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Application of High-Resolution UPLC–MS^E/TOF Confirmation in Forensic Urine Drug Screening by UPLC–MS/MS

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Abstract

The transition from presumptive (immunoassay) drug screening to definitive screening has continued in the practice of analytical toxicology. Development of a ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) screening method for over sixty drugs and metabolites (analytes) in urine has been reported by the authors and has been applied in probation, drug court, social services, chemical dependency, pain management and addiction medicine casework. Testing by the definitive screening method has increased both the rate and diversity of initial-positive drug findings, due to the lower positive thresholds and wider panel of analytes. Use of definitive screening in forensic casework, however, requires retesting of initial-positive analytes using a second method based upon a different analytical technique with at least equivalent sensitivity and selectivity. Consequently, a UPLC–MS^E/TOF method for confirmation of the initial-positive analytes has been adapted; the method is for targeted confirmation and is based upon an alternate mass spectrometry technology and column separation. Both the initial screen and the confirmatory analysis employ threshold accurate calibration for normalization of matrix effects, without the use of stable isotopes. Validation and application of the complete workflow, in forensic urine drug testing casework, is reported.

Introduction

Advances in urine drug monitoring are being driven, not only by the proliferation and misuse of new pharmaceutical and illicit agents, but also by advances in analytical technology and development of consensus on optimum testing practices for urine drug testing. The long-standing practice of a workflow comprising immunoassay screening for common drug classes followed, in forensic cases and some clinical cases, by selective drug and drug metabolite confirmation is evolving. Screening and confirmation terminology is being redefined using presumptive versus definitive testing criteria without a mandated sequence in the testing process (1). The American Medical Association (AMA) defines a positive presumptive test as an indicator of possible, but not definitive, presence of drugs or drug classes. The AMA further categorizes presumptive methods to include immunoassay, enzymatic methods, chromatographic methods without mass spectrometry, mass spectrometry without chromatographic separation or without adequate chromatographic resolution of structural isomers or isobars. By definition, definitive methods are qualitative or quantitative mass spectrometry techniques used to identify individual drugs and distinguish between structural isomers, thus providing a specific identification of individual drugs and drug metabolites in either the initial or confirmatory testing phases. AMA guidelines also allow definitive methods to be used in the initial testing process without prior presumptive testing. More recently, definitive methods have been further sub-categorized based upon method applications with or without the use of either a matrix normalization technique or a matrix-matched quality control protocol (2, 3).

In the clinical setting, an expert panel from the American Academy of Pain Management has published a consensus report regarding the use of definitive methods for monitoring urine drug profiles in patients receiving opioids for chronic pain (4). The panel recommends use of definitive urine drug monitoring as the most accurate method for assessing baseline opiate use and opioid misuse; they also recommend that presumptive testing should not be used unless mandated by institutional or payer policy. The consensus report cites increased specificity and sensitivity of definitive methods/techniques compared to presumptive screening and a reduced likelihood of false results when definitive screening is practiced. Another consensus group sponsored by the American Society of Addiction Medicine and the Center for Lawful Access and Abuse Deterrence focused on the proper use of urine drug tests. This consensus report also cited enhanced specificity, accuracy and reliability of definitive initial testing and stated that definitive testing may be more reasonable and necessary based upon patient history, medical response and clinical assessment (5).

A developing consensus on the value of initial definitive testing is analytically supported by the continuing development and validation of definitive methods for use in urine drug screening programs. Urine drug screening by nominal mass methods using coupled liquid chromatography-mass spectrometry technology continue to be reported in the literature with multi-analyte panels ranging up to 177 analytes (6–24). A number of high-resolution mass spectrometry (HRMS) methods employing liquid chromatography have also been reported for urine drug screening (25–31). Since acquisition dwell time is not an analyte-limiting factor in full scan HRMS acquisition methods, screening for an unlimited number of analytes is now possible. Definitive screening expands not only the number of tested analytes but also allows a reduction in the positive threshold concentration in urine screening which reduces the incidence of false negative results when compared with screening programs employing presumptive methods (32–34).

In forensic casework it is important to note that an expanding volume of positive analyte findings and an increase in the number of analytes tested is likely to increase the confirmatory workload and require additional confirmatory methods for the new analytes that were not previously detected in presumptive screening programs. Gabapentin for example is the most frequent finding in our definitive drug screening program and is not being tested routinely by immunoassay screening protocols (6). In forensic casework a second test for confirmation of positive drug findings is recommended and current standards of practice also point to the use of different methods for initial and confirmatory testing as a general matter of scientific and forensic principle (35). Due to the lower positive thresholds used in definitive screening the confirmation method must not only employ an alternate method with analyte selectivity but must have an analytical sensitivity equal to or greater than the definitive screening method.

Our group has previously developed and reported a qualitative ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method for definitive screening (7). The screening method was based upon a threshold accurate calibration (TAC) technique that normalizes matrix effects and eliminates the need for analyte-specific stable isotopes. We now report an expanded qualitative screening panel for additional analytes; in addition, we have adapted and validated an ultra-performance liquid chromatography–time-of-flight mass spectrometry in MS^E mode (UPLC–MS^E/TOF) method for targeted confirmation of initial-positive screened analytes. The confirmation method also employs the TAC technique and for forensic defensibility employs an MS technology and chromatographic separation phase that is different from the screening method.

Materials and Methods

Chemicals, supplies and reference material

High-performance liquid chromatography (HPLC) grade methanol, LC–MS grade acetonitrile and ACS grade isopropanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). Reagent grade ammonium formate (97%) and formic acid (>98%) were obtained from Sigma-Aldrich (St Louis, MO, USA). Morphine-3-beta-D-glucuronide (M3G) was purchased from Cerilliant (Round Rock, TX, USA), methapyrilene HCl from Supelco Analytical (Bellefonte, PA, USA) and reference material for the analytes listed in Table I were obtained from either Cerilliant and/or Grace Alltech® (Deerfield, IL, USA). Purified beta glucuronidase (IMCSzyme, activity ≥50,000 U/mL) and beta glucuronidase buffer (IMCSzyme) were obtained from and certified by Integrated Micro-Chromatography Systems (Columbia, SC, USA). Leucine enkephalin was purchased from Sigma-Aldrich.

Sample collection plates (96-well, 2 mL-square well) were obtained from Waters Corporation (Milford, MA, USA). Analyte-free urine, used for preparation of calibrator and quality control (QC) samples, was obtained from laboratory volunteers (n = 10) and the absence of detectable analytes was verified by UPLC–MS/MS analysis prior to use. Deionized water was produced by combined ultra-filtration (AquaPure, Schenectady, NY, USA) and reverse osmosis (Alpha Water Systems, Montague, NJ, USA) treatment.

