Simultaneous Determination of Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, and 6-Acetylmorphine in Urine, Serum, Plasma, Whole Blood, and Meconium by LC–MS–MS

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for simultaneous analysis of six major opiates in urine, serum, plasma, whole blood, and meconium is described. The six opiates included are codeine, morphine, hydrocodone, hydromorphone, oxycodone, and 6-acetylmorphine (6-AM). The method was compared to an in-house gas chromatography (GC)–MS method and an LC–MS–MS method performed by another laboratory. The sample preparation time was decreased by eliminating the glucuronide hydrolysis and derivatization required for GC–MS analysis, as well as by adapting the solid-phase extraction to elute directly into autosampler vials. These improvements illustrate the advantages of an LC–MS–MS method over a GC–MS method for opiates. The structural similarity of these six opiates and others in the opiate class causes a high potential for interference and false-positive results. Twelve opiate analogues and metabolites were evaluated for interference. The potential for interference was reduced by altering the MRM transitions chosen for the six opiates. The increased specificity of LC–MS–MS decreased the interference rate in urine to 3.9% compared to 13.6% on the in-house GC–MS method. The rate of positivity for 6-AM in meconium is described for the first time. In urine, 11.0% of morphine positive specimens were also positive for 6-AM compared to 8.3% in serum/plasma and 0.9% in meconium. Although 6-AM is infrequent in meconium, it provides a definitive proof of illegal heroin abuse by the pregnant mother. This method has been routinely used in our laboratory over the last 6 months on more than 1500 patient specimens.

Introduction

Opiates are among the most widely abused drugs in every social and economic strata of society. Although highly addictive, they are commonly prescribed after surgery, trauma, or for chronic pain relief. To evaluate patient compliance, and for the protection of the program, pain management clinics require frequent drug testing of patients under their direction. The patient may sign an agreement to take the opiate prescribed and no other opiate prescribed by another physician or obtained illegally. If a test result is positive for another opiate or negative for the prescribed opiate, the patient may be required to leave the program and be denied access to the drug(s) necessary to control their pain. It is therefore important that the laboratory prevent false-positive and false-negative opiate results and minimize reports for which a specific opiate cannot be identified because of interference. This is a challenge because of the close structural similarity of opiates and their metabolites. For example, analytical interferences lead to indeterminate results for one or more opiates in 13.6% of patient specimens analyzed in a year by the previous in-house gas chromatography–mass spectrometry (GC–MS) method (Appendix).

The use of liquid chromatography–tandem mass spectrometry (LC–MS–MS) is increasing in the clinical laboratory for drugs-of-abuse testing (1,2). Because of its increased sensitivity and specificity, LC–MS–MS is useful for simultaneous analysis of a wide variety of drugs. This makes it an ideal screening test to replace EIA methods commonly used for drug screening (3–10). LC–MS–MS is quickly becoming the gold standard for the determination of benzodiazepines, a class of drugs with a large number of structurally related compounds (11,12). In addition, the benefits of LC–MS–MS have also been reported in the analysis of cocaine (13,14), ritalin (15), and nicotine (16) abuse.

Because of these publications, we were interested in investigating LC–MS–MS to see if greater analytical specificity and sensitivity over our GC–MS opiate analysis could be achieved. Although the advantages of LC–MS–MS for opiates analysis have also been reported for urine (17), plasma (18), and hair (19), none of these papers included all six analytes in all matrices of interest to our laboratory service.

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Experimental

Reagents and supplies

Calibration standards, internal standards and analytes for the interference study were purchased from Cerilliant (Austin, TX). A calibration stock solution was prepared in acetonitrile at 0.1 ng/µL in morphine, hydromorphone, codeine, oxycodone, 6-acetylmorphine (6-AM), and hydrocodone. An internal standard stock solution was prepared in acetonitrile at 0.25 ng/µL in morphine-d_6, hydromorphone-d_3, codeine-d_6, oxycodone-d_6, 6-AM-d_6, and hydrocodone-d_3. All stocks were stored at –72°C until opened and then stored at 4°C while in use. All other solvents were reagent grade and purchased from Fisher Scientific (Pittsburgh, PA). Trace-B columns, 35 mg, were supplied by SPEWare (San Pedro, CA).

Sample specimens

Blank urine was prepared by collecting drug-negative urine in a bottle containing sodium fluoride. The final solution was filtered, and diluted to 20 mg/dL creatinine with nanopure water (deionized water that has been run through a Barnstead NANOpure infinity purifier). Blank plasma was prepared by combining expired samples from ARUP's blood bank and dialyzing. Blank meconium was pooled from excess patient specimens that tested negative for opiates by EMIT.

Positive patient samples were de-identified to protect personal health information in accordance with University of Utah IRB approval. Serum, plasma, and whole blood specimens that were positive by a reference laboratory LC–MS–MS method were stored at –72°C for up to 1 year and thawed at 4°C prior to analysis. Whole blood and plasma were obtained in gray stoppered tubes (potassium oxalate, sodium fluoride) and serum was obtained in plain red tubes. Urine and meconium patient specimens that were positive or had interference by GC–MS were stored at –72°C for up to 1 year and thawed at 4°C prior to analysis.

Apparatus

Homogenization of meconium samples was performed with an Omni Tissue Tearor (Fisher Scientific). Initial solvent evaporation was achieved with a centrifugal vacuum evaporator (CVE) system, (Jouan model RC10.10). Extraction was performed on a 48-place positive pressure manifold (Cera, SPEWare). The CEREX 48-place sample concentrator station (SPEWare) was used for final solvent evaporation. Samples were eluted into max recovery autosampler vials (VWR).

Sample preparation

Urine, serum, plasma, and whole blood. Multi-analyte calibrators were prepared with blank urine and the calibration stock solution to produce final concentrations of 2, 5, and 20 ng/mL for all analytes. A negative urine control, positive urine control at 5 ng/mL, and a positive plasma control at 5 ng/mL were also included. Patient samples were prepared with 1 mL of specimen. The internal standard stock solution was added to each calibrator, control and patient specimen to produce a final concentration of 25 ng/mL in all internal standards.

Two milliliters of 0.1M sodium phosphate buffer (pH 6) was added to each tube. After vortex mixing, samples were centrifuged at 0°C, 3500 rpm for 5 min. This low temperature and high speed is critical to prevent serum, plasma and whole blood samples from clogging the SPE column. The samples were loaded onto the Trace-B columns at approximately 4 drops/s. The samples were then washed at 1 drop/s with 1 mL each of sodium bicarbonate buffer (pH 9), water, 0.1M acetic acid, methanol, and ethyl acetate. The columns were dried at 25 psi for 5 min. The samples were eluted into 2 mL max recovery autosampler vials with 1 mL 70:25:5 ethyl acetate/isopropanol/ammonium hydroxide. Samples were dried in the autosampler vials by raising the CEREX 48 place sample concentrator to 40°C for approximately 12 min. The residue was reconstituted with 200 µL acetonitrile, capped, vortex mixed, and analyzed.

Meconium. Meconium samples (1.00 ± 0.02 g) were weighed into 16 × 50 polypropylene tubes, followed by addition of 3 mL of methanol and 100 µL of the internal standard stock solution. Calibrators were prepared with the calibration stock solution to produce concentrations of 2, 5, and 20 ng/g. The samples were homogenized until uniform, then centrifuged at 14,000 rpm and 0°C for 15 min. The supernatant was transferred to 16 × 100-mm culture tubes and the solvent was evaporated to ~ 1 mL under vacuum in the CVE at 60°C (20,21).

Two milliliters of 0.1M sodium phosphate buffer (pH 6) was added to each tube and the meconium samples were then extracted in the same manner as the urine and blood samples. The final residue was reconstituted with 150 µL acetonitrile, capped, vortex mixed, and analyzed.

