Journal of Analytical Toxicology, 2017;41:536–546
doi: 10.1093/jat/bkx035
Advance Access Publication Date: 24 May 2017
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Screening with Quantification for 64 Drugs and Metabolites in Human Urine using UPLC–MS-MS Analysis and a Threshold Accurate Calibration

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Abstract

Drug and metabolite (analytes) identification together with quantification is an important analytical tool in forensic and clinical toxicology. We report the development and validation of a definitive detection and quantification method (UPLC–MS-MS) for initial screening of 64 analytes in urine. The principle of the method is a quantitative extension of a recently reported threshold accurate calibration (TAC) technique which employs a rapid dual-specimen analysis i.e., with and without addition of a reference-analyte standard for normalization of matrix effects. Analytes include pharmaceutical and illicit agents from opiate and opioid agonist, opiate-antagonist, benzodiazepine, amphetamine, designer amphetamine, cathinone, cocaine, hallucinogen, gabapentinoid and sedative drug classes. Using a 96-well plate format, the protocol employs glucuronidase hydrolysis, 27-fold urine dilution and a 3 min UPLC–MS-MS acquisition. Subsequent data management includes calculation of a normalized TAC ratio response and weighted least squares calibration. The method utilizes analyte-specific calibration ranges from 2.5 to 1,500 ng/mL with quality control (QC) monitoring of transition-ion ratio, calibrator re-analysis, injection precision and multi-level QC analysis. Method precision, bias, calibration linearity, detection limit, carryover, crossover studies and external proficiency performance were evaluated based on pre-established criteria. The validated method provides an alternative to stable-isotope internal standardization methods of quantification and is applicable to screening with quantification in routine toxicology practice.

Introduction

In the fields of forensic and clinical toxicology the trend continues toward replacement of presumptive immunoassay screening of drug classes with definitive screening by liquid chromatography–mass spectrometry technology (LC–MS). Advances in LC-MS technology along with simplification and speed of sample preparation and analysis contribute to the increasing us of definitive methods in initial drug testing. An important indicator of this evolving trend is the proliferation of multi-analyte definitive methods (1–19) resulting from method development and validation work being conducted worldwide. In the application of definitive methods of urine drug screening, experience to date has shown a reduction in false negative results by definitive screening, due in part to lower positive thresholds in definitive compared to immunoassay methods (20, 21). Multi-analyte definitive screening also allows selective detection of pharmaceutical and illicit agents across many drug classes (8–10, 15, 16, 22–33) while immunoassay methods provide only presumptive identification of a drug or more often a drug class (34–36). Improved specificity of MS detection has allowed for a simplification of sample preparation and rapid chromatographic separation has

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improved workflow in definitive testing as reported in numerous dilute-and-inject procedures (8, 9, 18, 19, 33, 37, 38). The extent of definitive screening adoption in current practice varies from a well-established use in medical examiner toxicology (23–26, 28, 39) and anti-doping screening (18, 40, 41) to a growing use in pain management compliance and abuse surveillance (42–45).

Recent work from our group has shown the importance of matrix normalization in definitive methods of multi-analyte screening in urine (1). Our results showed that matrix effects from co-eluting agents may vary significantly from one urine sample to the next and may result in varying degrees of ion suppression or enhancement depending upon the analyte. Analysis of 42 analyte-negative urine samples supplemented with 33 common analytes at an equivalent concentration demonstrated this sample-dependent variability in matrix effect (1). Normalization of matrix effect by use of stable-isotope internal standardization (SIIS) is a widely used technique in quantitative drug testing and use of analyte-specific SIIS is considered best practice. Analyte-specific SIIS is not, however, used widely in large analyte panel screening by definitive methods, due in part to SIIS cost and availability, especially for newer psychoactive substances and metabolites. We have, therefore, developed an alternate approach to analyte-specific SIIS called threshold accurate calibration (TAC) technique. The fundamental principle underlying the TAC technique is the use of the analyte as a matrix-normalizing internal standard in a dual analysis system i.e., first analysis without and second analysis with the addition of a defined/known concentration of reference analyte. Definitive testing with the TAC technique has been validated for accurate threshold detection of analytes in the semi-quantitative screening mode and is currently used in our routine clinical and forensic toxicology practice.

While the majority of multi-analyte screening methods employing LC–MS technology are semi-quantitative, drug and metabolite quantification is an additional analytical tool that may needed in forensic and clinical toxicology casework. Only a limited number of quantitative multi-analyte screening methods by definitive technology have been reported (33, 46–48). These methods employ the SIIS technique for matrix normalization but the number of analytes with analyte-specific SIIS varies from only 26–74%. The aim of this analytical work is to extend the application of the TAC principle in the quantitative analysis of drugs in urine and to evaluate the accuracy of TAC-based quantitation by both external proficiency challenges and comparison of results with established methods of drug quantitation. The following is a report of our method development, optimization and validation for simultaneous identification and quantification of 64 analytes.

Materials and Methods

Chemicals, supplies and reference material

American Chemical Society grade methanol, HPLC grade acetonitrile and isopropanol were purchased from Fisher Scientific (Fairlawn, NJ). Reagent grade ammonium formate (97%) and formic acid (>95%) were obtained from Sigma-Aldrich (St Louis, MO) and methapyrilene from Supelco Analytical (Bellefonte, PA). Morphine-3-beta-D-glucuronide (M3G) was purchased from Cerilliant (Round Rock, TX), methapyrilene HCl from Supelco Analytical (Bellefonte, PA) and the analytes listed in Table I were obtained from either Cerilliant and/or Grace Alltech® (Deerfield, IL). Purified beta glucuronidase (IMCSzyme, activity ≥50,000 U/mL) and beta glucuronidase buffer (IMCSzyme) was obtained from and certified by Integrated Micro-Chromatography Systems (Columbia, SC).

Sample collection plates (96 well, 2 mL-square well) were obtained from Waters Corporation (Milford, MA). Analyte-negative urine is used for preparation of calibrator and quality control (QC) samples were obtained from laboratory volunteers and the absence of detectable analytes was verified by TAC screening analysis (1). Deionized water was produced by combined ultra-filtration (AquaPure, Schenectady, NY) and reverse osmosis (Alpha Water Systems, Montague, NJ) treatment.