Standards, controls and reagents

Two stock standards were prepared in methanol using separate sources of reference material stored at −10°C. Stock standards were used to prepare working calibrator, controls and fortification reagents. On the day of analysis, a working calibrator was prepared with threshold concentration of each analyte by a 100-fold dilution of stock standard in analyte-free urine. Analyte-specific calibration thresholds ranged from 10 to 1,000 ng/mL as specified in Table I. Quality control samples for validation studies were also prepared on the day of analysis by dilution of a second source of stock standard with analyte-free urine to achieve analyte-specific concentrations at 40, 75, 125 and 500% of threshold concentration. A fortification reagent was prepared at a concentration needed to achieve 300% of threshold concentration in the final sample preparation. A 3% methanol solution was prepared and used to maintain equivalent volume and methanol content for both neat and fortified test samples.

A stock hydrolysis control containing 10 µg/mL of M3G was prepared in methanol and stored at −10°C. Hydrolysis control was prepared on the day of analysis by dilution of the stock hydrolysis control with analyte-free urine to achieve at a free-morphine concentration equivalence of 400 ng/mL based upon the proportionate molecular weight difference of free and glucuronidated morphine. As an injection recovery standard, a stock solution of methapyrilene was prepared in methanol at a concentration of 10 µg/mL and was stored at −10°C. On the day of analysis, a working recovery reagent containing 200 ng/mL of methapyrilene was prepared by dilution of stock methapyrilene in water. The working recovery reagent was then mixed in equal volume with a buffered hydrolysis reagent containing 60% beta glucuronidase buffer and 40% purified beta glucuronidase for preparation of a mixed recovery and hydrolysis reagent.

Table I. Analyte threshold, retention time and MS acquisition conditions for UPLC–MS/MS and UPLC–MS^E/TOF methods

Analyte	Positive threshold (ng/mL)	MS/MS screening parameters							MS^E/TOF confirmation parameters					
		Retention time (min)	Precursor ion (*m/z*)	CV (V)	Prod. 1 (*m/z*)	CE (eV)	Prod. 2 (*m/z*)	CE (eV)	Retention time (min)	Molecular ion (*m/z*)	Frag. 1 (*m/z*)	Frag. 2 (*m/z*)	Frag. 3 (*m/z*)	Frag. 4 (*m/z*)
Alprazolam	100	2.47	309.1	50	281.2	27	205.2	42	8.42	309.0902	281.0715	274.1213		
Alprazolam, α-hydroxy	100	2.42	325.2	51	216.2	41	297.2	23	7.7	325.0851	297.0664	204.0339		
Amphetamine	100	1.23	136.1	20	91.2	15	119.1	8	2.2	136.1121	91.0543	119.0856		
Benzoylecgonine	100	1.74	290.2	39	168.1	19	105.1	30	2.86	290.1387	168.1020	105.0335	150.0914	
Buprenorphine	10	2.09	468.4	60	55.1	70	84.2	70	6.95	463.3109	414.2639	396.2170	58.0652	85.0886
Buprenorphine, nor	10	1.94	414.2	70	57.1	43	101.1	40	5.08	414.2639	101.0961	396.2534		
Carisoprodol	100	2.28	261.2	18	176.2	9	55.2	29	8.33	261.1809	176.1282	97.1012	200.1646	158.1176
Clonazepam	100	2.38	316.1	50	270.2	25	214.3	35	8.03	316.0484	270.0555	241.0528	214.0419	
Clonazepam, 7-amino	100	1.76	286.2	32	121.2	28	222.2	22	3.62	286.0742	222.1026	121.0761	250.0975	
Codeine	100	1.41	300.1	50	165.3	35	153.2	35	1.68	300.1594	215.1067	225.0911		
Dextromethorphan	100	2.06	272.2	50	147.1	30	171.1	40	6.25	272.2009	215.1431	213.1274	171.0799	147.0799
Dextrorphan/Levorphanol	100	1.68	258.2	65	133.1	35	157.1	40	3.4	258.1853	157.0656	199.1118		
Diazepam	100	2.56	285.1	55	154.2	29	193.2	29	10.3	285.0789	193.0886	222.1152	154.0419	
Diazepam, nor	100	2.51	271.1	55	140.1	29	165.1	27	8.9	271.0633	140.0262	208.0995	165.0215	243.0683
EDDP	100	2.08	278.3	47	186.2	33	249.2	23	7.29	278.1904	234.1278	249.1512	186.1278	
Ephedrine	100	1.13	166.1	25	133.1	20	148.1	12	1.81	166.1227	148.1121	117.0699	133.0886	115.0543
Ethylone	10	1.37	222.1	40	107.2	25	121.2	20	2.42	222.1125	174.0914	204.1020	149.0234	
Fentanyl	10	2.03	337.2	18	105.2	38	188.2	22	6.27	337.2275	188.1434	105.0699	216.1383	
Fentanyl, nor	10	1.64	233.2	38	84.2	18	56.2	25	3.32	233.1649	84.0808	177.1387	150.0914	
Flurazepam	100	2.11	388.2	45	315.2	21	100.2	29	6.7	388.1587	315.0695	288.0586	225.0949	
Flurazepam, OH ethyl	100	2.43	333.1	45	109.1	28	194.1	22	8.34	501.1917	297.1037	289.0539	184.0524	259.0433
Gabapentin	1,000	1.24	172.1	32	55.1	25	137.1	15	1.77	172.1332	154.1227	137.0961		
Hydrocodone	100	1.44	300.4	60	199.2	35	128.1	55	2.3	300.1594	199.0754	241.0857	171.0804	
Hydromorphone	100	1.08	286.4	60	185.3	30	157.2	40	1.12	286.1438	185.0598	227.0701	199.0754	
Ketamine	10	1.63	238.1	35	125	25	179	18	3.16	283.0994	125.0153	179.0623	207.0572	220.0888
Ketamine, nor	10	1.58	224.1	25	125	25	207	12	3	224.0837	125.0153	207.0572	179.0623	
Lorazepam	100	2.41	321.1	41	275.2	21	303.2	21	8.26	321.0192	275.0138	229.0528	138.0106	
MDA	100	1.32	180.4	20	163.1	15	105.1	20	2.25	180.1019	163.0754	135.0441	105.0699	133.0648
MDEA	100	1.49	208.3	30	163.2	13	105.2	25	2.95	208.1332	163.0754	135.0441	105.0699	133.0648
MDMA	100	1.40	194.2	27	163.2	13	105.2	25	2.51	194.1176	163.0754	135.0441	105.0699	
MDPV	10	1.77	276.2	35	175.2	20	135.2	30	4.44	276.1594	126.1278	175.0754	135.0441	205.0860
Meperidine	100	1.78	248.2	15	174.2	18	220.2	22	4.62	248.1645	220.1333	174.1278	131.0856	70.0652
Meperidine, nor	100	1.79	234.2	45	56	22	160.1	15	4.55	234.1489	160.1121	188.1070	131.0856	
Mephedrone	10	1.46	178.2	25	160.2	15	145.2	20	3.08	178.1227	160.1121	145.0886		
Meprobamate	100	1.93	219.2	42	158.2	16	97.2	12	4.82	219.1340	158.1176	97.1012		
Methadone	100	2.30	310.3	33	105.1	28	57.2	25	8.44	310.2166	265.1587	105.0335	219.1169	223.1118
Methamphetamine	100	1.34	150.2	27	91.2	17	119.2	11	2.47	150.1277	91.0543	119.0856		
Methapyrilene	-	1.82	262.3	25	97.1	35	217.3	20	4.94	262.1372	217.0794	97.0107	119.0604	
Methylone	10	1.25	208.2	30	160.2	20	132.3	30	2.04	208.0968	160.0757	147.0441	190.0834	

