-D6	50
Hydromorphone-3-glucuronide	Morphine-6G-D3	50
Lorazepam	α-Hydroxyalprazolam-D5	30
Lorazepam-glucuronide	α-Hydroxyalprazolam-D5	30
MDA	MDA-D5	100
MDEA	MDEA-D5	100
MDMA	MDMA-D5	100
Meperidine	Meperidine-D4	30
Meprobamate	Gabepentin-D10	200
Methadone	Methadone-D3	30
Methamphetamine	Methamphetamine-D5	50
Midazolam	Nordiazepam-D5	50
Morphine	Morphine-D3	50
Morphine-3-glucuronide	Morphine-6G-D3	50
Morphine-6-glucuronide	Morphine-6G-D3	50
Naloxone	Oxycodone-D6	50
Naltrexone	Oxycodone-D6	50
Norbuprenorphine	Buprenorphine-D4	10
Norbuprenorphine-3β-D-glucuronide	Buprenorphine-D4	10
Nordiazepam	Nordiazepam-D5	50
Norfentanyl	Norfentanyl-D5	5
Norhydrocodone	Hydrocodone-D6	50
Normeperidine	Normeperidine-D4	30
Noroxycodone	Oxycodone-D6	50
Norpropoxyphene	Propoxyphene-D11	50
NPD (norbuprenorphine degradent)	Propoxyphene-D11	50
O-Demethyl Tramadol	O-desmethyltramadol-D6	50
Oxazepam	Oxazepam-D5	50
Oxazepam-glucuronide	Oxazepam-D5	50
Oxycodone	Oxycodone-D6	50
Oxymorphone	Oxymorphone-D3	50
Oxymorphone-3-glucuronide	Morphine-6G-D3	50
PCP (Phencyclidine)	PCP (Phencyclidine)-D5	30
Pregabalin	Gabepentin-D10	200
Propoxyphene	Propoxyphene-D11	50
Sufentanil	Fentanyl-D5	5
Temazepam	Nordiazepam-D5	50
Temazepam-glucuronide	Nordiazepam-D5	50
Tramadol	Tramadol-13C, D3	50
Triazolam	Oxazepam-D5	50
α-Hydroxyalprazolam	α-Hydroxyalprazolam-D5	30
α-Hydroxymidazolam	Alprazolam-D5	30
α-Hydroxytriazolam	α-Hydroxytriazolam-D4	50
Zaleplon	Zolpidem-D7	30
Zopiclone	Zopiclone-D4	10
Zolpidem	Zolpidem-D7	30
Zolpidem-Phenyl Carboxylic acid	Zolpidem-D7	30
Amitriptyline	Imipramine-D3	100
Desipramine	Imipramine-D3	100
Imipramine	Imipramine-D3	100
Nortriptyline	Imipramine-D3	100
Butalbital	Butalbital-D5	100
Phenobarbital	Phenobarbital-D5	100
Pentobarbital	Pentobarbital-D5	100
Secobarbital	Secobarbital D5	100
THC-COOH	THC-COOH-D3	50
THC-COOH glucuronide	THC-COOH glucuronide-D3	50

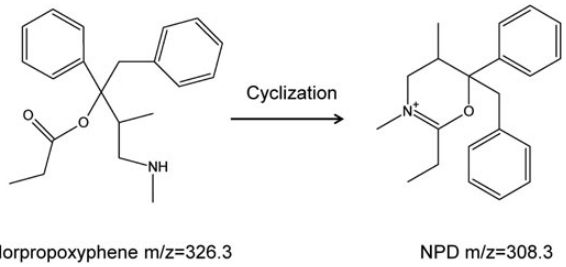

Figure 2. Conversion of norpropoxyphene to its cyclic form NPD.

determination was based on combination of high ion intensity, avoiding isobaric transitions, and low noise background in the presence of urine matrix. For most of the analytes, two MRM transitions were selected to monitor: the primary transition (quantifier) for concentration determination and the secondary transition (qualifier) for confirmatory analysis. The exceptions are amphetamine, buprenorphine-3β-D-glucuronide, norbuprenorphine and norbuprenorphine-3β-D-glucuronide, for which only one pair of ion transition was monitored. This was due to either the signal of non-primary ion transitions being too low or high noise background associated with mobile phases and/or urine matrix. MRM transitions and associated compound tuning parameters (such as declustering potential and collision energy) are included in Table I. IS transitions and compound tuning parameters are presented in Table II.