Table I. MRM Transitions for Opiates

	Quantitative			Qualitative			
Compound	MRM (*m/z*)	Cone (V)	Collision (eV)	MRM (*m/z*)	Cone (V)	Collision (eV)	RT (min)
Morphine-d_6	292.1 > 153.1	40	40	292.1 > 165.1	40	40	4.10
Morphine	286.1 > 153.1	40	40	286.1 > 157.1	40	35	4.10
Hydromorphone-d_3	289.1 > 185.1	40	30	289.1 > 157.1	40	40	4.82
Hydromorphone	286.1 > 185.1	40	30	286.1 > 153.1	40	40	4.82
Codeine-d_6	306.1 > 165.1	45	45	306.1 > 153.0	45	45	5.70
Codeine	300.1 > 165.1	45	45	300.1 > 153.0	45	45	5.74
Oxycodone-d_6	322.1 > 262.2	30	25	322.1 > 218.2	30	45	6.19
Oxycodone	316.1 > 256.2	30	25	316.1 > 212.2	30	45	6.23
6-AM-d_6	334.1 > 165.1	40	40	334.1 > 211.1	40	30	6.39
6-AM	328.1 > 165.1	40	40	328.1 > 211.1	40	30	6.42
Hydrocodone-d_3	303.1 > 199.1	45	30	303.1 > 171.1	45	40	7.04
Hydrocodone	300.1 > 199.1	45	30	300.1 > 171.1	45	40	7.13

Instrumental analysis

Instrumental analysis was performed on a Waters/Micromass Quattro Micro LC–MS–MS system equipped with a Waters Alliance® HT HPLC system. The instrument was operated in

multiple ion monitoring (MRM) mode with an ESI probe in positive electrospray ionization mode.

A Waters Nova-Pak® CN HP analytical column (3.9 × 100 mm, 4-µm particle size) was heated to a constant 30°C in the column heater. The LC flow was isocratic at 0.525 mL/min with 15% HPLC grade acetonitrile and 85% 2mM ammonium formate buffer at pH 3.0.

The MS source temperature was maintained at 120°C, and the desolvation temperature was 400°C with a nitrogen desolvation gas flow of 800 L/h. The resolution of both quads were maintained at unit mass resolution with a peak width at half height of 0.7 amu. Argon was used as the collision gas. The masses monitored along with the respective cone voltage, collision energy, and retention times for all analytes are listed in Table I.

Results and Discussion

The first advantage of LC–MS–MS was a reduction in the lower limit of quantitation from 200 ng/mL to 2 ng/mL, a 100-fold increase in analytical sensitivity for five opiates: codeine, morphine, hydrocodone, hydromorphone, and oxycodone. This significant increase in sensitivity allowed the elimination of the glucuronide conjugate hydrolysis step. The free forms range from 10 to 35% (codeine) to 100% (hydrocodone) of the total (free plus hydrolyzed glucuronide), requiring better sensitivity if only the free form is measured (22). Eliminating the hydrolysis improved the assay in three ways. First, the extraction time was significantly reduced. Second, a heroin metabolite, 6-AM, could be analyzed along with free morphine for definitive evidence of heroin use. If samples are hydrolyzed, 6-AM must be tested separately. Third, omitting the hydrolysis step reduced matrix complexity by eliminating degradation of non-target sample components leading to interferences. Although no specific interference was eliminated, the final extract was much cleaner, and the frequency of instrument maintenance decreased.

Another advantage of LC–MS–MS was a decrease in direct costs and sample preparation time in three main areas. First, expensive derivatization that is crucial to the sensitivity and specificity of the opiate GC–MS assay is not necessary in the LC–MS–MS assay. Second, because of the increased specificity of LC–MS–MS by using multiple reaction monitoring (MRM) mode, a less rigorous sample preparation can be used. The volume of solvents was reduced and several steps in the extraction were omitted. Third, the analytical run-time was decreased by 20%, resulting in a nearly 2 h reduction of instrumental analysis for a daily run of 60 samples.

Accuracy and precision

Figures 1 and 2 show MRM chromatograms of a spiked urine sample at 2 ng/mL of each opiate. The analytical measurement range was determined by preparing decreasing concentrations and increasing concentrations in urine. These samples were measured in triplicate on three different days. The limit of detection (LOD) was the lowest concentration that could be accurately identified by the presence of all MRM transitions and qualitative ion mass ratios within 25% of the ion mass ratio established by the middle calibrator. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were the lowest and highest concentrations, respec-

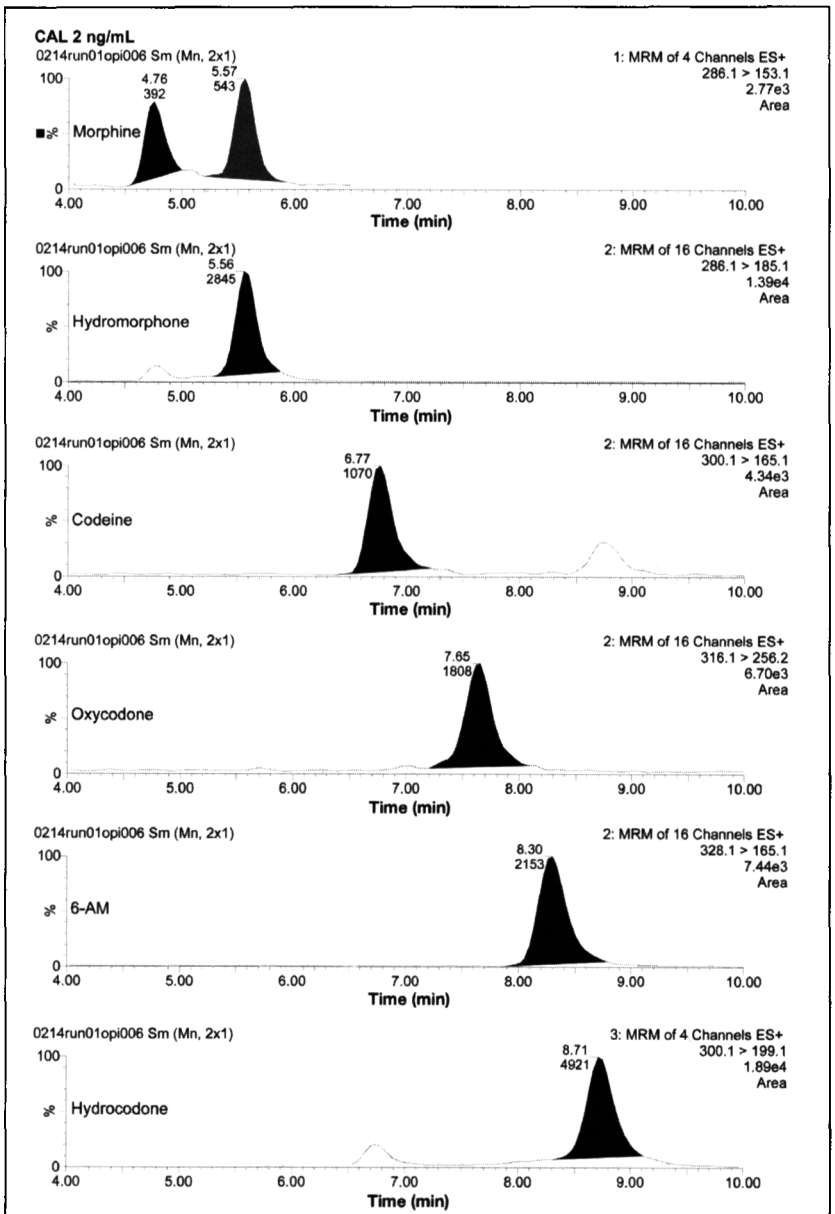

Figure 1. MRM chromatograms for morphine, hydromorphone, codeine, oxycodone, 6-AM, and hydrocodone (2 ng/mL).

tively, for which the total imprecision was less than 15% and the accuracy was within 85–115%. The LOD, LLOQ, and ULOQ determined by this method were 1, 2, and 2500 ng/mL, respectively.

At 1 ng/mL, all transitions were present and ion mass ratios were within range for all analytes. At 0.05 ng/mL, although all transitions were still present for all the analytes, the ion mass ratios were not always within range. In the 9 replicates evaluated, the number of samples with ion mass ratios outside 25% ranged from 1 out of 9 (hydromorphone) to 6 out of 9 (morphine).