Table continues

Table I. Continued

Analyte	Positive threshold (ng/mL)	MS/MS screening parameters							MS^E/TOF confirmation parameters					
		Retention time (min)	Precursor ion (*m/z*)	CV (V)	Prod. 1 (*m/z*)	CE (eV)	Prod. 2 (*m/z*)	CE (eV)	Retention time (min)	Molecular ion (*m/z*)	Frag. 1 (*m/z*)	Frag. 2 (*m/z*)	Frag. 3 (*m/z*)	Frag. 4 (*m/z*)
Methyphenidate	100	1.72	234.2	30	84.2	18	56.2	40	3.96	234.1489	84.0808	174.1278	154.0414	
Midazolam	100	2.12	326.2	55	291.3	26	244.3	26	6.53	326.0855	291.1167	244.0324	209.0636	
Mitragynine	10	2.11	399.2	35	110.2	35	174.1	32	7	399.2278	367.2011	123.0804	285.1598	226.1438
Mitragynine, 7-OH	10	1.83	415.1	35	110.1	32	190.1	28	4.5	415.2227	397.2122	190.0863		
Morphine	100	0.81	286.4	50	44.1	31	165.3	40	0.98	286.1438	165.0699	201.0911	185.0598	229.0860
Morphine, 6-acetyl	10	1.41	328.1	45	58.2	35	165.1	35	2.1	328.1544	211.0754	165.0699	268.1333	193.0648
Naloxone	100	1.30	328.2	48	253.2	25	310.1	20	1.57	328.1544	310.1438	253.1098	268.1333	212.0707
Naltrexone	100	1.40	342.1	50	55.1	38	270.1	25	1.98	342.1700	324.1595	267.1254		
Oxazepam	100	2.43	287.1	39	241.2	23	269.1	13	7.8	287.0582	241.0528	269.0477	231.0684	
Oxycodone	100	1.40	316.3	40	241.3	30	212.3	40	2.08	316.1544	298.1438	256.1333	241.1098	
Oxycodone, nor	100	1.37	302.2	43	198.2	45	227.2	20	2.07	302.1387	284.1282	227.0941	187.0754	
Oxymorphone	100	0.96	302.2	43	227.2	31	198.2	45	1.02	302.1387	284.1282	227.0941	242.1176	
Oxymorphone, nor	100	0.68	288.1	42	184.1	42	213.1	30	0.94	287.1158	270.1125	228.1019	227.0703	254.1019
Phencyclidine	10	1.98	244.3	19	86.2	11	91.2	27	5.87	244.2060	159.1169	91.0543	86.0965	81.0699
Phentermine	100	1.43	150.1	18	91	18	133.1	10	2.82	150.1277	91.0543	133.1012		
Pregabalin	500	1.14	160.1	30	55.1	22	97.1	15	1.82	160.1332	142.1227	99.0441	126.0913	
PVP, alpha	10	1.65	232.2	45	91.1	22	105.1	25	4.32	232.1696	161.0961	105.0335	91.0544	126.1277
Ritalinic acid	100	1.60	220.2	28	84.2	20	56.2	38	2.72	219.1259	84.0808	174.1277	129.0699	133.1012
Tapentadol	100	1.73	222.2	40	107.2	25	121.2	20	4.02	221.1780	107.0492	121.0648	135.0805	
Temazepam	100	2.46	301.1	30	255.2	22	177.2	40	9.6	301.0739	255.0684	193.0886	228.0575	
Tramadol	100	1.70	264.3	31	58.1	21	–	–	3.9	264.1958	58.0652	246.1853		
Tramadol, *N*-desmethyl	100	1.73	250.2	30	44	15	–	–	4.14	250.1802	232.1703			
Triazolam	100	2.43	343.2	56	308.2	26	239.2	44	8.61	343.0512	308.0824	315.0325		
Zaleplon	100	2.31	306.2	55	236.2	28	264.2	20	7.02	306.1350	236.0931	264.1244	260.0931	
Zolpidem	100	1.87	308.2	45	92.1	48	235.2	35	4.8	308.1758	235.1230	263.1172	221.1074	

In UPLC–MS^E/TOF analysis, the molecular and fragment ions *m/z* are based upon elemental composition.

EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; MDA: 3,4-Methylene dioxy amphetamine; MDMA: 3,4-Methyl enedioxy methamphetamine; MDPV: Methylenedioxypyrovalerone; PVP, alpha: α-Pyrrolidinopentiophenone.

Matrix effect was determined by analysis of multi-sourced analyte-free urine samples fortified at 300% of analyte-specific threshold concentration. An aqueous control containing equivalent concentration of analytes was also prepared and tested by analysis for evaluation of matrix effect.

For UPLC-MS/MS analysis, mobile phase solvent A contained 2 mM ammonium formate in water with 0.1% formic acid and solvent B contained 2 mM ammonium formate in methanol with 0.1% formic acid. An initial-mobile phase reagent was prepared by mixing two parts of mobile phase B with 98 parts mobile phase A. For the UPLC–MS^E/TOF method, mobile phase A contained 5 mM ammonium formate pH 3.0 in water with 0.1% formic acid and mobile phase B was 0.1% formic acid in acetonitrile. An initial-mobile phase reagent containing 13 parts of mobile phase B and 87 parts of mobile phase A was prepared.

Analytical Methods

TAC technique

The TAC technique of sample preparation was used in both the UPLC–MS/MS and UPLC–MS^E/TOF methods to achieve matrix effect normalization and threshold accuracy for analytes. The technique was based upon a sample preparation and analysis principle described previously for either qualitative drug testing (7) or quantitative drug testing (6). The technique involves preparation of calibrator, QC and unknown samples "with" (fortified sample) and "without" (neat-sample) fortification reagent addition. Dual samples (50 µL aliquots) of calibrator, QC or unknown were added to paired analysis wells in a 96-well plate for neat-sample and fortified sample analyses. The fortification reagent (50 µL) was added to the fortified sample wells and an equal volume of a 3% methanol solution added to the neat well, followed by additions of the mixed recovery and hydrolysis reagent (50 µL) to all wells. The plate was covered, mixed by rotation on the benchtop and then incubated for 1-hr in a 55°C oven. After cooling, 500 µL of initial-mobile phase was added to all analysis wells to achieve a 13-fold dilution of the urine specimen. Following either UPLC–MS/MS or UPLC–MS^E/TOF analysis a TAC ratio was used to matrix-normalize the ion response and was calculated from the ion response data for the paired-sample analysis by the following formula: [(neat ion response)/(fortified ion response – neat ion response)].