All 73 analytes detected in a positive mode and 6 analytes detected in a negative mode were well separated with a bi-phenyl reversed phase HPLC column. A bi-phenyl HPLC column with high hydrophobic retention and aromatic selectivity has been relatively widely used in pain management drug panels (2, 23, 28). The smoothed (two-point) chromatograms of standard mixtures in positive and negative modes are shown in Figure 1A and B, respectively. THC-COOH and its glucuronide, when made in aqueous solution, are known to stick to sample container surfaces made with different materials (29). This sample loss presents a hurdle in standard material preparations, such as calibrators and QCs, leading o inaccuracy and reproducibility issues. A simple and effective solution to this conundrum was to prepare THC-COOH and its glucuronide in methanol or methanol-based solution (8, 21, 23). In the current study, to avoid any other potential compound adsorption to container surfaces, both positive and negative standards were prepared in 50% methanol.

Assay validation

The linearity range was designed based on individual cutoff concentrations, with a dynamic range spanning 0.2× to 8× cutoff of each analyte (Table IV). As summarized in Table IV, all standard curves were linear with a linear regression coefficient *R* of

Table IV
Linearity, Cutoffs, LOD, LLOQ, Stability, Accuracy and Carryover in Assay Validation

Compound[a]	R^2[b]	Cutoff (ng/mL)	Linear range (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)	Stability[c] at 4°C	Accuracy[d]	Carry over (%)
2-Hydroxyethylflurazepam	0.998	20	4–160	0.2	2.5	102.9	100.4	ND
6-Acetylmorphine (6-MAM)	0.999	20	4–160	1.5	2.5	98.0	101.7	0.4
6β-Naltrexol	0.998	10	2–80	NQ	0.3	112.4	129.1	ND
7-Aminoclonazepam	0.993	10	2–80	0.2	1.3	76.8	95.7	ND
7-Aminoflunitrazepam	0.996	5	1–40	0.2	0.6	78.4	100.0	0.1
Alprazolam	1.000	10	2–80	0.4	1.3	105.8	94.4	0.1
Amphetamine	0.997	50	10–400	3.4	6.3	103.8	104.5	0.1
Benzoylecgonine	0.999	50	10–400	NQ	3.1	108.5	106.2	ND
Buprenorphine	0.996	10	2–80	0.3	2.5	111.8	92.9	ND
Buprenorphine-3β-D-glucuronide	0.999	10	2–80	0.1	0.6	103.9	104.0	ND
Carisoprodol	0.996	100	20–800	3.1	12.5	103.4	94.5	ND
Chlordiazepoxide	0.998	20	4–160	0.8	2.5	108.9	102.7	0.1
Clonazepam	0.999	10	2–80	0.4	0.6	92.4	65.8	0.1
Codeine	0.997	20	4–160	0.9	2.5	99.9	109.2	ND
Codeine-6-glucuronide	0.996	20	4–160	0.2	2.5	99.7	98.6	ND
Diazepam	1.000	20	4–160	0.2	0.3	100.7	94.4	ND
EDDP	0.998	10	2–80	NQ	0.6	99.9	105.5	0.1
Fentanyl	0.997	2	0.4–16	NQ	0.5	94.3	97.4	ND
Flunitrazepam	0.999	5	1–40	0.2	0.6	88.3	78.9	ND
Flurazepam	0.998	10	2–80	NQ	0.6	99.4	80.9	ND
Gabapentin	1.000	500	100–4,000	NQ	62.5	102.7	97.4	0.1
Hydrocodone	0.999	20	4–160	0.8	1.3	107.2	101.8	ND
Hydromorphone	0.998	20	4–160	0.1	1.3	91.4	97.9	ND