With a calibration curve at 2, 5, and 20 ng/mL, an experimentally determined ULOQ at 2500 ng/mL illustrates the excellent linear range of this method. The low calibration curve ensures that samples close to the cutoff are always accurately identified as positive or negative, while high samples are still able to be quantified. The linear range of the method is re-evaluated semi-annually by analyzing samples at the LOD, LLOQ, and the ULOQ in triplicate. In addition, national proficiency testing samples have been analyzed by this method. Both of these checks ensure that the assay continues to perform as validated across this wide linear range.

Samples at the LLOQ were prepared in serum/plasma, whole blood and meconium and extracted in triplicate on three different days to verify the accuracy and precision in all matrices. The accuracy and precision of each analyte at the LOQ in all matrices studied are given in Table II. Imprecision was less than 15% and accuracy was within 85–115%, except for morphine and 6-AM in whole blood. Even during the short (3 day) time frame of this study, 6-AM in whole blood was hydrolyzed to morphine and the concentrations of 6-AM decreased while the concentrations of morphine increased. To further evaluate the stability of 6-AM in blood products, samples were prepared at approximately 50 ng/mL in 6-AM and re-tested at frequent intervals while being stored at 4°C. Whole blood and plasma samples were obtained from grey stoppered vacuum tubes. Serum was obtained from red stoppered vacuum tubes. The results from this study are illustrated in Figure 3. Only in whole blood did the 6-AM significantly decrease over the two-week study time. Because of this observation, we now recommend that clients interested in blood 6-AM identification submit a serum or plasma specimen rather than whole blood.

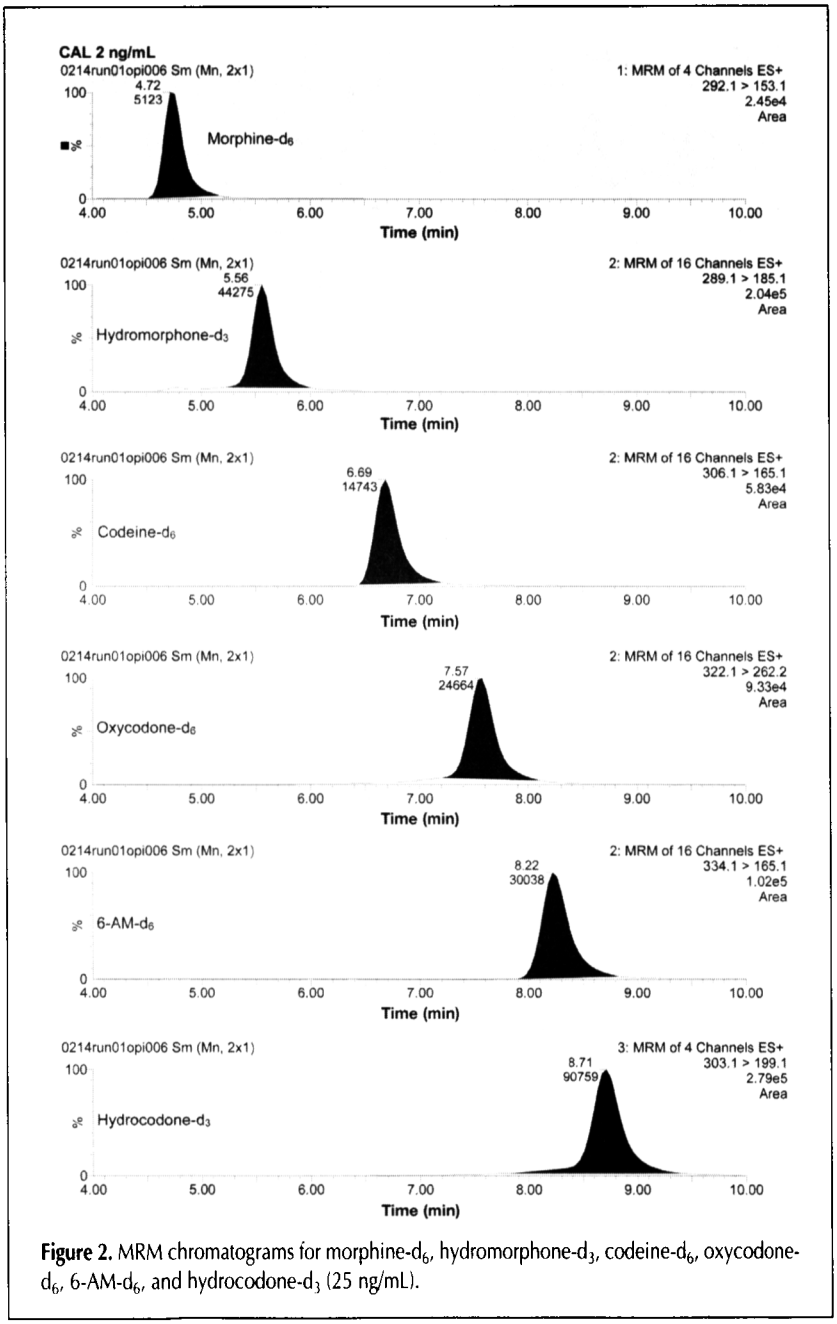

Figure 2. MRM chromatograms for morphine-d_6, hydromorphone-d_3, codeine-d_6, oxycodone-d_6, 6-AM-d_6, and hydrocodone-d_3 (25 ng/mL).

Recovery

The total recovery in both urine and meconium was determined by preparing two sample sets, each containing 3 aliquots at 20 ng/mL (20 ng/g in meconium). Internal standard was added at the beginning of the extraction as usual to set one. The internal standard for set two was not added until all after extraction steps were performed. The average recovery was calculated by dividing the average concentration of the set two by the average concentration of set one. The SPE recovery in meconium was found by extracting a third set of three blank meconium samples up to just before the SPE method (homogenizing, pouring off, and drying down). Just prior to placing on the SPE column, they were spiked at 20 ng/g and the internal standard was added at the end, along with all the other recovery samples.

The SPE extraction was optimized to allow elution with only 1 mL of solvent directly into autosampler vials. This increases the efficiency of the extraction as well as reducing potential sample mix-up. The original GC–MS method used 3 mL of 98:2 ethyl acetate/ammonium hydroxide, but when the analytes were eluted with only 1 mL of this, the recovery of mor-

phine and hydromorphone from urine were both < 50%. By adding isopropanol to the elution solvent, the recoveries were all 84% or better, as indicated in Table III.

This opiate meconium extraction was an improvement over the GC–MS extraction, where all the recoveries were < 20%. The meconium SPE recovery is useful to determine if the recovery loss is due to the solid meconium clogging up the SPE columns, or if it is due to the pouring off and drying down steps necessary for meconium analysis. The meconium SPE recoveries are all similar to the urine recoveries, indicating that future improvements upon the meconium extraction must be within the first few steps of the extraction.

Carryover

Carryover was evaluated by extracting a high sample in urine and meconium at 50,000 ng/mL of each opiate. A positive control was analyzed in triplicate before the high sample, then the high sample was injected followed immediately by the positive control. After injecting solvent until all traces of the analytes were removed, the positive control was again analyzed in triplicate. The difference between the first triplicate average and the sample immediately following the high was the total carryover. The difference between the second triplicate average and the first triplicate average was vial contamination, and the remaining carryover was caused by the autosampler, flow path and LC column. Homogenizer carryover during the preparation of meconium samples was evaluated by homogenizing a high sample, following standard washing protocol, then rinsing with methanol. Internal standard was added to the methanol rinse and the methanol was dried and analyzed by the method. Results for carryover are given in Table IV.

Vial contamination for both matrices was negligible, but autosampler, flow path and LC column contamination was measurable. Although the values are all < 8 ng/mL, since the limit of quantitation was 2 ng/mL, this is a critical value. To eliminate false-positive results, all patient samples with a concentration between 2 and 20 ng/mL following a high sample (> 2500 ng/mL) are re-injected to evaluate potential carryover. All meconium samples with a concentration between 2 and 20 ng/g following a high sample (> 2500 ng/g) must be retested to eliminate the possibility of homogenizer contamination from the high specimen.