The TAC technique utilizes a single calibrator to establish the positive threshold or cut-off for the assay. Thus, each analytical run includes a calibrator which is subject to the same dual analysis procedure, i.e., analyzed neat and following fortification. The TAC ratio for this calibrator is used as the reference and to establish if samples are above or below the positive threshold concentration, hence the TAC ratio for all samples are compared to the TAC ratio of the reference calibrator. Samples with a TAC ratio equal to, or greater than, the calibrator TAC ratio were positive for the analyte, subject to acceptable UPLC–MS/MS acquisition/integration criteria. In a sample without analyte, the ratio is very low with the calculation approaching zero. In samples with very high analyte concentration compared to threshold, the ratio may be orders higher than the calibrator ratio.

UPLC–MS/MS method

UPLC–MS/MS analysis of the diluted sample prepared by the TAC technique was performed using a Waters ACQUITY UPLC I-Class (FTN) system in combination with a Xevo® TQD tandem mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separation was achieved using an ACQUITY UPLC® BEH Phenyl column (1.7 µm, 2.1 mm × 50 mm; Waters) equipped with an ACQUITY UPLC® BEH Phenyl VanGuard™ pre-column (1.7 µm, 2.1 mm × 5

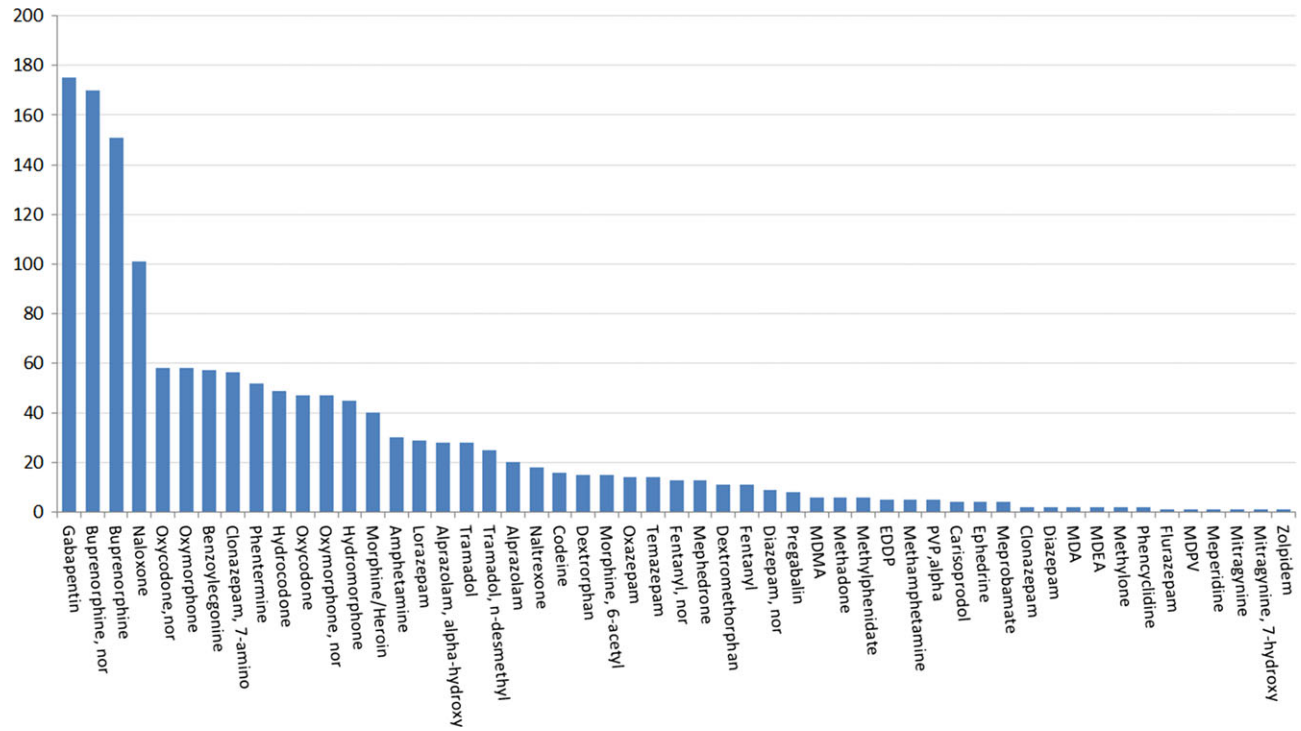

Figure 1. Frequency plot of positive drug and metabolite findings in 1,000 samples from court-related casework.