Reagents and controls

Two sources of stock multi-analyte reference solutions were prepared in methanol from separate sources of reference material and stored at −10°C for use in the preparation of calibrator and control material as well as reference-analyte spiking reagent. Stock concentrations were prepared at 1.0, 2.5 and 10 µg/mL concentrations, respectively, for analytes with analyte-specific of calibration ranges of low (5–150 ng/mL), medium (20–375 ng/mL) and high (50–1,500 ng/mL), as specified in Table II. Multi-analyte calibrators were prepared on the day of analysis by dilution of stock reference material with analyte-negative urine at the analyte-specific calibrator sets for the low (2.5, 5, 20, 50, 100 and 150 ng/mL), medium (6.25, 12.5, 50, 125, 250 and 375 ng/mL) and high (25, 50, 200, 500, 1,000, 1,500 ng/mL) calibration ranges. QC samples were prepared on the day of analysis. QC preparation involves dilution of the stock multi-analyte reference solution with analyte-negative urine to achieve three concentration levels (I, II and III) for the low (4, 12, 120 ng/mL), medium (10, 30 and 300 ng/mL) and high (40, 120, 1,200 ng/mL) calibration-range analytes.

Hydrolysis control was prepared on the day of analysis by dilution of the stock hydrolysis control containing M3G with analyte-negative urine to achieve at a free-morphine concentration equivalence of 400 ng/mL. On the day of analysis a multi-analyte spike reagent was prepared by dilution of stock reference solution in water to concentrations of 200, 500 and 2,000 ng/mL of reference analyte for low-, medium- and high-calibration range analytes. As an injection recovery standard a stock solution of methapyrilene was prepared in methanol at 10 µg/mL and stored at −10°C. On the day of analysis a working recovery reagent containing 200 ng/mL of methapylilene 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 prepared of a mixed recovery and hydrolysis reagent. A stock solution for hydrolysis control containing 10 µg/mL of M3G was prepared in methanol and stored at −10°C.

Matrix effect was determined by neat analysis of multi-sourced negative urine samples supplemented at three concentrations levels (A, B and C) for low (20, 50 and 100 ng/mL), medium (50, 125 and 250 ng/mL) and high (200, 500 and 1,000 ng/mL) calibration-range analytes. Aqueous controls containing equivalent concentration of reference analytes was also prepared and tested by neat analysis.

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. A starting mobile phase reagent was prepared by mixing two parts mobile phase B with 98 parts mobile phase A.

Analytical method

Technique principle

A novel technique (TAC) is used to achieve matrix effect normalization and linear-range accuracy for the 64 analytes, based upon a

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Table I. Analyte MS-MS acquisition conditions

Analyte	Precursor Ion (*m/z*)	CV (V)	Product 1 (*m/z*)	CE (V)	Product 2 (*m/z*)	CE (V)
Alprazolam	309.1	50	281.2	27	205.2	42
Alprazolam, alpha-hydroxy	325.2	51	216.2	41	297.2	23
Amphetamine	136.1	20	91.2	15	119.1	8
Benzoylecgonine	290.2	39	168.1	19	105.1	30
Buprenorphine	468.4	60	55.1	70	84.2	70
Buprenorphine, nor	414.2	70	57.1	43	101.1	40
Carisoprodol	261.2	18	176.2	9	55.2	29
Clonazepam	316.1	50	270.2	25	214.3	35
Clonazepam, 7-amino	286.2	32	121.2	28	222.2	22
Codeine	300.1	50	165.3	35	153.2	35
Dextromethorphan	272.2	50	147.1	30	171.1	40
Dextrorphan	258.2	65	133.1	35	157.1	40
Diazepam	285.1	55	154.2	29	193.2	29
Diazepam, nor	271.1	55	140.1	29	165.1	27
Ethyliden-1,5-dimethyl-3,3-diphenylpyrrolidine	278.3	47	186.2	33	249.2	23
Ephedrine	166.1	25	133.1	20	148.1	12
Ethylone	222.1	40	107.2	25	121.2	20
Fentanyl	337.2	18	105.2	38	188.2	22
Fentanyl, nor	233.2	38	84.2	18	56.2	25
Flurazepam	388.2	45	315.2	21	100.2	29
Flurazepam, hydroxyethyl	333.1	45	109.1	28	194.1	22
Gabapentin	172.1	32	55.1	25	137.1	15
Hydrocodone	300.4	60	199.2	35	128.1	55
Hydromorphone	286.4	60	185.3	30	157.2	40
Ketamine	238.1	35	125.0	25	179.0	18
Ketamine, nor	224.1	25	125.0	25	207.0	12
Lorazepam	321.1	41	275.2	21	303.2	21
3,4-Methyleneamphetamine	180.4	20	163.1	15	105.1	20
3,4-Methylenedioxy-*N*-ethylamphetamine	208.3	30	163.2	13	105.2	25
3,4-Methylenedioxymethamphetamine	194.2	27	163.2	13	105.2	25
Methylenedioxypyrovalerone	276.2	35	175.2	20	135.2	30
Meperidine	248.2	15	174.2	18	220.2	22
Meperidine, nor	234.2	45	56.0	22	160.1	15
Mephedrone	178.2	25	160.2	15	145.2	20
Meprobamate	219.2	42	158.2	16	97.2	12
Methadone	310.3	33	105.1	28	57.2	25
Methamphetamine	150.2	27	91.2	17	119.2	11
Methapyrilene	262.3	25	97.1	35	217.3	20
Methylone	208.2	30	160.2	20	132.3	30
Methyphenidate	234.2	30	84.2	18	56.2	40
Midazolam	326.2	55	291.3	26	244.3	26
Mitragynine	399.2	35	110.2	35	174.1	32
Mitragynine, 7-hydroxy	415.1	35	110.1	32	190.1	28
Morphine	286.4	50	44.1	31	165.3	40
Morphine, 6-acetyl	328.1	45	58.2	35	165.1	35
Naloxone	328.2	48	253.2	25	310.1	20
Naltrexone	342.1	50	55.1	38	270.1	25
Oxazepam	287.1	39	241.2	23	269.1	13
Oxycodone	316.3	40	241.3	30	212.3	40
Oxycodone, nor	302.2	43	198.2	45	227.2	20
Oxymorphone	302.2	43	227.2	31	198.2	45
Oxymorphone, nor	288.1	42	184.1	42	213.1	30
Phencyclidine	244.3	19	86.2	11	91.2	27
Phentermine	150.1	18	91	18	133.1	10
Pregabalin	160.1	30	55.1	22	97.1	15
PVP, alpha	232.2	45	91.1	22	105.1	25
Ritalinic acid	220.2	28	84.2	20	56.2	38
Tapentadol	222.2	40	107.2	25	121.2	20
Temazepam	301.1	30	255.2	22	177.2	40
Tramadol	264.3	31	58.1	21		
Tramadol, *N*-desmethyl	250.2	30	44	15		
Triazolam	343.2	56	308.2	26	239.2	44
Zaleplon	306.2	55	236.2	28	264.2	20
Zolpidem	308.2	45	92.1	48	235.2	35
Zopiclone	389.2	25	217	32	245.1	15