Table II. Accuracy and Precision at the Limit of Quantitation*

	Urine		Serum/Plasma		Whole Blood		Meconium	
Compound	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Within-run accuracy and imprecision†								
Morphine	3.4	100.3	3.4	105.8	11.3	128.7	4.9	109.0
Hydromorphone	4.2	107.8	4.3	110.9	6.3	103.5	4.1	108.0
Codeine	3.9	106.1	7.3	113.6	9.5	105.2	3.2	107.2
Oxycodone	4.9	97.8	8.8	114.6	5.6	102.3	4.4	113.5
6-AM	4.3	109.3	3.4	111.7	5.0	63.5	10.2	110.0
Hydrocodone	5.8	110.3	3.6	103.8	4.4	100.3	3.4	95.6
Between-run accuracy and imprecision‡								
Morphine	4.0	104.1	4.4	106.3	16.3	145.8	4.0	106.9
Hydromorphone	6.2	106.5	2.8	111.4	10.3	103.6	1.3	108.5
Codeine	8.0	98.3	9.6	103.3	11.0	100.1	1.4	108.3
Oxycodone	8.3	98.4	11.7	104.5	7.4	97.8	9.1	104.2
6-AM	4.9	108.7	6.4	109.2	12.1	56.9	9.1	104.6
Hydrocodone	11.3	103.3	4.0	105.6	6.3	101.6	2.6	97.1
Total imprecision								
Morphine	5.2		5.6		19.8		6.3	
Hydromorphone	7.4		5.1		12.1		4.3	
Codeine	9.0		12.1		14.5		3.5	
Oxycodone	9.7		14.7		9.3		10.1	
6-AM	6.6		7.3		13.1		10.2	
Hydrocodone	12.7		5.4		7.7		4.3	
Analytical Measurement Range§								
LOD	1							
LLOQ	2							
ULOQ	2500							

* 2 ng/mL in urine and blood; 2 ng/g in meconium.
† For within-run values, $n = 3$ samples analyzed in a single run.
‡ For between-run values, $n = 3$ runs.
§ ng/mL in urine, serum/plasma, and whole blood; ng/g in meconium.

Method comparison

Serum, plasma, and whole blood. Patient samples (serum/plasma, $n = 57$, whole blood, $n = 26$) were de-identified and tested by this LC–MS–MS method and compared to an LC–MS–MS method of a commercial reference laboratory. Both methods evaluated only the unconjugated drug. Because of the limited quantity of positive samples available, 15 spiked samples were prepared and included in the set. The comparison data for serum, plasma, and whole blood are plotted in Figure 4A and B. The correlation included specimens between 1 and 1200 ng/mL. The magnified portions of the curves indicated that the assay correlated well, even at low levels of < 50 ng/mL.

Urine and meconium. Patient samples (urine, $n = 116$, meconium, $n = 32$) were de-identified and extracted by this LC–MS–MS method and compared to an in-house GC–MS method. The GC–MS method measures both the free and conjugated forms by hydrolyzing the conjugates to the free form before analysis, whereas the LC–MS–MS method includes only the free form. The patient results were compared by reviewing the positivity rate between the two methods instead of a direct linear regression (Table V). Only hydrocodone, which does not form a glucuronide, and oxycodone, which has a low percentage of glucuronide, could be directly compared in patient samples by linear regression (Figures 4C and 4E). Fifteen samples were spiked with the free form of each analyte to allow a direct correlation in addition to the positivity rate of the samples (Figures 4D and 4F). The cutoff for

meconium samples by LC–MS–MS was set at the LOQ, 2 ng/g, but the cutoff for urine samples by LC–MS–MS was raised to 5 ng/mL to avoid the possibility of false positives.

Four urine samples that were positive for morphine by GC–MS could not be identified by LC–MS–MS because of interference, but four additional morphine positive samples were identified. Three of these additional samples were also positive for codeine. Since codeine metabolizes to morphine, these results are justifiable positives. The fourth could be due to poppy seed ingestion because the codeine was less than 20% of the morphine. All of the meconium GC–MS positive morphine samples were correctly identified by LC–MS–MS, plus two additional ones that were below the LOD of the GC–MS assay.

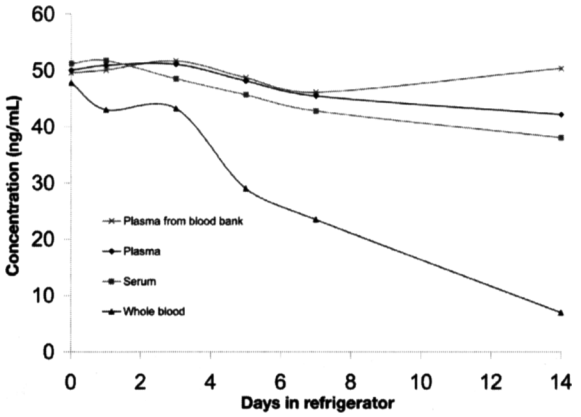

Figure 3. Stability of 6-acetylmorphine in blood samples while stored at 4°C.

Table III. Recoveries of all Target Opiates in Urine and Meconium (%)

Compound	Urine (total)*	Meconium (total)*	Meconium (SPE)†
Morphine	87.2	48.6	86.6
Hydromorphone	84.5	38.3	68.0
Codeine	90.8	50.4	84.6
Oxycodone	89.3	51.4	86.0
6-AM	88.4	53.0	85.7
Hydrocodone	86.9	51.1	84.8

* Recovery of all extraction steps.
† Recovery of only the final SPE extraction step in the meconium method.

Table IV. Carryover Contamination

	Urine (ng/mL)		Meconium (ng/g)		
Compound	Vial	Column	Vial	Column	Homogenizer
After a 50,000 ng/mL sample					
Morphine	0.11	8.75	0.19	5.93	8.20
Hydromorphone	0.87	6.39	0.00	3.87	7.73
Codeine	0.08	2.94	0.00	1.10	6.39
Oxycodone	0.00	2.86	0.21	0.19	7.17
6-AM	0.17	2.48	0.07	0.04	7.19
Hydrocodone	0.16	1.85	0.15	1.05	7.23
After a 25,000 ng/mL sample					
Morphine	0.03	4.80	0.00	5.75	1.96
Hydromorphone	0.00	2.45	0.30	3.10	1.87
Codeine	0.05	0.26	0.23	1.93	1.41
Oxycodone	0.00	0.00	0.00	0.00	1.67
6-AM	0.00	0.82	0.00	0.42	1.61
Hydrocodone	0.00	0.84	0.00	1.07	1.38

In urine, all of the samples positive by GC–MS for hydromorphone were also positive by LC–MS–MS. Twenty-two of the additional 24 samples positive for hydromorphone by LC–MS–MS were also positive for hydrocodone. Since hydrocodone metabolizes to hydromorphone this supports the additional positives. In meconium, all samples positive by GC–MS for hydromorphone were positive by the LC–MS–MS method. All four of the additional LC–MS–MS hydromorphone positive samples were also positive for hydrocodone.

All of the urine and meconium samples that were positive for codeine by GC–MS were also positive by LC–MS–MS except one meconium sample, which could not be accurately identified because of a co-eluting peak. The two additional urine codeine specimens that were positive by LC–MS–MS, had interference when analyzed by GC–MS. The one additional meconium sample positive for codeine was below the LOD (40 ng/g) of the GC–MS method.

Three of the urine samples positive for oxycodone when analyzed by GC–MS were negative by LC–MS–MS. However, these samples were also negative by an oxycodone immunoassay screen and negative by an independent reference laboratory. All of the GC–MS interference with oxycodone was eliminated on the LC–MS–MS. One of the meconium GC–MS positive sample was negative by LC–MS–MS. Although this sample could not be sent to an independent reference laboratory for testing, the same interference that causes false positives on the GC–MS in urine would also likely explain that seen in meconium. All 17 of the additional LC–MS–MS positive urine specimens were positive by the oxycodone screen. Ten of the additional LC–MS–MS positive urine specimens produced interference on the GC–MS method. The other 11 additional positive specimens were below the LOD of the GC–MS assay.