Hydromorphone-3-glucuronide	0.997	20	4–160	1.1	2.5	94.7	99.1	ND
Lorazepam	0.999	20	4–160	0.2	1.3	95.0	83.8	ND
Lorazepam-glucuronide	0.998	40	8–320	1.8	5.0	104.9	85.6	ND
MDA	0.999	100	20–800	2.5	3.1	105.2	100.5	ND
MDEA	0.999	100	20–800	NQ	6.3	100.9	101.8	ND
MDMA	0.999	100	20–800	2.4	12.5	103.4	102.5	ND
Meperidine	0.997	10	2–80	0.4	1.3	107.1	99.6	ND
Meprobamate	0.999	500	100–4,000	NQ	125.0	106.9	78.0	0.2
Methadone	0.999	10	2–80	0.1	0.3	101.2	93.4	ND
Methamphetamine	1.000	50	10–400	0.8	3.1	100.6	99.1	ND
Midazolam	0.997	20	4–160	NQ	1.3	111.5	111.6	ND
Morphine	0.998	20	4–160	NQ	2.5	107.6	101.3	ND
Morphine-3-glucuronide	0.995	20	4–160	0.1	2.5	103.6	102.2	ND
Morphine-6-glucuronide	0.997	20	4–160	0.2	2.5	96.6	91.5	0.1
Naloxone	0.997	100	20–800	3.7	25.0	111.0	105.9	0.0
Naltrexone	0.995	10	2–80	0.6	2.5	114.0	110.1	0.1
Norbuprenorphine	0.996	10	2–80	0.8	1.3	84.2	83.4	ND
Norbuprenorphine-3β-D-glucuronide	0.997	10	2–80	0.3	2.5	100.4	93.1	ND
Nordiazepam	0.999	20	4–160	0.3	0.6	99.3	91.4	ND
Norfentanyl	0.998	5	1–40	0.1	1.3	105.0	100.7	ND
Norhydrocodone	0.998	20	4–160	0.3	0.6	109.1	60.0	0.1
Normeperidine	0.998	10	2–80	0.2	0.6	97.0	97.1	0.1
Noroxycodone	0.997	20	4–160	0.1	1.3	116.2	80.1	ND
Norpropoxyphene and NPD	0.999	20	4–160	1.1	2.5	107.6	97.5	ND
O-Demethyl Tramadol	0.999	50	10–400	NQ	3.1	102.8	97.9	0.1
Oxazepam	0.999	20	4–160	0.4	2.5	95.3	90.4	ND
Oxazepam-glucuronide	0.998	20	4–160	3.6	5.0	106.9	100.9	ND
Oxycodone	0.997	20	4–160	0.5	1.3	103.5	85.3	ND
Oxymorphone	0.998	20	4–160	0.6	2.5	96.9	90.5	ND
Oxymorphone-3-glucuronide	0.997	20	4–160	0.9	2.5	98.0	105.1	ND
PCP (Phencyclidine)	0.997	10	2–80	0.3	0.6	100.7	91.5	ND
Pregabalin	0.999	500	100–4,000	2.4	7.8	100.5	93.4	ND
Propoxyphene	0.999	20	4–160	NQ	0.6	101.3	95.4	0.1
Sufentanil	0.998	5	1–40	NQ	0.6	99.6	100.0	ND
Temazepam	0.999	20	4–160	NQ	0.6	107.6	103.0	ND
Temazepam-glucuronide	0.999	20	4–160	2.2	5.0	106.4	88.8	ND
Tramadol	0.999	50	10–400	0.2	1.6	107.4	84.2	ND
Triazolam	1.000	20	4–160	0.4	1.3	104.3	106.2	0.1
α-Hydroxyalprazolam	0.998	10	2–80	0.5	2.5	97.9	99.5	ND
α-Hydroxymidazolam	0.999	20	4–160	1.0	2.5	96.8	106.2	0.1
α-Hydroxytriazolam	0.995	20	4–160	NQ	2.5	103.0	112.2	0.2
Zaleplon	0.999	10	2–80	0.2	0.3	112.1	104.6	ND
Zopiclone	0.997	10	2–80	0.1	1.3	100.4	97.4	0.1
Zolpidem	1.000	10	2–80	0.1	0.6	104.0	99.0	ND
Zolpidem-Phenyl Carboxylic acid	0.997	10	2–80	0.1	0.3	123.2	156.8	0.1
Amitriptyline	0.999	50	10–400	NQ	3.1	98.5	85.5	0.1
Desipramine	0.999	50	10–400	0.1	6.3	100.3	89.2	0.1
Imipramine	0.999	50	10–400	NQ	3.1	107.3	91.7	0.1
Nortriptyline	0.998	50	10–400	0.6	3.1	98.2	88.0	0.1
Butalbital	0.998	100	20–800	8.4	12.5	96.9	96.4	ND
Phenobarbital	0.995	100	20–800	3.1	12.5	92.0	106.6	ND
Pentobarbital	0.998	100	20–800	10.4	25.0	84.6	101.6	ND