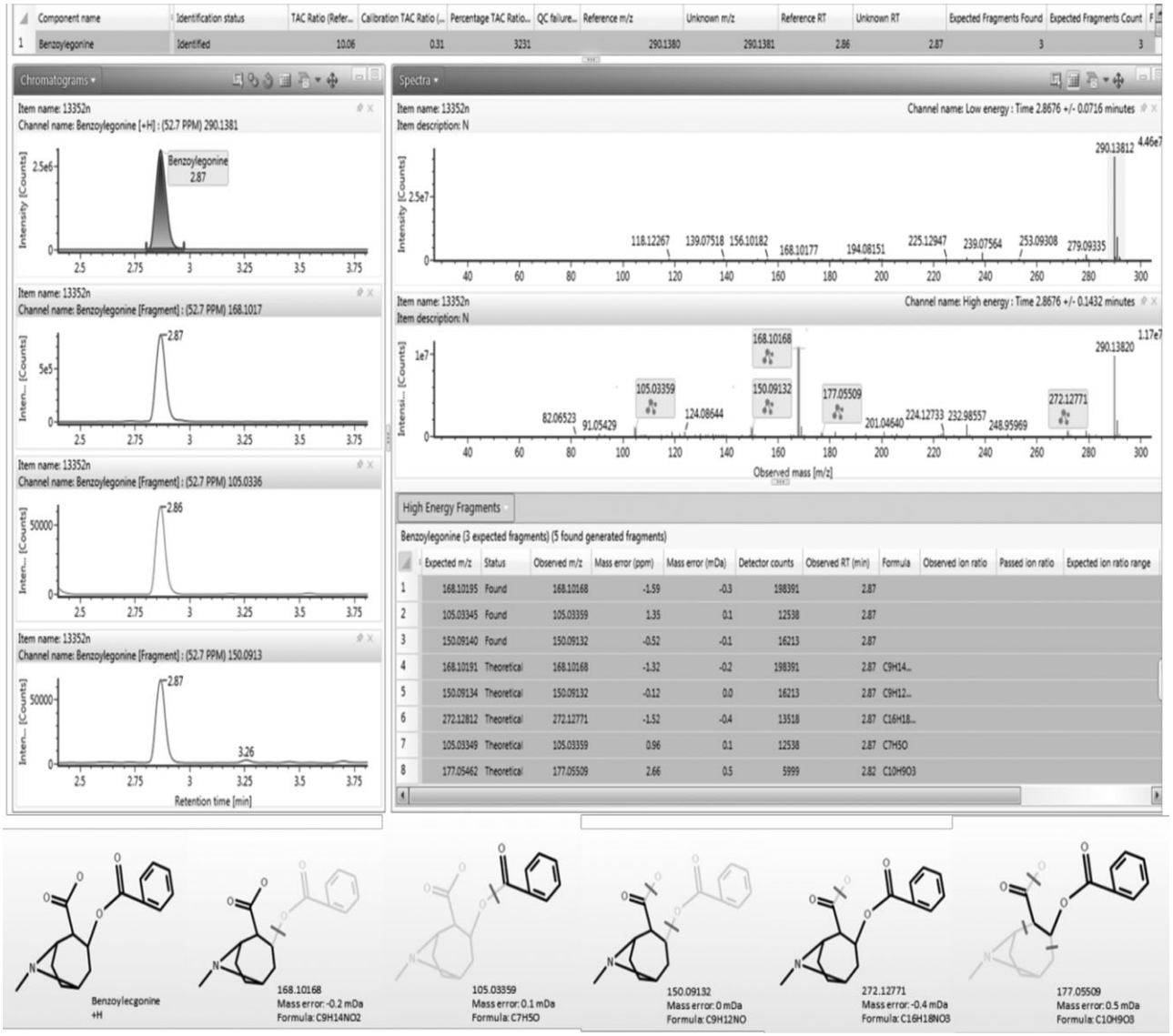

Figure 2. Representative UPLC–MS^E/TOF confirmatory data for a benzoylecgonine positive case. Case sample results include a display of comparative calibrator and case TAC ratios in the component summary (top), extracted ion chromatograms for molecular and fragment ions (left panel), and low and high energy mass spectra (right panel). The high energy fragments table lists both the found "expected" ions (i.e., matched to library) as well as *in silico* generated (theoretical) ions; their corresponding proposed structures are displayed along the bottom.

mm; Waters) and maintained at 45°C. The flow rate was 0.6 mL/min, and a gradient elution was applied as follows: 2% B (0–0.5 min), 2–70% B (0.5–2.2 min), 70–90% B (2.2–2.7 min) and 90%-2% B (2.7–3.0 min) with a 0.3 min hold. The injection wash solvent contained isopropanol:acetonitrile:water (1:1:1).

The mass spectrometer was operated in electrospray-positive ionization mode using the following MS/MS conditions: capillary voltage (0.55 kV), source temperature (150°C), desolvation gas (nitrogen delivered at a flow rate of 1,000 L/h and at a temperature of 550°C) and collision gas (argon) pressure of 0.5 Pa. Multiplereaction monitoring (MRM) was performed for the analytes and recovery standard by using two transitions, with the exception of tramadol and *N*-demethyl tramadol-desmethyl tramadol where only one transition was available. Cone voltage and collision energy were optimized to give the maximum response for each transition (Table I). Parameters were arranged into 64 individual acquisition windows as shown in Figure 1 with dwell times optimized to provide accurate profiling of the chromatographic peaks (typically >15 points/peak). Target transition–ion ratios (i.e., qualifier response/quantifier response) were determined from the average ratio obtained with the calibrator and QC analyses.

Data were processed initially using TargetLynx™ (Waters), which integrated peak-area responses for each MRM transition. Acceptability of acquisition/integration results was based on the following analytical criteria: analyte retention time to be within 0.05 min of predicted and transition–ion ratios to be within 20% of target for ratio ≥0.50, within 25% of target for ratio <0.5–0.20, within 30% of target for ratio <0.20–0.10 and within 50% of target for ratio <0.10. For paired-sample injection of neat and fortified samples, verification of ±20% tolerance for injection volume

variability was monitored by analysis of ion area responses with the recovery standard methapyriline. Additional data handling for TAC calculations was achieved by export of the TargetLynx summary data into Microsoft Excel, which used quantifier ion response to calculate TAC ratio.

UPLC–MS^E/TOF method

Sample preparation was performed as described for the UPLC–MS/MS method using an additional urine sample aliquot obtained from the primary specimen container. Chromatographic separation was performed using a Waters ACQUITY UPLC I-Class with chromatography on an ACQUITY HSS C18 analytical column (1.7 mm, 2.1 mm × 150 mm) maintained at 50°C. Analytes were separated in an analytical run time of 15 min by gradient elution, using a mixture of mobile phase A (5 mM ammonium formate pH 3.0 in water with 0.1% formic acid) and mobile phase B (0.1% formic acid in acetonitrile). The chromatographic system was interfaced with a G2-XS QTOF mass spectrometer (Waters) using electrospray in positive mode with MS^E acquisition performed at both low (6 eV) and ramped (10–40 eV) collision energies (36). The molecular and fragment ions for all targeted analytes are listed in Table I. Acquisition data were processed using the UNIFI™ software with custom calculations for TAC analysis. Analytical and threshold criteria for a positive confirmation included acceptable quality control, retention time, injection recovery performance, detection of analyte molecular ion (within 5 ppm) and at least one fragment ion (within 5 ppm), and a TAC ratio (based on response of molecular ion) equal to or exceeding the working calibrator TAC ratio.

Method validation plan

The method validation plan was based upon Clinical Laboratory Standards provided by New York State's Department of Health (37). Method accuracy and precision were determined by at least 10 quality control analyses, with control preparation and analysis on separate days and with control concentrations at 40, 75, 125 and 500% of threshold concentration. The percent matrix effect was determined in aqueous versus urine based control samples at 300% of threshold. Matrix effect was calculated using the following formulae: [(B/A – 1) × 100%] where A represents the ion response without urine matrix present and B represents the ion response in urine matrix. A wide range of matrix effects was anticipated with a dilute-and-inject method of sample preparation and the observed matrix affect was determined as acceptable if all analytical validation criteria were met. Analyte-free urine from multiple donor collections (n = 10) were also analyzed to evaluate the presence of any co-eluting interferents in the UPLC–MS/MS method. Hydrolysis was performed according to previously evaluated and optimized conditions of 1-hr incubation at 55°C (7). For each analytical run the efficiency of this step was verified by inclusion of a glucuronide-conjugated analyte (M3G) at morphine-equivalent concentrations of 400 ng/mL, using a ±20% acceptance criteria for the measured morphine-equivalent concentration. Assay limit of detection (LOD) is defined as 40% of threshold with assessment on each analysis run with the low-level quality control sample analysis. At least ten analytical runs were used to demonstrate that all detection and identification criteria were met at the LOD.

The range and frequency of drugs detected by the definitive screening method was evaluated by statistical analysis for 1,000 de-identified screening results obtained from probation, drug court, social services, chemical dependency, pain management and addiction medicine casework. Qualitative accuracy of the methods was also assessed by analysis of both proficiency testing specimens (Drug Monitoring for Pain Management Survey, College of American Pathologist, Northfield, IL) and de-identified positive case specimens using confirmation testing by comparison between the UPLC–MS/MS and UPLC–MS^E/TOF methods. Specimen and data handling for de-identified positive and analyte-free case specimens was based on an Albany Medical Center IRB reviewed protocol (# 4454).