In both urine and meconium, all of the patients samples identified as positive by GC–MS for hydrocodone were also identified by LC–MS–MS. All the additional samples identified by LC–MS–MS could be justified by either interference in the original GC–MS data or concentrations on the LC–MS–MS that were lower than the GC–MS cutoff. Because hydrocodone does not form a glucuronide conjugate, the LC–MS–MS would allow detection of a larger number of positive hydrocodone samples due to the lower cutoff.

Specificity

Because of the structural similarity between drugs and metabolites in the opiate family, there is a high potential for interference. Twelve related compounds were evaluated for potential interference with the six opiates of interest. The relative retention times of these compounds, as well as the major

MRM transitions are listed in Table VI.

A positive control (5 ng/mL) for each of the six opiates analytes was spiked with a low level (200 ng/mL) and a high level (5000 ng/mL) of each of the compounds in Table VII. Samples containing 200 ng/mL did not affect the results of the positive control. In the samples spiked with 5000 ng/mL, five of the opiate compounds were falsely elevated.

The first two compounds, morphine-3-glucuronide and heroin, increased only the concentration of its metabolite, morphine and 6-AM, respectively. High morphine-3-glucuronide raised the concentration of the morphine from 5 ng/mL to 10 ng/mL and high heroin raised the concentration of 6-AM to 22 ng/mL. Both of these are most likely due to a partial hydrolysis during sample preparation.

Two other compounds, oxymorphone and norcodeine, are much more critical. Oxymorphone is a prescribed drug as well as a metabolite of oxycodone. Norcodeine is only a metabolite of codeine. Oxymorphone elevated the morphine concentration to 15 ng/mL, and norcodeine elevated the hydromorphone concentration to 60 ng/mL. This observation illustrated the importance of using secondary transitions, even when analyzing samples in MRM mode. Despite multiple adjustments to the LC analysis, these compounds still co-eluted with the opiate analytes. Expected transitions for morphine and hydromorphone were still present in high amounts of oxymorphone and norcodeine, so it was impossible to choose a single transition that would not be affected. Instead secondary transitions were chosen and ion mass ratios (IMR) between the primary and secondary transitions were established so that in the presence of high oxymorphone or norcodeine, the IMR would be out of range. Even though the presence of high amounts of oxymorphone and norcodeine would falsely elevate the concentration of morphine and hydromorphone, respectively, the IMR would be out of range and an indeterminate result would be reported instead of a false positive.

The fifth interfering compound was dihydrocodeine, which raised the hydrocodone concentration from 5 ng/mL to 11 ng/mL. This was unusual because the retention time of dihydrocodeine was 5.74 min, far away from the retention time of hydrocodone at 7.72 min. The most likely explanation for this observation is contamination of stock dihydrocodeine with hydrocodone. This was verified by analyzing samples of dihydrocodeine from two different suppliers: Cerilliant and Cambridge Isotope Laboratories. At 5000 ng/mL, the dihydrocodeine stock solutions from both suppliers also contained from 5 to 8 ng/mL of hydrocodone. Further work to investigate the presence of hydrocodone in pharmacological preparations of dihydrocodeine and the presence of hydrocodone in patients on dihydrocodeine is necessary.

The 116 urine patient samples that were selected for the correlation study were also evaluated for the ability of LC–MS–MS to resolve interferences. When analyzed by LC–MS–MS, the interference rate went down to 4.3% compared to a rate of 24.1% by GC–MS (Table VII). The interference in the GC–MS method with hydromorphone, codeine, oxycodone and hydrocodone was completely resolved with LC–MS–MS. The only interference observed in the LC–MS–MS method was the expected morphine interference due to oxymorphone in samples with high concentrations of oxycodone. There were no samples with hydromorphone interference due to norcodeine in samples with high concentrations of codeine. This is most likely due to the high rate of conjugated norcodeine, and without hydrolysis, free norcodeine is not present in sufficient concentrations to interfere with hydromorphone.

To evaluate whether this improvement of specificity remains when evaluating a random assortment of patient samples, data from approximately 5000 patient specimens analyzed over a period of 18 months in the production laboratory was analyzed. During the year prior to implementing the new LC–MS–MS

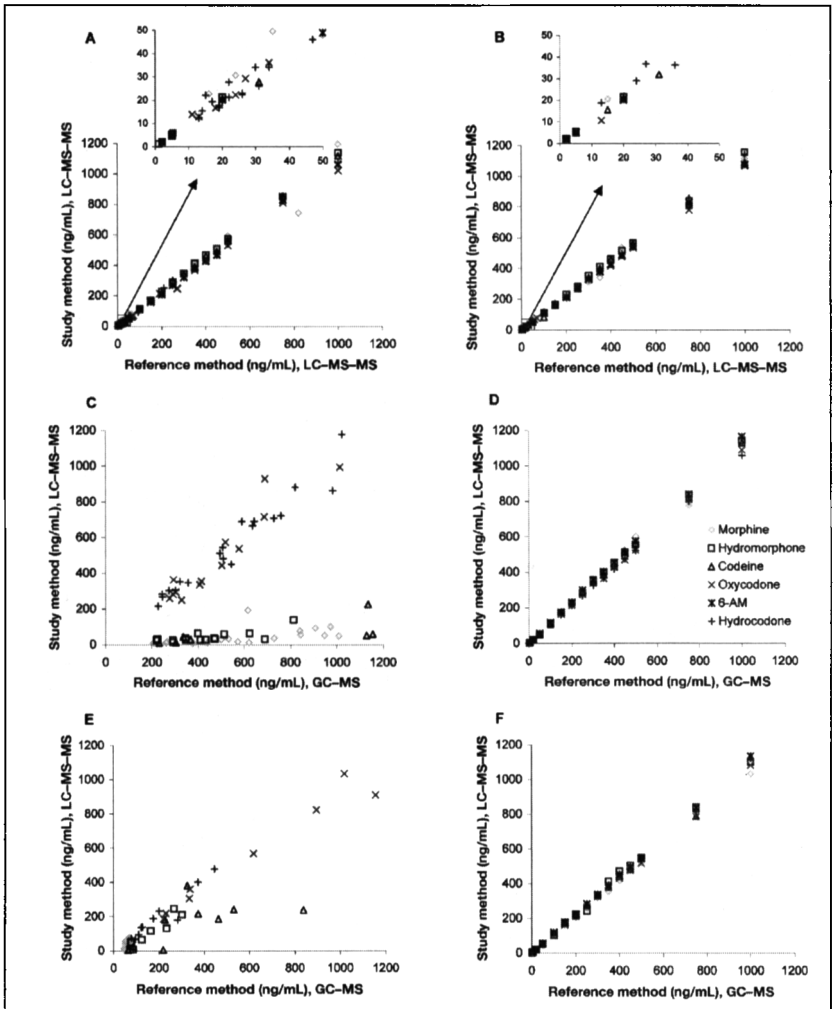

Figure 4. Correlation data in serum/plasma, $n = 57$ (A), whole blood, $n = 26$ (B), patient urine specimens, $n = 116$ (C), spiked urine samples, $n = 15$ (D), patient meconium specimens, $n = 32$ (E), spiked meconium samples, $n = 15$ (F).

method, 13.6% of patient samples had interference with one or more analytes and 11.1% of the 6-AM samples were reported with interference. Because 6-AM was evaluated in a separate aliquot and only performed at the client's request, the interference with 6-AM was not included in the total. In the six months since implementing the LC–MS–MS method, only 3.9% patient results could not be reported due to interferences. Unlike the correlation samples, the analyte with the highest interference was hydromorphone at 2.5%. All of these samples were positive for hydrocodone and not for codeine. Because they were negative for codeine, this interference cannot be from norcodeine, but is possibly due to some endogenous compound or an unidentified metabolite of hydrocodone.