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Table II. Validation data summary for linearity, precision, bias, matrix effect, calibration linearity and analyte carryover

Analyte	Analytical range, ng/mL				Precision			BIAS			Matrix			Calibration		Carryover	
	Neg Ẋ				%CV			%			EFFECT %			R^2		%	
	LLQ	ULQ	LOD	+3.3SD	I	II	III	I	II	III	A	B	C	Mean	SD	Mean	SD
Alprazolam	50	1,500	25	22	9.4	9.7	11.8	3.6	−1.1	−1.1	−20	24	20	0.9975	0.0019	0.55	0.44
Alprazolam, alpha-hydroxy	50	1,500	25	22	18.5	14.6	8.5	3.0	−0.3	0.1	24	67	62	0.9955	0.0024	0.62	0.39
Amphetamine	50	1,500	25	9	6.2	4.1	6.3	−0.9	−1.7	2.0	−48	−26	−22	0.9955	0.0024	0.34	0.21
Benzoylecgonine	50	1,500	25	12	8.0	6.8	9.2	−1.8	−2.6	1.0	−24	6	11	0.9961	0.0028	0.50	0.26
Buprenorphine	5.0	150	2.5	2.2	18.3	10.9	14.9	−6.7	0.5	−6.8	7	58	25	0.9941	0.0044	0.58	0.46
Buprenorphine, nor	5.0	150	2.5	2.0	15.8	10.3	9.5	3.7	−0.5	−2.4	7	53	49	0.9961	0.0033	0.38	0.46
Carisoprodol	50	1,500	25	9.3	8.3	7.6	9.0	−0.6	−2.8	1.0	−8	36	29	0.9954	0.0046	0.38	0.20
Clonazepam	50	1,500	25	27	13.8	10.3	7.3	5.4	−2.5	−0.2	−48	−18	−19	0.9960	0.0027	0.74	0.48
Clonazepam, 7-amino	50	1,500	25	18	10.0	5.1	6.6	8.8	−3.5	4.1	−21	10	11	0.9957	0.0033	0.72	0.32
Codeine	50	1,500	25	24	7.6	12.8	12.5	−1.3	−0.6	−2.6	−10	20	24	0.9956	0.0025	0.45	0.49
Dextromethorphan	50	1,500	25	12	8.6	8.6	8.6	0.2	−0.8	−2.8	−10	27	21	0.9963	0.0040	0.47	0.25
Dextrorphan	50	1,500	25	13	8.2	7.7	6.5	0.3	−2.4	−1.9	−23	9	7	0.9952	0.0024	0.42	0.26
Diazepam	50	1,500	25	26	16.2	7.9	11.0	4.5	−0.8	2.0	−27	12	12	0.9951	0.0037	0.60	0.43
Diazepam, nor	50	1,500	25	21	8.9	6.7	7.9	−0.9	−5.7	−0.5	−19	19	19	0.9973	0.0026	0.87	0.33
Ethyliden-1,5-dimethyl-3, 3-diphenylpyrrolidine	50	1,500	25	11	10.5	8.9	7.2	0.0	−1.2	−3.3	−4	38	30	0.9956	0.0030	0.48	0.25
Ephedrine	50	1,500	25	10	7.1	6.1	7.2	−3.6	−1.1	−0.3	−29	0	1	0.9977	0.0017	0.29	0.20
Ethylone	12.5	375	6.3	3.5	13.6	7.1	6.8	−1.7	0.4	−1.3	−32	−4	−8	0.9955	0.0034	0.09	0.17
Fentanyl	5.0	150	2.5	1.3	11.7	10.3	7.0	−1.2	−0.1	−2.8	−35	5	5	0.9958	0.0032	0.33	0.28
Fentanyl, nor	5.0	150	2.5	2.0	15.4	6.6	7.9	6.2	−2.7	−1.5	−50	−26	−28	0.9968	0.0022	0.33	0.44
Flurazepam	50	1,500	25	8.3	6.8	7.7	8.6	0.6	−1.8	−2.2	−15	24	15	0.9962	0.0035	0.57	0.18
Flurazepam, hydroxyethyl	50	1,500	25	15	6.3	6.8	7.2	1.0	−0.4	0.1	−6	46	40	0.9971	0.0021	0.63	0.27
Gabapentin	50	1,500	25	15	15.7	9.4	8.6	−4.5	−3.5	−0.8	−12	29	23	0.9816	0.0326	0.43	0.36
Hydrocodone	50	1,500	25	13	9.8	6.6	10.1	0.9	−3.8	0.9	−21	17	4	0.9949	0.0043	0.51	0.30
Hydromorphone	50	1,500	25	16	11.4	6.9	11.5	5.8	0.2	−6.7	−7	13	6	0.9955	0.0023	0.43	0.37
Ketamine	50	1,500	25	11	7.6	4.2	11.1	−1.4	−2.3	−0.8	−41	−16	−16	0.9968	0.0023	0.28	0.24
Ketamine, nor	50	1,500	25	11	6.1	4.9	8.9	−1.5	−2.6	−0.7	−57	−39	−38	0.9963	0.0026	0.31	0.26
Lorazepam	50	1,500	25	24	12.5	10.5	12.0	3.6	−1.9	−5.8	19	82	68	0.9950	0.0026	1.05	0.47
3,4-Methyleneamphetamine	50	1,500	25	22	12.5	9.7	5.6	3.4	2.9	2.6	−39	−11	−7	0.9956	0.0029	0.61	0.45
3,4-Methylenedioxy-*N*-ethylamphetamine	50	1,500	25	11	8.2	7.5	5.2	−2.1	−2.2	0.7	−35	−9	−10	0.9968	0.0026	0.32	0.23
3,4-Methylenedioxy methamphetamine	50	1,500	25	17	12.2	7.4	7.3	−0.3	−2.6	2.4	−37	−13	−13	0.9961	0.0035	0.37	0.38
Methylenedioxypyrovalerone	12.5	375	6.3	3.6	10.8	11.4	9.0	4.7	−5.1	−2.1	−23	−2	0	0.9959	0.0060	0.16	0.18
Meperidine	50	1,500	25	10	7.5	5.8	5.2	0.1	−2.1	2.7	−24	6	6	0.9970	0.0031	0.32	0.22
Meperidine, nor	50	1,500	25	12	7.8	5.8	10.4	−1.8	−2.2	−1.9	−26	4	7	0.9968	0.0024	0.54	0.26
Mephedrone	12.5	375	6.3	8.7	11.3	8.9	8.6	−0.7	0.2	1.6	−68	−56	−56	0.9974	0.0021	0.04	0.19
Meprobamate	50	1,500	25	20	12.0	7.3	12.6	−4.3	−2.9	−1.0	−10	30	30	0.9964	0.0030	0.26	0.44
Methadone	50	1,500	25	12	9.0	10.1	7.9	1.3	−4.3	−4.6	−11	40	38	0.9951	0.0063	0.66	0.28
Methamphetamine	50	1,500	25	10	7.1	5.5	5.4	−0.8	−0.6	0.9	−28	2	2	0.9964	0.0022	0.32	0.24
Methylone	12.5	375	6.3	6.9	13.9	13.3	6.7	2.5	0.0	−2.0	−30	−7	0	0.9973	0.0028	0.08	0.14
Methyphenidate	50	1,500	25	12	7.9	6.6	7.8	0.8	−2.0	1.5	−33	−4	−5	0.9958	0.0024	0.42	0.26
Midazolam	50	1,500	25	16	8.6	7.0	6.4	0.1	−2.4	0.3	−44	−12	−15	0.9966	0.0037	0.43	0.35
Mitragynine	5.0	150	2.5	1.6	7.3	10.2	10.4	1.8	1.4	−3.7	11	58	40	0.9964	0.0034	0.64	0.35
Mitragynine, 7-hydroxy	5.0	150	2.5	1.5	16.3	11.5	10.5	−5.4	4.0	−0.1	−28	11	6	0.9959	0.0021	0.31	0.35
Morphine	50	1,500	25	19	16.6	9.5	6.5	5.6	0.8	0.4	−10	27	22	0.9974	0.0024	0.38	0.40
Morphine, 6-acetyl	5.0	150	2.5	2.3	18.5	10.8	13.8	3.0	−6.6	5.5	−23	−3	8	0.9960	0.0055	0.03	0.05
Naloxone	50	1,500	25	11	8.7	7.0	7.4	−4.1	−2.3	−0.9	−5	28	30	0.9972	0.0022	0.28	0.23
Naltrexone	50	1,500	25	13	6.2	4.3	8.7	0.3	−1.0	3.8	−25	2	9	0.9965	0.0031	0.51	0.25
Oxazepam	50	1,500	25	21	14.1	10.0	7.3	1.2	−1.0	2.3	25	87	79	0.9962	0.0025	0.96	0.46
Oxycodone	50	1,500	25	14	12.8	9.8	12.1	−5.0	1.4	1.7	−35	−8	−12	0.9957	0.0024	0.39	0.27
Oxycodone, nor	50	1,500	25	19	12.6	8.9	9.3	0.4	−0.5	0.3	−58	−35	−33	0.9956	0.0034	0.36	0.37
Oxymorphone	50	1,500	25	13	6.7	7.3	7.1	−1.6	−3.9	−1.3	−30	−1	3	0.9962	0.0025	0.27	0.24
Oxymorphone, nor	50	1,500	25	10	6.9	5.5	9.7	−1.3	−1.8	−2.1	−47	−26	−19	0.9918	0.0136	0.26	0.21
Phencyclidine	12.5	375	6.3	2.7	9.5	11.2	13.9	2.1	−1.1	−5.2	−38	−8	−10	0.9946	0.0056	0.18	0.13
Phentermine	50	1,500	25	9.4	5.2	6.5	7.8	1.2	−0.7	0.6	−34	−5	−2	0.9969	0.0044	0.37	0.21
Pregabalin	50	1,500	25	14	13.6	8.8	5.7	−3.6	−2.8	1.3	106	139	119	0.9943	0.0045	0.54	0.31
PVP, alpha	12.5	375	6.3	3.4	7.2	8.9	12.3	−0.7	−0.7	2.0	−35	−2	−8	0.9951	0.0036	0.54	0.76