(*continued*)

Table IV Continued

Compound[a]	R^2[b]	Cutoff (ng/mL)	Linear range (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)	Stability[c] at 4°C	Accuracy[d]	Carry over (%)
Secobarbital	0.997	100	20–800	4.6	12.5	103.2	107.0	0.2
THC-COOH	1.000	50	10–400	NQ	3.1	103.8	96.5	0.1
THC-COOH glucuronide	0.999	50	10–400	1.0	1.6	101.5	94.0	ND

NQ, not quantifiable; ND, not detectable.

[a]All calibrators and QCs are stored at −20°C for 1 year.

[b]Linear regression coefficient was determined from the standard curve of each analyte.

[c]Stability is defined as the percentage of observed day-7 concentration normalized to that of day-0 specimen.

[d]Accuracy is defined as observed concentration in fortified negative urine normalized to the nominal concentration.

≥0.990 (with a range of 0.994–1.000). For all analytes, LODs were lower than LLOQ. With some analytes, LODs were not quantifiable, producing a false value of below zero (designated as 'NQ' in Table IV). LLOQs of all analytes were at least 25% of the cutoff concentration and were comparable or lower than those in previous reports of simultaneous multiple drug quantitation LC–MS-MS methods (2, 8, 15, 16). Detailed information of LODs and LLOQs are included in Table IV.

Urine drug testing cutoffs for pain management must be determined with care. If they are too high, false negative may result; if too low, the risk of reporting a positive drug test due to inadvertent exposure (such as pharmaceutical impurities) is increased (30, 31). Unlike immunoassay-based urine drug testing with relatively high cutoffs due to limited sensitivity, LC–MS-MS are capable of detecting drugs at low concentrations (see Table IV for LOD and LLOQ). Some reference laboratories offering LC–MS-MS drug panels use LLOQ as their cutoffs, which are the thresholds at which reliable quantitation can be made. To avoid potential false positives, comparable cutoffs were used in our assay although the LLOQs are at least 25% or lower of the cutoff values for each analyte.

The analytes were found to be stable in urine for at least 7 days at 4°C ($n = 3$), with a range of recovery 77–123% (acceptance range 100 ± 25%). Although no significant carryover was observed between calibrator 5 and calibrator 1, it is recommended to be aware of potential carryover during routing sample analysis, especially for acute intoxicated patients whose urine drug concentration could be much higher than those tested in carryover studies.

Both of the positive mode and negative mode methods were found to be specific and no interference peaks were observed in 10 drug-negative urine controls. The same drug-negative urine specimens were used in the matrix effect investigation with a post-column infusion method. No substantial matrix effect or ion suppression was observed for any of the analytes with any of the negative urine specimens (data not shown). As presented in Table IV, accuracy of most analytes in spike-and-recovery experiment fell in the acceptance range 80–120%. The exceptions are 6β-naltrexol (129.1%), clonazepam (65.8%), flunitrazepam (78.9%), meprobamate (78.0%), norhydrocodone (60.0%) and zolpidem-phenyl carboxylic acid (156.8%). It is noteworthy that none of the six analytes had their own deuterium-labeled IS used in quantitation (Table III). Instead, shared ISs of different analytes from the same drug class that had close retention time were applied. Therefore, the observed matrix effect might be contributed from that fact the IS used for those analyes were not eluted at the same retention time. Including the analyte-specific IS might be warranted to alleviate their matrix effects. Ideally, isotope-labeled IS compound should be used for its own non-labeled ones. However, ISs are not always commercially available and can also be costly. It is a common practice to have different analytes with close retention time share IS in multiple pain drug panel method development (2, 8, 15, 16). With the observed matrix effect for those six analytes, extra care should be taken in patient result interpretation. The combined results of the parent drugs or metabolites of the six analytes, namely naltrexone, 7-aminoclonazepam, 7-aminoflunitrazepam, carisoprodol, hydrocodone and zolpidem, which were also included in our panel and less subjective to matrix effect, could provide more reliable information in final interpretation.