Table V. Positive Patient Samples

	GC–MS with Hydrolysis		LC–MS–MS without Hydrolysis			
Compound	Positive	Interference	Positive	New positive*	Negative†	Interference
Urine						
Cutoff	200 ng/mL				5 ng/mL	
Morphine	30	2	30	4		4
Hydromorphone	18	14	42	24		
Codeine	17	1	19	2		
Oxycodone	32	14	46	17	3	
Hydrocodone	47	5	71	24		
Meconium						
Cutoff	40 ng/g				2 ng/g	
Morphine	12		14	2		
Hydromorphone	9		13	4		
Codeine	10		10	1		1
Oxycodone	10		11	2	1	
Hydrocodone	9		18	9		

* Positive by LC–MS–MS, but negative by GC–MS.
† Negative by LC–MS–MS, but positive by GC–MS.

Table VII. Percent of Interference by Analyte in Urine (%)

	Correlation Samples		Historical Results	
Compound	GC–MS (n = 116)	LC–MS–MS (n = 116)	GC–MS (n = 3339)*	LC–MS–MS (n = 1571)†
Morphine	1.7	4.3	0.8	0.5
Hydromorphone	12.1	0	5.4	2.5
Codeine	0.9	0	0.3	0.5
Oxycodone	12.1	0	6.7	0.2
6-AM	0	0	11.1	0.1
Hydrocodone	4.3	0	0.4	0.1
Combined	24.1	4.3	13.6	3.9

* All samples analyzed in the production laboratory 1 year prior to beginning analysis by LC–MS–MS.
† All samples analyzed by the production laboratory 6 months after implementing analysis by LC–MS–MS.

Ion suppression

To evaluate ion suppression, drug negative specimens in meconium, urine, serum/plasma and whole blood were extracted and injected while infusing solution at 0.2 ng/µL for all six analytes and internal standards at 10 µL/min (24). Because each analyte is quantitated by its deuterated internal standard, ion suppression is not as critical for accurate quantitation. However, to avoid low area counts resulting in poor chromatography, ion suppression was evaluated in all matrices of interest. Figure 5 shows that the ion suppression at the retention times of interest was minimal.

6-AM

The heroin metabolite 6-AM is commonly analyzed in urine samples. Although it is rapidly metabolized to morphine, and thus is difficult to identify, when it is found, it is definitive indication of heroin abuse. We could find no previous data analyzing the frequency of 6-AM in meconium. The majority of meconium opiate analyses utilize hydrolysis, and thus are not able to identify the 6-AM metabolite from the same aliquot. Meconium sample volume is small and repeat testing is often not possible. By eliminating the hydrolysis, the 6-AM metabolite can now be identified along with other opiates from a single aliquot in meconium.

After 6 months of analyzing samples by this method, 180 of 1641 (11.0%) morphine positive urine samples were also positive for 6-AM, compared to 2 of 24 (8.3%) in serum/plasma and 2 of 234 (0.9%) in meconium. The low positivity rate in meconium may be due to differences in maternal and neonatal metabolism as well as continued hydrolysis while stored in the neonatal intestine during gestation. Although the frequency of positivity in meconium appears much lower, it may be helpful when positive.

Conclusions

Analysis of opiates in urine, serum, plasma, whole blood and meconium by LC–MS–MS provides many benefits over a GC–MS analysis. Specificity is increased, with a decreased interference rate of 3.9% on this LC–MS–MS method compared to 13.6% on the GC–MS method. The variety of LC columns available allows the possibility of further reducing this interference rate by use of other LC columns. Increased sensitivity allows lower cutoffs and elimination of hydrolysis without sacrificing drug identification. The low cutoffs enabled identification of the correct analyte for all patient specimens analyzed by LC–MS–MS even without hydroysis. The sample preparation time is decreased by eliminating hydrolysis and derivatization required for the GC–MS analysis as well as by adapting the extraction to elute directly into autosampler vials. The elimination of hydrolysis also allows identification of the heroin

Table VI. Retention Time of Related Opiate Compounds

Compound	RT (min)	Primary MRM (*m/z*)
1: morphine-3-glucuronide	1.86	462.1 > 286.1
2: normorphine	3.99	272.1 > 181.1
3: noroxymorphone	4.16	288.1 > 213.1
morphine-d_6	4.40	292.1 > 153.1
morphine	4.40	286.1 > 153.1
4: oxymorphone	4.53	302.0 > 227.1
hydromorphone-d_3	5.08	289.1 > 185.1
hydromorphone	5.08	286.1 > 185.1
5: norcodeine	5.39	286.1 > 165.1
6: noroxycodone	6.08	302.1 > 187.1
7: dihydrocodeine	5.74	302.2 > 199.1
codeine-d_6	6.04	306.1 > 165.1
codeine	6.08	300.1 > 165.1
oxycodone-d_6	6.76	322.1 > 262.2
oxycodone	6.80	316.1 > 256.2
6-MAM-d_6	7.24	334.1 > 165.1
6-MAM	7.29	328.1 > 165.1
hydrocodone-d_3	7.72	303.1 > 199.1
hydrocodone	7.76	300.1 > 199.1
8: levorphanol	12.35	258.2 > 157.1
9: dextrorphan	12.28	258.2 > 157.1
10: heroin	12.80	370.1 > 165.1
11: 6-acetylcodeine	12.94	342.1 > 225.1
12: dextromethorphan	28.25	272.3 > 215.3

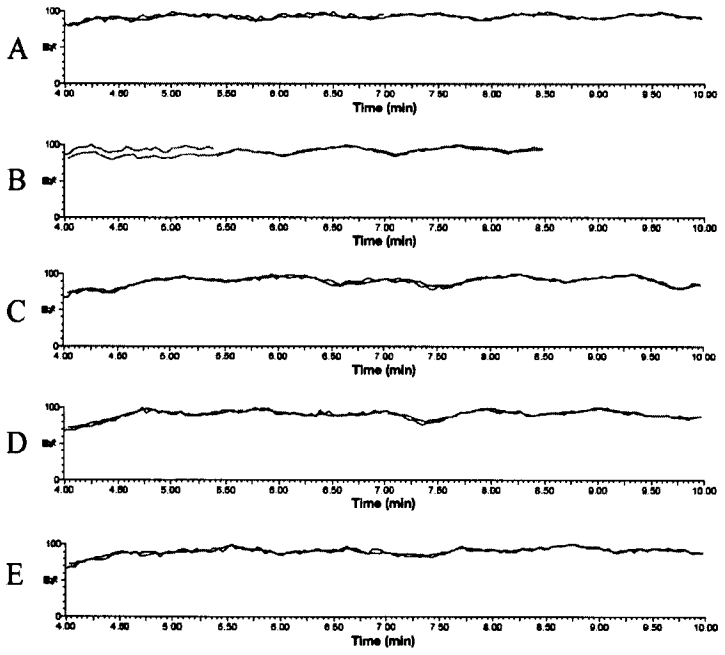

Figure 5. Illustration of ion suppression. Infusion of all analytes with a sample at 0.2 ng/µL while injecting samples of extracted blank matrix: solvent (A), meconium (B), urine (C), serum/plasma (D), and whole blood (E).

metabolite, 6-AM, from the same aliquot. In urine, 11.0% of morphine positive specimens were also positive for 6-AM compared to 8.3% in serum/plasma and 0.9% in meconium. Although the 6-AM is infrequent in meconium, it provides a definitive case for illegal heroin abuse in pregnant women.

Acknowledgment

Funding, instrumentation, and physical facilities to conduct this research was provided by the ARUP Institute for Clinical and Experimental Pathology and ARUP Laboratories, Inc. We also acknowledge valuable consultation with our colleagues, Phillip Dimson and Chen-Chih Chen, SPEWare, Inc.

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Manuscript received March 31, 2006;
revision received June 6, 2006.

Appendix

Supplemental Information: Confirmation Test for Opiates in Urine by Electron Impact Gas Chromatography–Mass Spectrometry, SIM mode

Reagents, Calibrators, and Controls

Label all reagents, calibrators, and controls with contents, date prepared, expiration date, and initials of preparer. To facilitate identification, the calibrators have a red sticker and the IS a blue sticker.