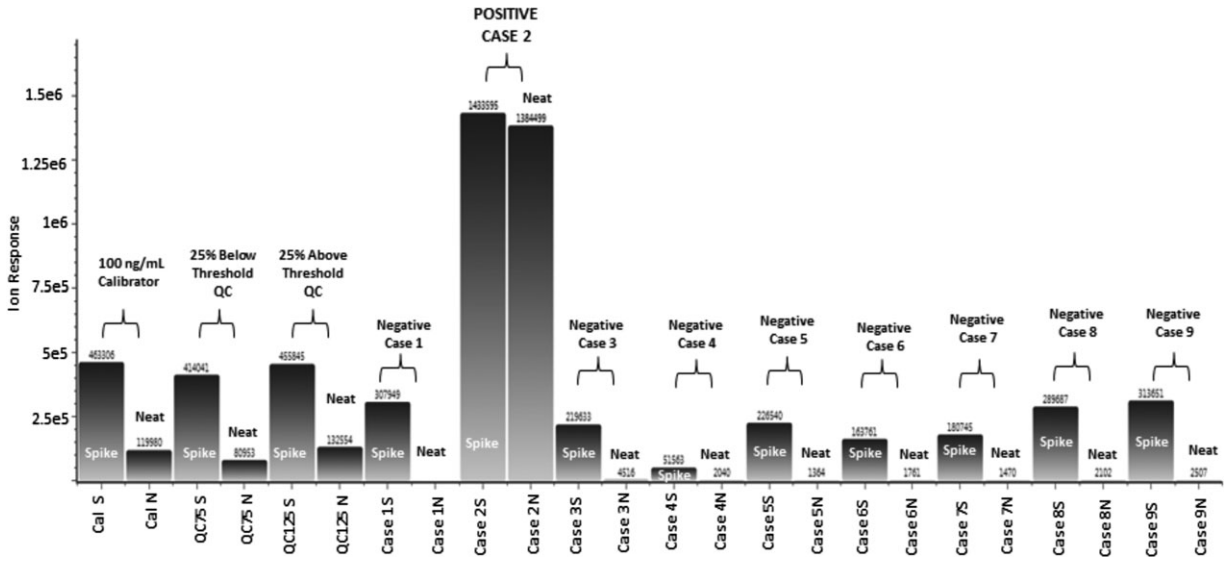

Figure 3. Representative cross-sample summary view of ion response for calibrator, quality control, and nine case samples for benzoylecgonine analysis by UPLC–MS^E/TOF. Sequential analysis of fortified and neat samples from each test sample is shown with an example of above threshold detection of benzoylecgonine in a case sample.

Table II. Validation data for UPLC–MS/MS and UPLC–MS^E/TOF method precision and matrix effects

Analyte	UPLC–MS/MS			UPLC–MS^E/TOF		
	Precision, ng/mL (CV)		Matrix effect	Precision, ng/mL (CV)		Matrix effect
	QC 75%	QC 125%	% (SD)	QC 75%	QC 125%	% (SD)
Alprazolam	74 (7)	124 (9)	5 (6)	72 (13)	117 (8)	13 (4)
Alprazolam, α-hydroxy	75 (19)	143 (16)	27 (23)	79 (12)	116 (7)	35 (3)
Amphetamine	73 (7)	132 (7)	3 (13)	79 (10)	113 (17)	−9.6 (3)
Benzoylecgonine	74 (5)	129 (5)	−3 (6)	76 (9)	113 (9)	5.9 (4)
Buprenorphine	6.1 (19)	12.7 (21)	−19 (10)	7.8 (9)	11.2 (6)	16 (13)
Buprenorphine, nor	7.9 (11)	13.3 (11)	0 (8)	8.5 (15)	11.2 (8)	16 (8)
Carisoprodol	73 (4)	129 (6)	2 (5)	86 (12)	123 (11)	16 (4)
Clonazepam	77 (14)	128 (11)	−44 (11)	78 (15)	117 (9)	−28 (8)
Clonazepam, 7-amino	74 (8)	128 (7)	13 (12)	81 (9)	119 (6)	37 (16)
Codeine	75 (15)	132 (14)	−6 (10)	75 (11)	114 (13)	6.2 (2)
Dextromethorphan	74 (4)	130 (5)	−11 (6)	74 (12)	111 (14)	1.9 (2)
Dextrorphan/Levorphanol	75 (5)	131 (4)	−10 (6)	76 (10)	114 (11)	1.8 (2)
Diazepam	74 (6)	127 (6)	−4 (10)	75 (12)	117 (10)	4.7 (3)
Diazepam, nor	73 (6)	126 (7)	8 (9)	72 (11)	111 (13)	7.7 (7)
EDDP	74 (5)	129 (7)	−10 (6)	77 (10)	113 (12)	3.3 (1)
Ephedrine	74 (6)	128 (5)	−15 (5)	81 (14)	114 (10)	−4.6 (2)
Ethylone	7.7 (12)	13.1 (13)	−16 (8)	8.8 (2)	10.7 (1)	−19 (8)
Fentanyl	7.2 (11)	12.5 (11)	−9 (6)	7.9 (12)	11.6 (8)	−1.2 (3)
Fentanyl, nor	7.4 (7)	12.9 (8)	−8 (6)	8.1 (9)	12.0 (5)	14 (5)
Flurazepam	73 (5)	126 (5)	−2 (5)	75 (10)	112 (12)	5.5 (3)
Flurazepam, hydroxy ethyl	71 (7)	125 (8)	14 (8)	77 (13)	115 (14)	14 (3)
Gabapentin	720 (14)	1,280 (18)	1 (12)	790 (8)	1,120 (8)	9.7 (3)
Hydrocodone	75 (10)	131 (9)	−24 (11)	71 (18)	112 (16)	−12 (8)
Hydromorphone	74 (6)	130 (10)	−8 (10)	90 (14)	111 (12)	2.2 (11)
Ketamine	7.6 (10)	12.5 (12)	−10 (5)	7.8 (7)	12.8 (14)	2.2 (7)
Ketamine, nor	7.4 (13)	13.1 (11)	−2 (25)	8.4 (13)	12.1 (11)	16 (4)
Lorazepam	74 (11)	129 (14)	12 (10)	78 (12)	116 (11)	13 (5)
MDA	73 (7)	131 (10)	−20 (7)	86 (12)	113 (9)	−2.1 (3)
MDEA	75 (5)	129 (6)	−23 (11)	68 (8)	123 (10)	1.6 (3)
MDMA	74 (6)	128 (7)	−30 (9)	75 (6)	122 (9)	1.7 (2)
MDPV	7.6 (17)	13.3 (13)	−12 (7)	8.4 (15)	11.5 (8)	−3.5 (2.9)
Meperidine	73 (6)	128 (6)	−10 (5)	74 (12)	112 (15)	2.3 (1)
Meperidine, nor	74 (11)	130 (11)	−7 (5)	82 (6)	128 (19)	14 (8)
Mephedrone	7.8 (13)	13.6 (19)	−34 (9)	7.5 (15)	12.1 (9)	−20 (7)
Meprobamate	74 (9)	127 (8)	−6 (8)	82 (8)	121 (7)	6.1 (1)
Methadone	73 (5)	128 (6)	−11 (6)	73 (16)	111 (17)	18 (22)
Methamphetamine	75 (5)	130 (7)	−4 (10)	83 (7)	118 (4)	−4.1 (2)
Methapyrilene	–	–	−1 (23)	–	–	−2.1 (6)
Methylone	7.7 (13)	13.3 (12)	−19 (7)	7.5 (6)	12.3 (18)	−15 (6)
Methyphenidate	73 (5)	126 (7)	−35 (9)	76 (11)	112 (13)	−16 (3)
Midazolam	72 (6)	125 (7)	−8 (7)	77 (12)	113 (11)	8.3 (2)
Mitragynine	7.4 (15)	13.2 (13)	9 (25)	7.8 (6)	11.1 (6)	−14 (3)
Mitragynine, 7-hydroxy	7.2 (20)	13.5 (17)	−20 (11)	8.2 (11)	11.2 (7)	−26 (7)
Morphine	75 (12)	130 (10)	−9 (8)	78 (17)	119 (7)	−37 (15)
Morphine, 6-acetyl	7.7 (18)	14.2 (21)	−34 (13)	8.6 (15)	12.4 (8)	6.1 (1)
Naloxone	71 (5)	130 (10)	−1 (8)	84 (15)	118 (8)	11 (4)
Naltrexone	74 (9)	127 (9)	−25 (14)	82 (10)	117 (7)	11 (4)
Oxazepam	74 (14)	129 (14)	20 (18)	80 (8)	116 (9)	12 (6)
Oxycodone	76 (15)	129 (16)	−26 (14)	81 (13)	119 (16)	4.1 (1)
Oxycodone, nor	72 (13)	134 (15)	−10 (9)	86 (11)	121 (6)	−6.9 (2)
Oxymorphone	74 (7)	132 (8)	−15 (10)	85 (9)	118 (9)	11 (19)
Oxymorphone, nor	72 (10)	129 (13)	−24 (16)	90 (19)	112 (8)	−26.1 (28)
Phencyclidine	7.3 (9)	12.8 (10)	−27 (8)	8.4 (9)	11.9 (13)	−22 (8)
Phentermine	74 (8)	130 (6)	−38 (10)	76 (13)	112 (14)	−4.8 (3)
Pregabalin	370 (5)	655 (6)	−3 (14)	420 (13)	595 (5)	22 (4)
PVP, alpha	7.5 (5)	13.0 (6)	28 (23)	8.8 (15)	11.9 (6)	−16 (2)
Ritalinic acid	74 (6)	130 (5)	58 (27)	79 (14)	115 (13)	34 (3)
Tapentadol	72 (8)	130 (9)	−8 (9)	76 (11)	115 (12)	3.3 (2)