Table continues

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Table II. Continued

Analyte	Analytical range, ng/mL				Precision			BIAS			Matrix			Calibration		Carryover	
	Neg X̊				%CV			%			EFFECT %			R^2		%	
	LLQ	ULQ	LOD	+3.3SD	I	II	III	I	II	III	A	B	C	Mean	SD	Mean	SD
Ritalinic acid	50	1,500	25	13	6.3	7.7	8.6	1.2	−1.9	0.4	103	177	176	0.9969	0.0025	0.54	0.29
Tapentadol	50	1,500	25	21	10.6	6.7	9.0	1.0	−4.6	1.0	−27	3	4	0.9967	0.0019	0.49	0.44
Temazepam	50	1,500	25	18	10.3	8.1	5.5	−2.6	−4.4	−0.3	−7	48	43	0.9959	0.0028	0.64	0.24
Tramadol	50	1,500	25	16	9.0	8.6	4.9	0.0	−0.7	−1.9	−21	12	12	0.9960	0.0034	0.35	0.34
Tramadol, *N*-desmethyl	50	1,500	25	13	5.7	7.7	6.2	−0.7	−1.5	−1.2	−29	0	−2	0.9935	0.0093	0.45	0.28
Triazolam	50	1,500	25	26	11.3	9.9	8.7	5.4	−0.5	−0.8	−17	34	25	0.9948	0.0045	0.66	0.57
Zaleplon	50	1,500	25	15	7.3	8.2	6.4	2.5	−0.3	−0.6	−16	24	21	0.9969	0.0025	0.72	0.27
Zolpidem	50	1,500	25	12	8.4	6.0	6.7	0.8	−1.5	−0.4	−17	20	14	0.9966	0.0017	0.44	0.24
Zopiclone	50	1,500	25	15	17.0	12.0	18.8	−8.9	0.9	−6.1	−31	−1	−6	0.9958	0.0033	0.39	0.34

principle described in a prior report (1) and included as a Supplementary data, Figure. Briefly, each calibrator, QC and unknown sample is analyzed with (neat) and without (spiked) a reference-analyte addition. Following the addition of recovery and hydrolysis reagent to both the neat and spiked analysis mixtures, the dual-sample sets are incubate for hydrolysis then dilute and analyze by UPLC-MS-MS. The TAC ratio which is the matrix normalized ion response is calculated as [neat ion response/(spiked ion response − neat ion response)]. TAC ratio for each analyte is calculated from the ion response data for the dual-sample analysis by dividing transition-ion response in the neat analysis by spiked ion area which is calculated by subtraction the ion response in the neat analysis from the response in the spiked analysis. The analyte concentration is then calculated from a regression line obtained by a five point calibration of TAC ratio versus calibrator concentration, using a linear weighted least squares regression model.