Reagents

6.0M Hydrochloric acid (HCl)[1,3]

Add 1983 mL of concentrated HCl to a 4-L plastic bucket that is approximately half full of Type I water. Add sufficient water to bring the total volume to somewhat less than 4 L. Mix by stirring for 10 min. Transfer to a 4-L amber bottle, top off to 4 L with Type I water, and allow to equilibrate 2 h at room temperature. Label appropriately. Stable for 1 year.

0.1M Hydrochloric acid (HCl)[1,3]

Add 33.6 mL of concentrated HCl to a 4-L plastic bucket that is approximately half full of Type I water. Add sufficient water to bring the total volume to somewhat less than 4 L. Mix by stirring for 10 min. Transfer to a 4-L amber bottle, top off to 4 L with Type I water, and allow to equilibrate 2 h at room temperature. Label appropriately. Stable for 1 year.

Methanolic HCl

To 99 mL of methanol add 1 mL of concentrated HCl (Fisher Scientific, or other reliable supplier), and mix. Store in glass bottle. Label appropriately (black ink). Stable for 1 year.

6.0M Sodium hydroxide (NaOH)[1,3]

Dissolve 240 g sodium hydroxide in 1 L of Type I water. Mix by stirring for 10 min. Transfer to a 1 L amber bottle and equilibrate 2 h at room temperature. Label appropriately. Stable for 1 year at room temperature.

0.1M Acetate buffer

Add 19.0 mL acetic acid to 3.50 L of nanopure water while stirring. Add 5.04 g sodium acetate while stirring. Allow the solution to stir until it is homogeneous. Adjust the pH of the solution to 4.0 with glacial acetic acid. Add the glacial acetic acid one drop at a time and allow the solution to equilibriate between drops. Do not go below pH 4.0 during pH adjustment. Adjust the solution to 4.0 L with nanopure water. Transfer the solution to a glass bottle. Stable for 6 months at room temperature.

Methanol[2], high purity grade

Obtained from Fisher, or other reliable source.

Ethyl acetate[2], high purity grade

Obtained from Fisher, or other reliable source.

Certified negative urine pool (NUP).

Drug-negative urine is used as the matrix for calibrators and controls. It is prepared by the ARUP Reagent Laboratory. Prior to being placed in service, it is certified to be free of drugs covered in CDAT testing by the EIA initial test and the GC–MS confirmation methods, down to the limit of detection, for all drugs covered. When it is analyzed by GC–MS, the NUP is spiked with all opiate analytes included in the assay, to the equivalent of a low calibrator (300 ng/mL) and evaluated for interferences with the ions, resulting in a change in the ion mass ratio.

Derivatizing reagent[2,3,7]

MSTFA. N-Methyl-N-trimethylsilyl-trifluoroacetamide ($C_6H_{12}F_3NOSi$), is obtained from Campbell Supply Co. (Rockton, IL) or other reliable source. Used as supplied. Stable at 2–8°C for 6 months. *Note: flammable, irritant, and potential carcinogen—work under the hood.*

Elution solvent

Ethyl acetate[2], with 2% ammonium hydroxide (NH_4OH).

Reagent precaution list (superscripts in order of severity).

[1] Corrosive—avoid contact with skin—wash thoroughly with water.
[2] Flammable—avoid heat or flame.
[3] Irritant—avoid contact with skin—avoid inhaling fumes or powder.
[4] Oxidizer—potential for fire or explosions.
[5] Poison—do not ingest.
[6] Toxic—avoid prolonged contact or inhalation.
[7] Potential carcinogen.

Calibrators and internal standards

Calibrators

Reference materials for the opiates confirmed by this procedure may be obtained in liquid form, at a concentration of 1 µg/µL in methanol, or in powder form. Calibrators can be prepared from either. The description, which follows, utilizes liquid form reference material. A stock calibration standard of the same concentration in methanol may be prepared from weigh-out of a powder, taking into account the purity, and salt form in calculating the weigh-out amount.

OPI reference material solutions, 1 µg/µL for each analyte in methanol.

Codeine, hydrocodone, morphine, hydromorphone and oxycodone reference materials are obtained from Cerilliant, or other reliable source, in separate sealed ampules containing 1000 µg of each analyte in 1.0 mL of methanol. It is supplied

with a certificate of analysis. Store in the freezer. Stable 3 years, or to the manufacturer's outdate.

Combined OPI working calibration standard, 20 ng/µL of each analyte, in methanol

Quantitative step.

Determine the percent purity of the reference material, as indicated by the manufacturer in the accompanying literature, and make an appropriate adjustment to the volume of the five reference material solutions to prepare the combined working calibration standard.

To a 100-mL volumetric flask containing approximately 50 mL of methanol, add 2000 µL (or the adjusted amount) each of the five reference material solutions, and fill to volume with methanol. Mix thoroughly, and equilibrate 1 h at room temperature. Transfer to eight glass tubes, close with new Teflon-lined caps, label appropriately (red sticker), and store in the refrigerator. Stable for 3 months at refrigerator temperature, and 1 year at freezer temperature. Parallel check the combined working calibration standard before placing into service (see Quality Control section of the Mass Spectrometry SOP for details of the process).

This is the standard used directly to prepare the calibrators used in the analytical procedure.

Combined OPI calibrators, 200, 500, and 2000 ng/mL of each analyte, in urine. The combined working calibration standard is used to prepare the three calibrators in the analytical procedure. For details regarding their preparation in each run, see item 3 in the Analytical Procedure section.

Internal standards

Codeine-d_6, hydrocodone-d_3, morphine-d_6, hydromorphone-d_3, and oxycodone-d_6 reference material solutions, 1 µg/µL each in methanol. Obtained from Cerilliant or other reliable source, in separate ampules containing 1000 µg of each analyte in 1.0 mL of methanol, for a concentration of 1 µg/µL, with a certificate of analysis. Store in the freezer. Stable 3 years or to manufacturer's outdate.

Combined OPI working IS solution, 10 ng/µL

Quantitative step.

Quantitatively transfer the contents of 1 sealed ampoule each of the five IS reference material solutions to a 100-mL volumetric flask containing approximately 50 mL of methanol, and fill to volume with methanol. Mix thoroughly, and equilibrate 1 h at room temperature. Transfer to 10 silanized glass tubes, close with Teflon-lined caps, label appropriately (blue sticker), and store in the refrigerator. Stable for 3 months at refrigerator temperature, and for 1 year at freezer temperature.

This is the IS solution used in the analytical procedure. The final IS concentration in the calibrators, controls, and test specimens is 500 ng/mL.

Controls

Unextracted control (UNX CONT). The UNX CONT is prepared in the same manner as the 200 ng/mL calibrator in each batch, except no urine matrix is used.

Negative control (NEG CONT). Prepared from the certified blank urine pool. For details of preparation of the certified blank urine pool, see the SOP of the Reagent Laboratory.

Positive control (POS CONT). Note: the combined OPI positive working control solution must be a separate preparation from the combined working calibration standard, so that the controls are independent of the calibrators.

OPI positive control reference material solutions, 1 µg/µL each, in methanol. See 2.a.1 above for all analytes except the following.

Morphine-3β-D-glucuronide reference material is obtained from Cerilliant, or other reliable source, in separate sealed ampules containing 1.0 mg in 1.0 mL of methanol. It is supplied with a certificate of analysis. Store in the freezer. Stable 3 years, or to the manufacturer's outdate.

Combined OPI positive working control, 500 ng/mL of each analyte, in urine

Quantitative step.

Determine the percent purity of the reference material, as indicated by the manufacturer in the accompanying literature, and make an appropriate adjustment to the volume of the six reference material solutions to prepare the combined positive working control.