Table continues

Table II. Continued

Analyte	UPLC–MS/MS			UPLC–MS^E/TOF		
	Precision, ng/mL (CV)		Matrix effect	Precision, ng/mL (CV)		Matrix effect
	QC 75%	QC 125%	% (SD)	QC 75%	QC 125%	% (SD)
Temazepam	76 (5)	126 (5)	10 (8)	80 (17)	108 (17)	9.6 (5)
Tramadol	74 (6)	129 (8)	−2 (6)	79 (11)	117 (11)	3.5 (1)
Tramadol, *N*-desmethyl	74 (5)	129 (5)	−4 (5)	78 (10)	113 (12)	6.2 (1)
Triazolam	76 (10)	129 (9)	10 (6)	75 (10)	111 (13)	14 (3)
Zaleplon	74 (7)	129 (6)	1 (6)	78 (5)	117 (8)	17 (4)
Zolpidem	73 (4)	127 (5)	−2 (4)	80 (11)	119 (8)	7.7 (4)

The precision is based upon quality control data for 75 and 125% of threshold concentration in 24 UPLC–MS/MS and 10 UPLC–MS^E/TOF analytical runs. Matrix effect (mean and SD) was determined in 26 UPLC–MS/MS and three UPLC–MS^E/TOF studies in which analyte-free urine (A) and water (B) were both fortified at 300% threshold. Matrix effect (%) was calculated from molecular ion response by the formula $(A/B - 1) \times 100$.

Results and Discussion

The UPLC–MS/MS screening method is a modification of a previously reported method (7). Modifications include expansion of the targeted analytes and use of centrifugation with a higher sample dilution in place of a sample filtration method. The frequency of positive drug finding in 1,000 court-related and clinical cases, using the modified screening method, is displayed in Figure 1. Positive analyte findings totaled 1,486 with positive cases for 53 of the targeted analytes. The most frequent finding was gabapentin which was not previously included in our urine drug screening program. Additional new analytes found frequently include naloxone resulting from formulations of buprenorphine therapy and the nor metabolites of oxycodone and oxymorphone. Additional findings resulting from the expanded screening panel included positives for fentanyl, pregabalin, methylphenidate, dextromethorphan, phentermine, naltrexone, carisoprodol, meprobamate and mitragynine. In forensic urine casework, the expanded frequency and diversity of positive analyte findings have been a workload challenge with the traditional confirmatory approach based on individual drug-class methods. A single complementary confirmation method was therefore developed based upon modification of a UPLC–MS^E/TOF method previously reported for postmortem blood analysis (38). Modifications for the confirmatory method included use of a targeted-analyte search and report routine. Additionally, the MS^E/TOF analysis was programed to identify and report only the analytes in the screening panel, rather than the complete toxicology library comprising >1,300 substances.

Both screening and confirmation methods employ the TAC technique for sample preparation. It is well-documented that matrix effect can lead to variable ion responses when using methods based on LC–MS technology. In our report, this effect of urine matrix is evident in Figure 3 where we observe a variable response for negative case samples that have been subsequently fortified with an identical amount of benzoylecgonine. The figure clearly shows the inter-case difference in ion response. To address this matrix effect, it is common for stable isotope internal standards (SI-IS) to be used to compensate for this matrix phenomenon and to ensure accurate quantitation. Previously we have reported an alternative novel approach that does not require SI-IS (6, 7). The method relies upon the analysis of the sample twice, once neat and once after fortification with a known amount of analyte. The TAC ratio is calculated to provide a matrix-normalized ion response comparison of analyte present in the neat-sample to analyte added by fortification. The TAC technique allowed threshold accurate detection of analytes in both the screening and confirmation methods without the use of SI-IS.