Sample

Two samples (25 µL aliquots) of calibrator, QC or unknown were added to paired analysis wells in a 96-well plate for neat and spike analysis. Multi-analyte spike reagent (50 µL) was added to spiked wells and an equal of water was added to the neat wells, followed by additions of the mixed recovery and hydrolysis reagent addition (100 µL) to all wells. The plate was covered, mixed by rotation on the benchtop and then incubated at 55°C for 1 h in an oven. After cooling, 500 µL of starting mobile phase was added to all analysis wells then mixed and analyzed by UPLC–MS-MS.

UPLC–MS-MS analysis

UPLC–MS-MS analysis was performed using a Waters ACQUITY UPLC I-Class (FTN) system in combination with a Xevo® TQD tandem mass spectrometer (Waters Corporation, Milford, MA). Chromatographic separation was achieved using an ACQUITY UPLC® BEH Phenyl column (1.7 µm, 2.1 × 50 mm, Waters) equipped with an Acquity UPLC® BEH Phenyl VanGaurd ™ pre-column(1.7 µm, 2.1 × 5 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), collision gas (argon maintained at a pressure of 0.5 Pa) and RF 2.5 volts. Multiple reaction monitoring (MRM) was performed for the 64 analytes and recovery standard by using two transitions per analyte (with the exception of tramadol and n-desmethyltramadol where only one transition was available); cone voltage and collision energy was optimized to give the maximum response for each transition (Table I). Parameters were arranged into 65 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 processing

Data were processed initially using TargetLynx™ (Waters) which integrated peak-area responses for each MRM transition and verified acceptability of results with respect to QC criteria as follows: analyte retention time to be within 0.2 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 dual-sample injection of neat and spiked samples, verification of ±10% tolerence for injection volume variability was achieved by analysis of ion area responses with the recovery standard methapyriline. Additional data handling for TAC analysis was achieved by export of the TargetLynx summary data into Microsoft Excel, which used quantifier ion response to calculate TAC ratio as follows: TAC ratio = neat peak ion area/(spiked ion area − neat ion area). Calibration of the TAC ratio was calculated by a weighted least squares regression analysis of TAC response at five calibrator concentrations.

Method validation plan

The method validation plan was based upon both SWGTOX guidelines (49) and Laboratory Standards provided by New York State's Department of Health (50). Method accuracy and precision were determined by replicate analysis of Levels I, II and III QC data in 10 analytical runs. The percent matrix effect was determined in aqueous versus urine-based control samples at three concentration levels as described in the method section. Matrix effect was calculated using the following formulae: [(B/A − 1) × 100%] where A represents the ion response in urine matrix and B represents the ion response without urine matrix present. A wide range of matrix effect

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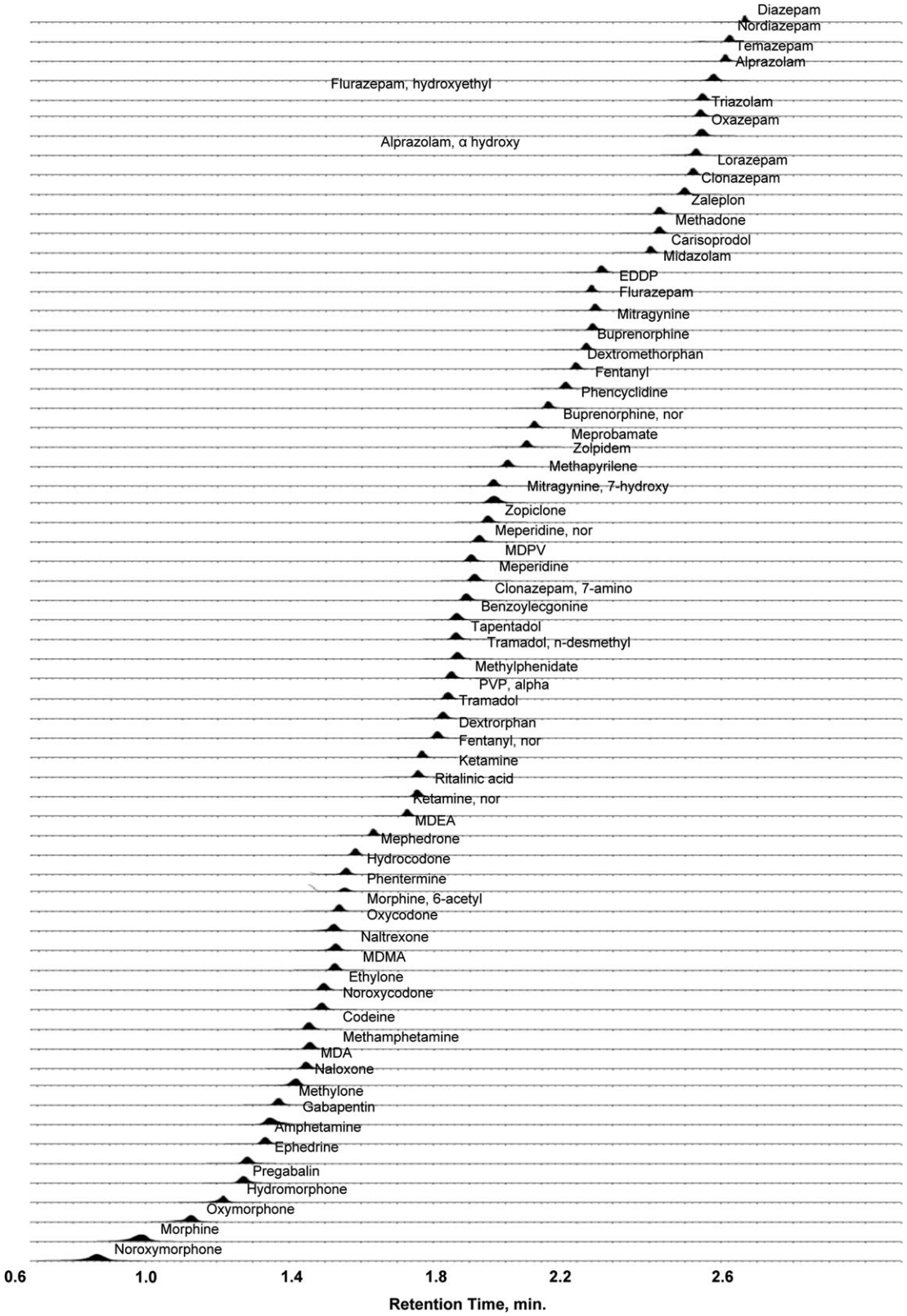

Figure 1. Extracted ion chromatogram obtained following analysis of the 50/125/500 ng/mL calibrator; the figure shows the quantifier MRM transition for 64 drugs and metabolites plus recovery standard in 3-min acquisition program.