Norpropoxyphene is a major metabolite of propoxyphene found in urine. However, norpropoxyphene has been reported to be unstable not only in urine matrix but also in methanol stock (8, 18, 19). There is substantial conversion of norpropoxyphene to a cyclic degradation product (norbuprenorphine degradent, NPD), as shown in Figure 2. As this structural conversion is constantly on-going, it is impossible to predict the exact amount of norpropoxyphene and NPD in the manufacture's original stock at a particular time point, which presents difficulties when preparing QCs and calibrators. To circumvent this issue, the total norpropoxyphene was calculated by summing up an intact and cyclized product (NPD). Because of the molecular weight difference of intact (326 Da) and NPD (308 Da), the peak area of NPD was first converted to that of norpropoxyphene by multiplying the molar normalizing factor (326/308). Then, the peak area of norpropoxyphene and NPD was summed and divided by their common IS peak area. This combined peak area ratio was then used for constructing standard curves and quantification of routine urine samples. This manual calculation was successful in generating linear calibration curves, accurate and precise validation results (Tables IV and V).

The intra-assay CV for QC-L ranged 1.7–8.8% and for QC-H ranged 1.8–7.5%; the inter-assay CV for QC-L ranged 2.1–12.2% and for QC-H ranged 1.9–10.2%. The accuracy which was measured by % bias was within ±20% acceptance criteria at both QC-L and QC-H levels from intra- or inter-assay experiments (Table V). To establish the assay dilution linearity for samples with high concentrations of analytes, a 10-fold dilution validation using 40× working solution diluted with Bio-Rad blank urine was performed. The inter-assay CV for the dilution study ranged 1.6–8.7% and the assay % bias was within ±20% except for THC-COOH and THC-COOH glucuronide (data not shown). As expected, significant negative % bias (around −35%) was observed with THC metabolites after 10-fold dilution with Bio-Rad urine, due to the adsorption effect discussed