Chromatographic separation conditions for UPLC–MS/MS and UPLC–MS^E/TOF analysis were also optimized for separation of the analytes and for differential analyte selectivity. Table I lists the retention time of analytes for the two chromatographic methods and contrasts the chromatographic separation using BEH phenyl and HSS C18 conditions in the screening and confirmation methods, respectively. Differential retention times between screening and confirmation chromatography was achieved for all analytes due largely to the different total chromatographic gradient run time of 3.3 min for screening and 15 min for confirmation. In addition, selectivity of the BEH phenyl and HSS C18 column phases resulted in significant reversals in retention-order among many of the analytes. The BEH phenyl column separation resulted in a relative enhanced retention for opiates including codeine, oxycodone, hydrocodone, 6-acetylmorphine naloxone and naltrexone. Other more retentive analytes on the BEH phenyl phase were benzoylecgonine and several benzodiazepines (oxazepam, midazolam, 7-aminoclonazepam, alprazolam). The HSS C18 was relatively more retentive for phenethylamines (amphetamine, methamphetamine, MDA, MDMA mephedrone) as well as for alpha PVP, carisoprodol, methadone and EDDP. The chromatographic columns and conditions for the screening and confirmation methods were determined to be sufficiently different in retention time and selectivity.

The application of dual definitive methods allows sensitive and selective analyte identification as well as confirmation in forensic casework. As shown in Table I, the same positive threshold criteria were used in both screening and confirmation testing. Table I also shows however that the method of analyte detection differs between the two analytical methods with the use of transition–ion analysis at nominal mass accuracy in initial screening and HRMS with a 5 ppm ion mass identification limit for the molecular species and up to four fragment ions in the confirmation analysis. The extent of molecular data available in the HRMS confirmatory method is further shown in a benzoylecgonine confirmation analysis (Figure 2). Extracted exact mass chromatograms show the identification of the molecular species (*m/z* 290.1387) at both low and ramped collision energies along with three fragment ions (*m/z* 168.1020, 105.0335, 150.0914) identified in the ramped collision energy spectrum. Automated theoretical fragmentation analysis (*in silico*) identified two additional fragment ions (*m/z* 272.1218 and 177.0546). The proposed structure and elemental composition of the molecular species and fragment ions are shown at the bottom of the figure. Similar molecular detail is available for each confirmed analyte, adding further certainty to the confirmation analysis.

Table III. Concordance of drug findings between UPLC–MS/MS and UPLC–MS^E/TOF in 114 court-related cases

Analyte	UPLC–MS/MS Screen Positive	UPLC–MS^E/TOF Confirmed Positive
Buprenorphine, nor	49	49
Buprenorphine	47	47
Gabapentin	44	44
Naloxone	35	35
Amphetamine	26	26
Benzoylecgonine	16	16
Tramadol	15	15
Tramadol, *N*-desmethyl	15	15
Morphine	15	15
Pregabalin	14	14
Hydromorphone	12	12
Hydrocodone	10	10
Oxycodone	10	10
Oxymorphone	9	9
Oxycodone, nor	9	9
Ritalinic acid	8	8
Codeine	8	8
Fentanyl, nor	8	8
Morphine, 6-acetyl	7	7
Fentanyl	6	6
Methylphenidate	5	5
Clonazepam, 7-amino	5	5
Oxymorphone, nor	4	4
Lorazepam	3	3
Dextrorphan/levorphanol	2	2
Detromethorphan	1	1
Diazepam, nor	1	1
Temazepam	1	1
Alprazolam, alpha hydroxy	1	1
Alprazolam	1	1
MDPV	1	1
Mephedrone	1	1
Phencyclidine	1	1
Naltrexone	1	1

In both methods, the normalization for matrix affect was achieved with the use of the TAC technique, which involved the analysis of each urine specimen with and without the addition of fortification reagent. Figure 3 shows the ion response for fortified and neat benzoylecgonine analysis of a calibrator with a threshold concentration of the analyte followed by similar analysis of above and below-threshold quality control as well as case samples. The variation in fortification analyte response in the cases without any ion response for benzoylecgonine in the neat analysis demonstrated the variable matrix effect between samples. The variability is consistent with a prior report by the authors in which intra-analyte variation in matrix effect was correlated with urine concentration assessed by either creatinine or specific gravity measurement (7). The TAC approach ensures matrix effect is normalized in analyte-positive cases by equivalent matrix effect in both the neat and fortified analyses.

Matrix effect was evaluated for the screening and confirmatory methods which both employ a simple hydrolysis and dilution preparation of urine samples. As shown in Table II, matrix effect was less than 20% for most analytes analyzed by the confirmatory method employing a 15 min chromatographic separation. Greater than 20% ion suppression was observed for clonazepam, noroxymorphone and ion enhancement was greater than 20% for morphine alpha-hydroxyalprazolam and ritalinic acid. The screening method tended to show a greater degree of ion suppression possibly due to the shortened retention times.

Threshold accuracy of the TAC technique for both methods was assessed, and Table II summarizes both accuracy and precision for analysis of quality control samples with multi-analyte concentrations prepared at 75 and 125% of threshold concentration. The 25% below threshold control resulted in an average of 74% (range 61–79) and 79% (range 68–90) of threshold for the screening and confirmatory methods, respectively. Coefficient of variation averaged 9.0% (range 4–20) and 11% (range 2–19) for screening and confirmation. The 25% above threshold control data averaged 130% (range 124–143) and 116% (range 107–128) of threshold for the screening and confirmatory methods with respective coefficient of variation averaging 10% (range 4–21) and 10% (range 1–19). Qualitative accuracy in the identification of above and below threshold concentration of analytes was achieved for all analytes in both methods.

Positive analyte findings by the UPLC–MS/MS screening and UPLC–MS^E/TOF confirmatory methods were also compared by co-analysis of 114 de-identified urine samples from court-related casework. A total of 391 concordant screening and confirmation positive findings were determined in court-related cases as shown in Table III, and no discrepant findings were determined. The analytes identified as positive in this cohort of case samples included over 50% of the analytes testing in the test panel. There were a total of 6,791 analyte-negative findings in the cohort, also with complete concordance between the screening and confirmatory methods. The high degree of selectivity and specificity of both the screening and confirmation method was evidenced by these concordant findings.

The accuracy of the screening and confirmation methods was further evaluated by both proficiency testing challenges and comparison with quantitative confirmation methods. Co-analysis of six specimens tested from the College of American Pathologists' 2017 testing program for Drug Monitoring and Pain Management also showed 100% qualitative identification performance and no false positives for the proficiency testing samples. Quantitative confirmation testing for 66 UPLC–MS/MS and UPLC–MS^E/TOF positive analytes was performed by referral of 23 specimens to MedTox Laboratories. The 66 positive analyte findings by qualitative UPLC–MS/MS screening and UPLC–MS^E/TOF confirmation showed 100% concordance with reference laboratory analysis with concentrations above the qualitative positive threshold. Analyte concentrations in this study varied widely and included concentration near threshold level.

Conclusions

Definitive urine drug screening by UPLC–MS/MS with an expanded panel of analytes has resulted in a significant increase in the volume and diversity of analyte confirmation testing in forensic casework. A single UPLC–MS^E/TOF method for analyte confirmation has been developed and validated for use in a forensically defensible testing. Differences in chromatographic selectivity and mass spectrometry analysis in the screening and confirmatory methods have been employed to further reduce uncertainty in the detection of drug use. The methods employ a novel technique for matrix normalization in a dilute-and-shoot sample preparation and are applicable to forensic as well as clinical casework.

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