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was anticipated with a dilute-and-inject method of sample preparation and the observed matrix affect was determined as accepted if all analytical validation criteria were met. Analyte-negative urine from multiple donor collections ($n = 10$) was 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 h incubation at 55°C (1). 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. The calibration model using a weighted least squares linear regression was evaluated over 10 analytical runs based upon calibrator re-analysis concentration with criteria of ±20% for re-analysis concentration and an $R^2 > 0.98$ for the regression analysis equation. Assay limit of detection (LOD) is defined in accordance with SWGTOX guideline approach (49) using the lowest non-zero calibrator concentration. Ten analytical runs were used to demonstrate that all detection and identification criteria were met at this calibration level. A statistical analysis of background in blank matrix samples was also analyzed to compare with the defined LOD. Ten sources of blank matrix sample were analyzed over 10 analytical runs and the average signal of the negative samples plus 3.3 times the standard deviation was calculated and compared to the defined LOD. The concentration of the second lowest calibrator is the lowest reportable concentration and is defined as lower limit of quantitation (LLQ). Ten LLQ concentration samples were analyzed over 10 analytical runs to demonstrate that all detection, identification, bias and precision criteria were met.

Quantitative accuracy of the method 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 an alternate quantitative LC–MS-MS methods performed by MEDTOX Diagnostics, Inc. (St. Paul MN). Specimen and data handling for de-identified positive and negative case specimens were based on an Albany Medical Center IRB reviewed protocol (#4454).

Results and Discussion

Analytical column and gradient mobile phase conditions were adapted from a prior study (1) for optimal retention of morphine and separation of isobaric agents during a rapid 3.3 min gradient and re-equilibration sequence. The MS-MS transition-ion conditions for 64 analytes and recovery standard are shown in Table I. The resultant quantifier ion chromatograms obtained with the optimized chromatographic and MS-MS conditions are represented in Figure 1. With optimization of mobile phase the phenyl column showed adequate separation of morphine from the void volume with baseline separation of the isobaric analytes morphine/hydromorphone, codeine/hydrocodone and methylphenidate/normeperidine. The analytical column life ranged from 3,000 to 4,000 injections with an average use of three pre-columns during the analytical column life. The total time for the dual-sample analysis including between injection gradient re-equilibration was <7 min, allowing for the analysis of 96 samples in an overnight UPLC–MS-MS run.

Fentanyl, nor Average RT = 1.66 min

Sample	TAC Ratio	Conc ng/mL	Range (±20%)	Conc Criteria	RT Criteria	Injection Recovery	Injection Criteria	Ion Ratio Failed	Ion Ratio Criteria
Cal 2.5	0.01	2.9	2.0-3.0	PASS	PASS	97	PASS	NO	PASS
Cal 5.0	0.02	4.7	4.0-6.0	PASS	PASS	100	PASS	NO	PASS
Cal 20	0.09	18	16-24	PASS	PASS	101	PASS	NO	PASS
Cal 50	0.23	47	40-60	PASS	PASS	102	PASS	NO	PASS
Cal 100	0.52	104	80-120	PASS	PASS	107	PASS	NO	PASS
Cal 150	0.76	151	120-180	PASS	PASS	96	PASS	NO	PASS
QC Neg	0.00	0.6	<2.5	PASS	PASS	95	PASS	NO	-
QC 4.0	0.02	4.0	3.2-4.8	PASS	PASS	96	PASS	NO	PASS
QC 12	0.05	9.9	9.6-14.4	PASS	PASS	101	PASS	NO	PASS
QC 120	0.61	122	98-144	PASS	PASS	101	PASS	NO	PASS
QC Hydrolysis	0.00	0.6	<2.5	PASS	PASS	100	PASS	NO	-

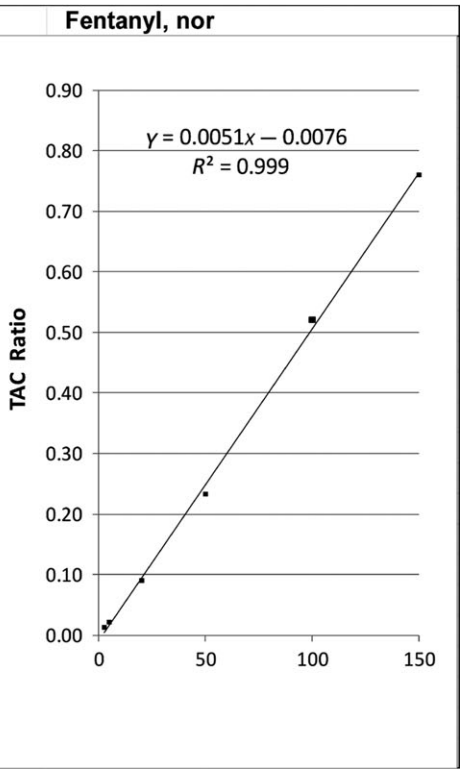

Figure 2. Representative analyte linearity along with calibration and QC analysis template. Peak-area responses for the MRM transitions as generated by TargetLynx are copied into the Excel template and the resultant analysis with regression graphics are automatically displayed. The following acceptance criteria were calculated for calibrators as well as QC samples: concentration, ion ratio, retention time and injection recovery.

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The concentration of reference-analyte added to the spiked analysis was optimized to achieve linearity for the 64-analyte assay. In the semi-quantitative screening application of TAC previously reported (1), a reference-analyte spike concentration equal to the threshold concentration was employed and achieved limited linearity at above and below threshold concentrations. To extend the linear range in the current quantitative application of the TAC technique, it was found that a reference-analyte spike concentration at or above the concentration of the highest calibrator maximized linearity with the linear weighted least square calibration model. Linearity is represented in Figure 2 which displays a representative Excel-calculation and a regression plot for norfentanyl, a low-calibration range analyte. The table within the figure displays the calculated TAC ratio from the ion acquisition data (not shown) along with the norfentanyl concentration interpolated from the regression line shown to the right of the figure. The Excel template also provides an easy pass/fail display for all analytical criteria including retention time, ion ratio and injection volume comparability for each neat and spiked analysis of calibrator and QC samples.