Table V
Precision and Accuracy at Two QC Levels

Compound	Intra-assay precision and accuracy				Inter-assay precision and accuracy			
	QC-L[a] CV (%)[b]	QC-L bias (%)[c]	QC-H[d] CV (%)	QC-H bias (%)	QC-L CV (%)	QC-L bias (%)	QC-H CV (%)	QC-H bias (%)
2-Hydroxyethylflurazepam	5.5	−1.0	3.5	2.3	6.2	−4.1	4.3	−1.4
6-Acetylmorphine (6-MAM)	5.9	−9.9	5.1	−2.8	8.2	−3.8	5.2	−1.3
6β-Naltrexol	5.1	9.5	3.5	6.6	4.3	19.5	5.0	15.1
7-Aminoclonazepam	2.4	0.6	4.3	1.2	4.1	1.2	3.0	1.3
7-Aminoflunitrazepam	5.5	−1.0	5.1	−3.3	6.3	−1.8	3.0	0.8
Alprazolam	3.6	−1.7	4.1	−0.6	4.3	1.9	4.2	−0.3
Amphetamine	3.2	−3.5	3.3	−3.8	4.4	−0.9	3.3	−1.1
Benzoylecgonine	2.4	−2.7	3.5	1.2	4.9	0.2	4.2	−2.0
Buprenorphine	7.9	10.1	7.5	4.9	8.5	6.4	5.6	2.3
Buprenorphine-3β-D-glucuronide	3.2	16.8	4.8	12.1	5.5	6.4	6.4	10.0
Carisoprodol	4.2	−2.2	3.3	−4.3	6.7	1.4	4.9	−0.7
Chlordiazepoxide	5.3	−12.2	4.4	−12.4	5.4	−10.2	5.4	−8.3
Clonazepam	3.6	−9.0	3.7	−10.5	4.3	−0.9	4.6	−2.5
Codeine	3.7	−3.2	6.5	3.1	5.8	−1.5	5.5	−1.0
Codeine-6-glucuronide	4.9	1.5	3.5	6.5	9.5	−5.8	9.7	−1.5
Diazepam	2.3	0.6	2.4	3.6	2.3	1.3	2.2	3.4
EDDP	4.3	2.3	4.5	5.3	5.0	3.1	4.8	4.7
Fentanyl	5.9	−4.2	3.8	−2.8	4.2	−5.5	4.0	−4.3
Flunitrazepam	5.0	8.2	3.3	10.4	4.2	13.2	1.9	10.7
Flurazepam	3.4	−10.1	4.7	−12.7	6.8	−5.7	7.5	−7.1
Gabapentin	3.5	−2.6	3.8	2.5	4.6	2.3	3.6	4.1
Hydrocodone	4.6	−5.7	4.3	0.4	4.2	−1.7	3.2	−0.4
Hydromorphone	5.7	−5.8	3.9	−1.9	9.7	−5.7	7.4	−2.4
Hydromorphone-3-glucuronide	4.1	3.9	3.5	6.0	8.5	−1.7	4.8	4.0
Lorazepam	4.4	−6.5	4.8	−6.3	5.3	−8.1	6.2	−7.8
Lorazepam-glucuronide	5.0	−2.5	4.6	−3.4	4.8	0.5	6.3	−1.6
MDA	2.9	0.4	3.3	2.2	2.9	0.3	3.8	1.5
MDEA	3.4	−0.5	2.9	3.6	2.5	2.1	2.6	2.3
MDMA	4.1	−3.8	4.5	−0.3	4.3	1.1	3.6	1.4
Meperidine	5.0	−5.0	4.2	−2.2	6.2	−1.1	3.7	−1.9
Meprobamate	3.1	−8.6	1.8	−5.0	6.9	−8.5	5.7	−4.1
Methadone	3.9	−3.4	2.8	−1.4	4.5	−1.9	3.6	−1.0
Methamphetamine	3.0	−5.5	3.8	−2.9	2.1	0.2	2.7	1.2
Midazolam	5.6	−5.0	3.9	−5.5	6.3	2.8	5.3	−0.4
Morphine	4.0	−2.6	3.0	−4.2	5.3	−2.4	4.5	−0.4
Morphine-3-glucuronide	4.4	4.9	3.5	10.0	7.2	−2.3	5.9	4.9
Morphine-6-glucuronide	4.9	3.8	5.0	3.3	5.8	−0.3	4.8	0.1
Naloxone	2.3	0.2	4.1	3.9	7.8	11.3	6.7	9.3
Naltrexone	3.6	−6.5	4.7	−3.3	12.3	1.7	7.0	6.2
Norbuprenorphine	8.3	2.2	5.7	4.6	7.6	8.4	4.5	5.1
Norbuprenorphine-3β-D-glucuronide	5.0	17.5	5.8	12.1	6.2	9.6	4.9	9.8
Nordiazepam	3.8	−5.3	2.8	−3.1	3.5	−4.1	3.8	−2.3
Norfentanyl	5.8	−2.4	4.7	−3.5	5.0	−0.2	4.6	0.2
Norhydrocodone	5.0	6.1	5.4	5.4	7.1	3.6	4.4	3.1
Normeperidine	5.7	−1.6	4.4	2.2	6.4	2.1	6.0	1.2
Noroxycodone	2.5	−4.9	7.0	−0.6	9.7	2.5	6.6	1.3
Norpropoxyphene and NPD	1.8	8.8	3.4	2.9	6.6	−0.8	4.1	−1.1
O-Demethyl Tramadol	3.3	−0.9	3.7	2.6	4.0	0.3	3.4	1.9
Oxazepam	3.7	−3.1	4.0	−0.6	3.6	0.2	3.3	−0.4
Oxazepam-glucuronide	6.1	−12.4	5.0	−9.3	5.0	−0.7	5.3	−0.6
Oxycodone	5.5	0.9	5.3	−0.1	8.1	−0.5	5.5	−1.3
Oxymorphone	4.9	6.1	5.1	2.9	4.3	2.9	4.4	3.2
Oxymorphone-3-glucuronide	4.4	1.4	3.5	15.0	8.9	−4.8	7.5	5.0
PCP (Phencyclidine)	3.8	−7.1	4.3	−4.3	5.2	−1.5	4.2	−2.6
Pregabalin	2.7	−7.4	2.9	−4.6	5.7	1.9	3.9	1.7
Propoxyphene	3.0	−2.3	3.8	0.5	5.2	−3.4	3.5	−0.8
Sufentanil	5.6	−6.8	3.3	−5.4	5.2	−4.1	4.3	−0.4
Temazepam	5.6	5.1	4.3	7.9	6.2	2.5	4.1	3.4
Temazepam-glucuronide	7.7	−0.9	4.1	−1.7	9.1	−0.1	6.7	3.4
Tramadol	3.0	−2.2	4.3	−0.3	3.4	0.3	2.4	1.9
Triazolam	2.7	−3.1	4.5	−2.4	5.2	5.4	4.8	1.7
α-Hydroxyalprazolam	6.8	−5.9	2.8	−5.7	10.5	−0.5	5.6	0.3
α-Hydroxymidazolam	5.1	−12.1	2.7	−8.9	6.5	−1.1	3.2	−2.6
α-Hydroxytriazolam	5.2	10.6	4.7	9.6	6.3	14.0	5.1	9.0
Zaleplon	3.5	15.5	2.5	19.8	6.3	14.3	6.8	13.4
Zopiclone	3.0	−7.4	2.8	−7.0	5.6	−8.1	3.2	−9.3
Zolpidem	3.4	−1.3	2.8	1.8	3.2	−0.2	3.8	1.0
Zolpidem-Phenyl Carboxylic acid	2.6	18.8	3.0	18.9	2.2	18.3	4.2	14.7
Amitriptyline	3.3	0.2	3.9	1.7	4.6	1.3	4.0	3.3
Desipramine	3.1	0.8	3.6	−0.2	3.7	0.1	3.1	2.2
Imipramine	3.6	−1.3	3.1	2.7	4.2	1.4	2.5	3.0
Nortriptyline	3.1	−1.2	3.0	2.1	5.1	4.4	4.4	3.7
Butalbital	7.2	5.0	4.2	9.1	10.4	11.0	10.2	11.4