Transfer approximately 50 mL of certified blank urine to a 100-mL volumetric flask. Using careful technique and an accurate pipet, transfer 80.8 µL (or the adjusted amount) morphine-3β-D-glucuronide reference solution, and 50 µL (or the adjusted amount) of each of the other five control reference solutions to the volumetric flask. Fill to the mark with certified blank urine. Mix thoroughly, and equilibrate 2 h at room temperature. Transfer to an appropriate container, label, and store in the refrigerator. Stable at refrigerator temperature for 3 months, and at freezer temperature for 1 year. Parallel check the combined positive working control before placing into service.

Instrumentation and Equipment

GC–MS

Instrumental analysis is performed on an Agilent Technologies 6890N/5973 electron impact GC–MS equipped with a capillary column, an autosampler, and controlled from a Hewlett-Packard Pentium 4, or compatible Pentium PC with at least 64 MB RAM, 4.3 GB HDD, 1.44 MB FDD, and CD ROM computer system (or similar configuration), using Agilent Technologies ChemStation® software. The GC–MS is operated in the selected ion monitoring (SIM) mode. The following operational conditions and software program parameters are used.

GC column

DB-5ms capillary, with 0.25-µm film thickness, 0.25-mm i.d., approximately 15-meter length.

Injection

Pulsed split mode (20:1 ratio), approximately 1 µL injection volume.

Carrier gas

Ultra high purity helium at a pressure pulse inject of 50 psi for 0.50 min, then 1.0 mL/min constant flow, with gas saver reduction to 20 mL/min at 3.0 min.

Temperatures

Column: 170°C (initial) for 0.2 min, 30°C/min to 230°C, hold 2.0 min; 60°C/min to 310°C, hold 2.5 min

Injector: 220°C
Transfer line: 280°C
MS source: 230°C
MS quad: 150°C
Mass spectrometer parameters.
Span: 0.3 amu
Solvent delay: 2.5 min
Area count threshold: 3% of largest peak

Group 1

Dwell time: 10 ms
Resolution: low mass
Start time: 3.7 min
End time: 4.65 min
Electron multiplier voltage (relative): 350 v
Ions monitored:

Codeine-d_6	377, 349
Codeine	371, 343
Hydrocodone-d_3	374, 237
Hydrocodone	371, 372, 234
Hydromorphone-d_3	432, 417
Hydromorphone	429, 430
Morphine-d_6	435, 436
Morphine	429, 401

Group 2

Dwell time: 30 ms
Resolution: low mass
Start time: 4.65 min
End time: 6.0 min
Electron multiplier voltage (relative):
350, 400 at 5.20 min
Ions monitored:

Oxycodone-d_6	393, 394
Oxycodone	387, 372

Note: the following parameters can be adjusted by the analyst at the beginning of the batch:

1. *Start/end times, because retention times change with column age and length.*
2. *Injection volume, 1–2 µL, to optimize area counts.*
3. *Sample split ratio, 20:50, to optimize area counts.*
4. *Electron multiplier voltage, to optimize area counts.*

Equipment

Solid phase separation column

Cerex Clin II (35 mg/3 mL). Obtained from SPEware Inc., San Pedro, CA.

Other equipment

Standard glassware, pipets, bead baths, centrifuges, hydrolyzing and dry-down equipment.

Analytical Procedure

Organization of the batch. Print out a worklist of specimens to be tested from PathNet. Label a series of 16 × 100 silanized tubes: 200 cal, 500 cal, 2000 cal, UNX CONT, NEG CONT, POS CONT, and one with the lab accession number of each specimen in the batch. Five opiate drugs are included in the opiate confirmation. Because multiple opiates are often present in the same specimen, some at extremely high concentrations, and others at low (but > 200 ng/mL), it is an advantage to prepare aliquots at full strength and diluted. Prior to setting up a batch of opiates, from the re-extraction log, find screen specimens which are positive for opiates, and for which the immunoassay result is > 300 milliabsorbance units. Aliquot each such specimen full strength, and at a 10× dilution, and insert them in the run.

Aliquot test specimens and POS CONT. Quantitative step. To each test specimen tube, quantitatively transfer 2 mL of specimen. *NOTE: when pipetting specimens, never return any urine, once removed, to the specimen bottle, and never insert anything but a new tip into a specimen bottle.* Add 2 mL of POS CONT to POS CONT tube.

Prepare calibrators, NEG CONT and UNX CONT. Quantitative step. Using good technique with a 2-mL MLA pipet, to the 200 cal, 500 cal, 2000 cal, and NEG CONT tubes, add 2 mL of blank urine. To the 200 cal, 500 cal, and 2000 cal tubes, add 20, 50, and 200 µL of the combined OPI working calibration standard, respectively, using Drummond pipets. To the UNX CONT tube, add 20 µL of combined OPI working calibration standard. Set aside the UNX CONT tube for the following steps (the UNX CONT becomes active again in step 7g, below).

Add IS. Quantitative step. Using a Drummond pipet, transfer 100 µL of the working IS solution to each tube. For quantitative accuracy it is important that the same amount of internal standard be added to each calibrator, control, and test specimen tube.

Hydrolyze glucuronide conjugates. To each calibrator, control, and specimen add 1.0 mL of 6.0 M HCl, cap, vortex briefly, and place in autoclave.

Press the ON button.

Insure autoclave has sufficient water for a liquid cycle but that it is not overfull (water above full line).

Load samples into chamber. *CAUTION: BE SURE TO USE STAINLESS STEEL RACK. RACKS MADE OF OTHER MATERIALS COULD MELT OR RUST IN THE AUTOCLAVE.*

Close and lock chamber door.

Select PACKS cycle (121°C, 17.5 psi, hold 30 min).

Confirm that TIME is set to 30 min and DRY is set to 20 min. If not, then perform the following.

Press PROGRAM/SET.
Adjust time with arrow keys.
Press PROGRAM/SET again.
Adjust dry time.
Press PROGRAM/SET to save.
Press START. Run time is approximately 1 h.
The cycle is complete when the COMPLETE indicator light comes on and three groups of three beeps sound.
Remove samples from the chamber immediately.
Leave chamber door open slightly and then press START again. This will begin the drying cycle.
Add 1 mL of 6.0M NaOH to each tube, vortex mix briefly, and spin at 2500 rpm for 5 min.

Cerex Clin II solid-phase extraction

Label the SPE columns with sample identifiers.
Load samples onto the columns at less than 2 psi.
Wash each column at less than 5 psi, with 3 mL of each of the following: water, *0.1M hydrochloric acid (at a flow of ≤ 1 mL/min)*, methanol, then 1 mL ethyl acetate.
Dry the columns for 15 minutes at 25 psi.
Dispose of waste into sink.
Elute the opiates with 3 mL of ethyl acetate/NH_4OH (98:2) into a new set of tubes pre-labeled with the sample IDs.
Include the UNX CONT in the following steps along with other tubes in the batch.
Add 100 µL of methanolic HCl to each tube prior to evaporation. DO NOT VORTEX MIX.

Evaporate elution solvent. Place the tubes in the dryer at 35 °C and 15 psi. Evaporate to dryness (~ 8 min). *DO NOT OVERDRY!*

Derivatization (perform under the hood)

To each tube add 50 µL ethyl acetate and 50 µL MSTFA, vortex mix for 5 s.
Cap each tube with a Teflon-lined cap, and vortex for 5 s. Place the tubes in the 60°C heat block for 20 min. After incubation, cool tubes to room temperature, and centrifuge for 10 s at approximately 1000 rpm (this is important for recovering the vaporized drug).
Transfer to autosampler vials. Label autosampler vials in the same manner and order as extraction tubes, and transfer tube contents to the vials.
CAUTION: TRANSFER STEP. To avoid error, always keep tubes and labeled autosampler vials in the order listed on the Batch Review form. Work with one aliquot at a time. Hold culture tube and corresponding autosampler vial in such a way as to be able to compare the labels on both containers. After assuring that the numbers on the tube and ALS vial match, make the transfer with a pipet.
Label an ALS vial as SOLVENT, and add approximately 100 µL of acetonitrile. This will become the first injection of the run, if the previous batch on the intended instrument used other than acetonitrile as a reconstituting solvent.

Perform instrumental analysis