The inter-urine effect on analyte recovery was assessed by supplementing urine specimens from multiple donors with concentrations of 40, 100 and 400 ng/mL for respective low-, medium- and high-calibration range analytes. Mean recovery within 20% of target concentration was determined across all analytes as shown in Figure 3.

The analytical range and performance parameters are listed for each analyte in Table II. The LLQ and ULQ are based upon the second lowest calibrator and highest calibrator concentrations, respectively. Also listed is the LOD which is defined as the lowest calibrator for each of analytes. The LOD was validated for all

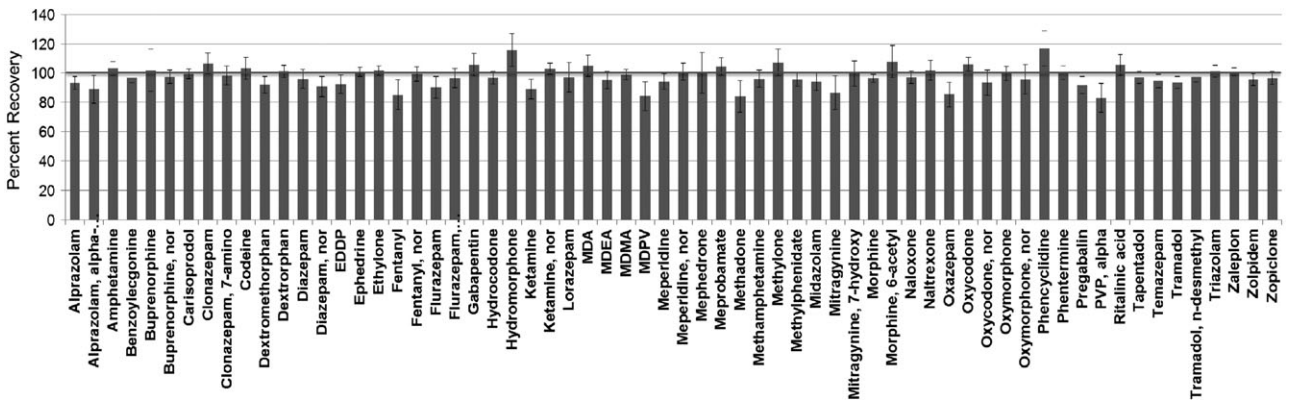

Figure 3. Analytical recovery (mean ± SD) of TAC-based method for analyte-negative urine (seven donors) supplemented with reference standards for 64 analytes at 400% of LOQ. Recovery averaged within 20% of target.

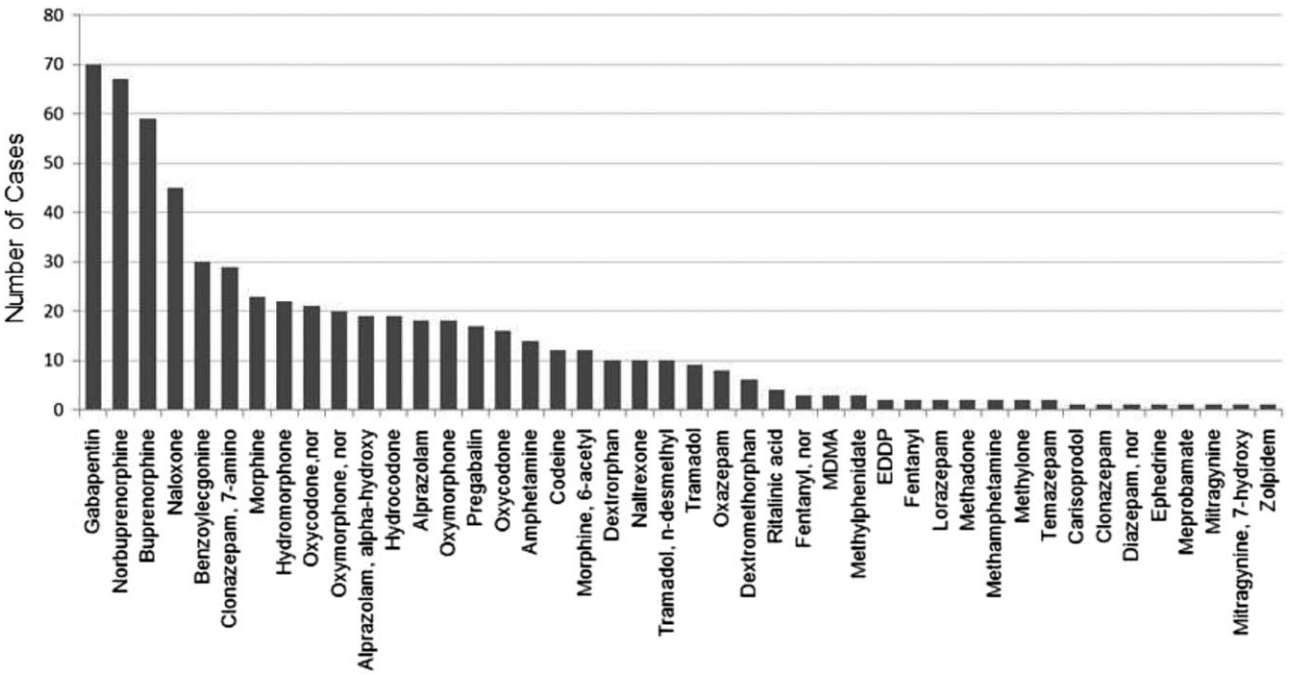

Figure 4. Drugs detected by the TAC-based method in 448 consecutive donor urines analysis from court-ordered casework.

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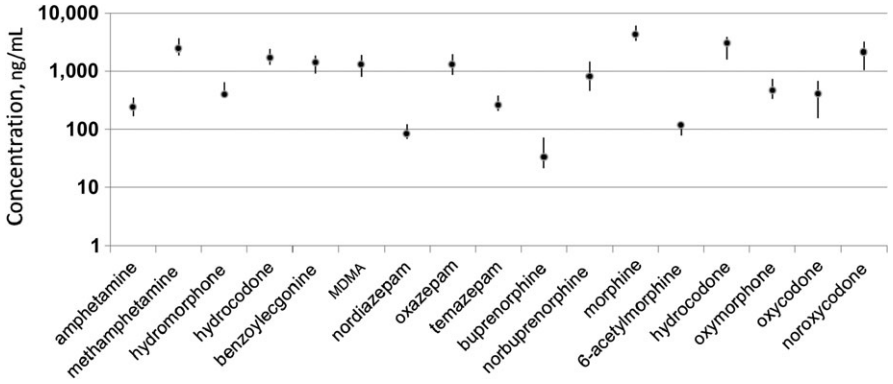