(*continued*)

Table V Continued

Compound	Intra-assay precision and accuracy				Inter-assay precision and accuracy			
	QC-L[a] CV (%)[b]	QC-L bias (%)[c]	QC-H[d] CV (%)	QC-H bias (%)	QC-L CV (%)	QC-L bias (%)	QC-H CV (%)	QC-H bias (%)
Phenobarbital	8.8	−0.9	5.4	4.6	7.7	0.9	6.5	3.3
Pentobarbital	4.5	19.9	6.3	18.5	11.3	10.1	8.3	8.5
Secobarbital	7.0	2.8	5.0	8.6	8.0	5.3	5.8	6.7
THC-COOH	2.1	0.5	3.1	1.2	3.6	2.9	3.5	1.7
THC-COOH glucuronide	2.9	7.4	2.4	8.7	3.0	8.6	3.4	8.7

[a]Low-level QC (QC-L) is at 50% of each analyte's cutoff concentration.
[b]Precision is defined as the percentage coefficient of variation (CV).
[c]Accuracy is defined as the difference of observed concentration from the nominal concentration (percentage bias).
[d]High-level QC (QC-H) is at three times of each analyte's cutoff concentration.

earlier. This dilution study result suggests that urine specimens can be diluted for all analytes except for the quantitation of THC metabolites.

Our assay was then evaluated by comparing patient sample testing results with those from a reference laboratory. The comparison results were summarized in Supplementary Table I. Twenty patient urine specimens that were screened positive for various drug classes by in-house immunoassays (Roche Cobas) were tested using our LC–MS-MS method and the reference laboratory screening and confirmation methods. Although the comparison results were largely consistent between all three methods, we did observe some inconsistencies, which are discussed below. VP3 was tested positive for amphetamine by immunoassay, but not detected by either the ARUP time-of-flight (TOF) MS or the in-house LC–MS-MS method. Instead, VP3 was tested positive for lorazepam-glucuronide only in our LC–MS-MS method, which is the only method of the three that can detect the conjugate of the sedative drug lorazepam. The VP3 specimen was from a patient from the emergency department who complained of severe insomnia lasting for a few days. Although the patient medical record did not indicate any medication taken before the hospital course, it is a reasonable guess that the patient could have taken a sedative or sleep inducing drug for his insomnia, for example, lorazepam or trazodone. Recently, trazodone metabolite *m*-CPP was found to have cross-reactivity with the Roche amphetamine II assay (32). Similarly, our in-house Roche amphetamine screening assay was found to be falsely positive with *m*-CPP of 10 μg/mL or higher (data not shown). Therefore, a possible explanation for the false-positive amphetamine result is that the patient might have been taking both trazodone and lorazepam for his insomnia, which resulted in positive amphetamine immunoassay and detection of lorazepam metabolite by LC–MS-MS. VP7 was tested positive for benzodiazepines and tricyclic antidepressant by the Roche immunoassay, but positive for tramadol by both ARUP TOF- MS and the in-house LC–MS-MS, showing the advantage of high specificity with mass spectrometry-based methods. In the analysis of specimen VP14, the immunoassay-positive benzodiazepine was detected as 7-aiminoclonazepam by in-house LC–MS-MS, but shown to be negative by the ARUP TOF method. Similar observation was made in VP10 analysis that was tested positive for barbiturates by Roche immunoassay, but negative by ARUP immunoassay. Consistent with Roche screening results, phenobarbital was detected positive by our LC–MS-MS method, showing the high sensitivity of urine drug screening by our LC–MS-MS.