Figure 5. Proficiency test results showing concentration by TAC-based method along with acceptable range. CAP PMDM 2016.

analytes in 10 analytical runs by replicate analysis of the lowest calibrator using the qualitative criteria for retention time, ion ratio and injection criteria. Additional validation of the selected LOD was performed by duplicate analysis of 10 sources of analyte-negative urine. The mean plus 3.3 times the standard deviation for the negative urines was calculated and reported to the immediate right of the LOD in Table II. The comparison of these negative urine control data with the selected LOD shows that LOD selection using the lowest calibrator technique does not significantly underestimate the LOD based on blank matrix analysis statistics. Method precision and bias was determined by replicate analysis of Levels I, II and III QC data over 10 analytical runs. Coefficient of variation averaged 10.5, 8.3 and 8.7% at the respective low-, medium- and high-control levels for all analytes and all precision data was within the established criteria of 20%. Assay bias ranged from −8.9 to 8.8% and was also within criteria for all assays. Linearity on dilution has also been validated for accurate quantitation of drugs and metabolites that exceed the calibration range of the assays in casework and proficiency specimens.

Additional validation study data is presented in Table II. Matrix effect varied widely between analytes and followed a previously reported trend where the early eluting analytes showing a trend toward ion suppression and the later eluting analytes trending toward ion enhancement (1). Notable exceptions are pregabalin and ritalinic acid with significant ion enhancement. An effect of analyte concentration on ion recovery was also observed. The data shows a trend for all analytes toward an increase in ion recovery with increasing analyte concentration. Calibration model evaluation demonstrated within criteria R^2 data as shown in Table II and calibrator concentrations determined by calculation from the regression equation were also within criteria during the 10 analytical runs. Carryover was also studied and was within 1.0% for all analytes.

The usefulness of definitive identification of drug use as an initial screening test is supported by routine casework data reported in Figure 4. In our court-related testing program definitive testing has replaced presumptive immunoassay screening and has enhanced the ability to detect the diversity of current drug use in forensic casework. The figure shows drug and metabolite identification in 448 consecutive case specimens tested by the 64-analyte definitive panel. Gabapentin, a drug that was not tested in our immunoassay screening program, was the most frequent drug finding by definitive testing with a 16% positive rate. Buprenorphine use was also prevalent (15% of cases) in our court-ordered case study and co-identification and quantification of norbuprenorphine and naloxone provided

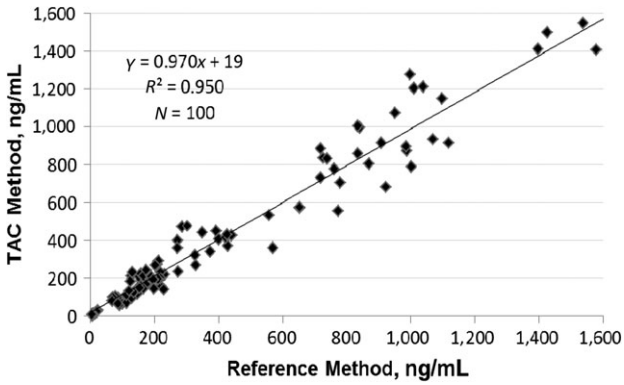

Figure 6. Correlation of quantification by TAC-based method versus validated LC–MS-MS methods using SIIS. Analytes include amphetamine, benzoylecgonine, buprenorphine, norbuprenorphine, 7-aminoclonazepam, diazepam, hydrocodone, hydromorphone, methylphenidate, methamphetamine, 6-acetymorphine, nordiazepam, oxazepam, oxycodone, oxymorphone, ritalinic acid, temazepam and triazolam.

additional information on drug metabolism as well as the combined use of buprenorphine-naloxone as part of the initial testing protocol. Additional case findings not routinely tested in the prior immunoassay screening program included: methyphenidate with ritalinic acid, fentanyl with norfentanyl, mitragynine with 7-hydroxy mitragynine, tramadol with *N*-desmethyltramadol, naltrexone, pregabalin and methylone. The identification of this additional drug use clearly demonstrates the value of the definitive method in reducing false negatives in forensic screening.

Quantitative accuracy of the method was evaluated by performance in external proficiency testing as well as by crossover analysis with confirmatory LC–MS-MS methods of testing in a reference laboratory. Figure 5 shows the results for three specimens tested in the College of American Pathologists' 2016 testing program for Drug Monitoring and Pain Management. The closed circle represents the concentration determined by the TAC-based definitive method and the vertical line represents the acceptable range as established by the College of American Pathologists. Proficiency testing results are within acceptable range for analytes tested in the program. In addition, specimens with a total of 100 analytes quantitated by the definitive screening method were tested by directed confirmatory methods performed by MedTox Laboratories Inc. (St Paul MN). Figure 6 shows the regressed relationship between the methods with

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a slope of 0.97 and an R^2 of 0.95. Accuracy of the quantification was demonstrated in both proficiency and crossover testing.

An advantage of TAC approach as used in the current method compared to the widely used analyte-specific stable-isotope technique for quantitation by LC–MS-MS is elimination of the significant cost of deuterated internal standards. Due to both cost and availability, most large multi-analyte screening and quantitation methods employing deuterated internal standardization do not employ analyte-specific internal standardization across all analytes and, therefore, risk quantitative inaccuracy for the known variability in matrix effect between urine samples. In support of this conclusion is the significant variability in absolute ion response for the same concentration of drug or metabolite supplemented into drug-free urine from multiple donors as observed when a matrix normalization technique is not employed. We also know from prior studies that matrix effect on ion response using electrospray interface varies between analytes and can also be dependent upon the concentration of urine and chromatographic retention time (1). Analyte-specific stable-isotope standardization is a well-established method of correcting for matric effect but TAC provides an alternative approach that may be advantageous for large multi-analyte panel testing and especially when deuterated standards are not available.

Conclusions

A UPLC–MS-MS method has been developed for concomitant identification and quantification for a panel of 64 drugs and drug metabolites in urine. Method performance studies have been conducted and meet validation criteria, including acceptable comparison to established methods of quantitation in casework and proficiency testing. Application of definitive urine drug screening and quantification based on sample dilution, rapid chromatographic analysis, matrix normalization by a TAC technique and customized data management is applicable to clinical and forensic toxicology casework. In forensic work, the expanding use of multi-analyte definitive testing in the initial drug identification phase points to the need for complementary confirmatory testing by alternate methodology with at least equivalent selectivity and sensitivity. The TAC principle of calibration may also be applicable in development of these confirmatory methods of testing.

Supplementary data

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

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