Table VI
CAP DMPM PT Specimens Results

ID	Expected drugs	Acceptable range[a] (ng/mL)	Mean[b] of quantitation methods (ng/mL)	HMH LC–MS-MS (ng/mL)	Accuracy[c] (%)
DMPM-1	6-MAM	169–434	304	380	127.9
	α-Hydroxyalprazolam	952–1,752	1,352	1,507	104.4
	Benzoylecgonine	477–777	626	653	106.9
	Carisoprodol	964–2,632	1,798	1,811	100.7
	Meprobamate	3,011–5,971	4,491	4,673	104.1
	Morphine	19,417–88,965	54,191	51,891	99.7
DMPM-2	Amphetamine	184–298	241	240	98.8
	Methamphetamine	1,627–3,200	2,413	2,820	118.4
	Lorazepam	1,139–2,447	1,842	1,470	74.7
	Oxycodone	503–979	741	635	84.9
	Oxymorphone	3,595–6,279	4,936	4,980	101.4

[a]Acceptable range according to the CAP 2014 DMPM-A proficiency testing.
[b]Mean concentration reported by participating peer groups that employ GC–MS or LC–MS-MS.
[c]Accuracy is defined as the percentage of observed concentration normalized to the mean concentrations.

As glucuronide hydrolysis is not included for the TOF-MS screening or in the opiate LC–MS-MS assay performed by ARUP, all glucuronide metabolites in screening and opiate glucuronides in confirmation/quantitation were not detected. This can lead to potentially false-negative screening results for some analytes that are mainly excreted in glucuronide forms, as seen for oxazepam, temazepam and codeine in VP1 analysis. Similarly, opiate quantitation by LC–MS-MS without hydrolysis were significantly underestimated as seen in the analyses of specimens VP2, 5, 8, 11, 12, 14 and 15 in Supplementary Table I. Besides higher sensitivity for glucuronide conjugates and avoiding various low de-glucuronidation efficiencies by glucuronidase, another advantage of directly detecting glucuronide conjugates is that it helps identify the adulterated urine specimens, as fortified drugs will not become glucuronide conjugated *in vitro*.

Two CAP DMPM PT samples were also tested with our method and quantitatively compared with the peer groups that employed GC–MS or LC–MS-MS methods for quantitation. As summarized in Table VI, all expected drugs were found to be positive in both specimens. More importantly, the determined concentrations by our method for all analytes ranged 74.7–127.9% compared with those of method mean from peer groups, which was within the PT program acceptance criteria ±30% or mean ±2 SD.

In comparison with other published LC–MS-MS pain management panels that comprise multiple drug classes (2, 8, 15, 16, 21,

33), our assay has the most complete drug list for compliance testing and monitoring illicit use (excluding novel psychoactive substances) (15). With comparable performances, such as linearity, sensitivity, specificity, accuracy, precision etc., the assay presented involves little sample preparation by employing dilute-and-shoot and including glucuronide conjugates. Those commonly included laborious steps, such as SPE and glucuronide hydrolysis that both can bring in more variations, are avoided in our assay. With these advantages, lower cost and better turnaround time might be achieved in routine pain management testing.

Conclusion

An LC–MS-MS method has been established and validated that allows simultaneous detection of 78 pain management drugs and metabolites in urine samples. This method requires minimal sample preparation using a dilute-and-shoot strategy. Although the suboptimal recovery rate was observed for six analytes due to the lack of available ISs, this method could considerably reduce overall analysis cost and time, which are important factors for its application to routine urine drug compliance testing in pain management.

Supplementary Data

Supplementary data are available at *Journal of Analytical Toxicology* online.

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