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Article

Article

A Single Method for 127 Recommended and Additional DUID Drugs in Blood and Urine by LC-MS/MS

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Abstract

Driving under the influence of drug (DUID) cases continue to challenge forensic toxicologists as both the volume and complexity of casework increases. Comprehensive DUID testing should also meet the drafted Academy Standards Board (ASB)/ American National Standard Institute (ANSI) standard and the National Safety Council's Alcohol, Drugs and Impairment Division (NSC-ADID) recommendations. A simple method using protein precipitation followed by filtration extraction with an 8 minute run time by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed, and a comprehensive ASB/ANSI validation was performed. Target drugs and metabolites were quantitatively assessed in blood and qualitatively assessed in urine. Included were 127 target analytes including cannabinoids (12), amphetamines (11), cocaine and metabolites (6), benzodiazepines (36), Z-drugs (5), opioids (27), anticonvulsants (3), first-generation antihistamines (6), muscle relaxants (2), dissociatives and hallucinogens (6), barbiturates (10), and miscellaneous substances (3). Limits of detection are appropriate for DUID and other forensic casework such as drug-facilitated crime (DFC) and postmortem investigations. To demonstrate applicability, 78 proficiency test blood and urine samples and 1,645 blood and urine samples from authentic cases samples demonstrated effective detection of target analytes in forensic casework. By increasing the analytical scope of multiple drug classes via a single method, this technique detects drugs that may have previously gone undetected, such as flualprazolam, etizolam, mitragynine, gamma-hydroxybutyric acid and psilocin and improves laboratory efficiency by reducing the number of tests required. The described method is, to the authors' best knowledge, the only published single procedure to meet all drugs listed in the drafted ASB/ANSI standard and recommended Tier 1 and traditional drugs from Tier 2 for DUID screening, while also achieving many drug scope and sensitivity recommendations for DFC and postmortem testing.

Introduction

Driving under the influence of drugs (DUID) is the act of driving while impaired by a drug, regardless of whether a drug is licit. Despite the illegality of DUID, 20% of night-time weekend drivers tested positive for drugs in a 2013–2014 National Roadside Survey (1). A survey conducted by the Substance Abuse and Mental Health Services Administration in the USA revealed that more than 12.6 million people drove under the influence of drugs in 2018 (2). In

California, 733 (19%) of all crash fatalities involved drugs in 2016, a 176% increase since 1995 (3). Further, these are likely conservative reports considering the often-minimal scope of drugs legally required for postmortem testing in motor vehicular deaths (i.e., alcohol, barbituric acid and amphetamine derivative) (4).

Drugs that are considered impairing and prevalent in DUID casework are typically divided into seven classes: central nervous system (CNS) depressants, CNS stimulants, hallucinogens, dissociative anesthetics, narcotic analgesics, inhalants and cannabis. Law enforcement agencies detect drug-induced impairment of drivers by training and certifying officers as Drug Recognition Experts (DREs) in the USA. The DRE's final assessment is often compared to the toxicology findings of the collected biological samples, typically blood or urine (5). All described drug classes are included in the recommended scope for DUID toxicology testing by the National Safety Council's Alcohol, Drugs and Impairment Division (NSC-ADID), as well as in the anticipated ANSI/ASB standard published by the Academy Standards Board (ASB) for development by American National Standard Institute (ANSI), which originated at the Organization of Scientific Area Committees for Forensic Science (OSAC) (6, 7).

Historically, methods used to identify and quantitate these various drug classes focused on one or just a few drug classes (8–11). Even if multiple drug classes were analyzed collectively, cannabinoids are still typically analyzed separately due to their lipophilicity and relatively low blood concentrations (9, 10, 12, 13).

This research aims to develop a single, comprehensive, multiclass, and rapid method that meets and exceeds the recommended scope and sensitivity of testing for DUID casework and, if feasible, other forensic casework, with the goal of increased efficiency and sustainability as casework and polydrug use increases.

Experimental

Chemicals, reagents and gases

Certified reference standards for analytes and isotopically labeled internal standards (ISTD) were obtained from Cerilliant (Round Rock, Texas, USA), Lipomed (Cambridge, Massachusetts, USA), Cayman Chemicals (Ann Arbor, Michigan, USA), Wyeth (Madison, New Jersey, USA), Toronto Research Chemicals (North York, Ontario, Canada), USP (Rockville, Maryland, USA), and Alltech (Deerfield, Illinois, USA). Analyte stocks were provided by the manufacturers or subsequently prepared in-house at 0.1, 1.0 or 10 mg/mL. Optima LC–MS grade methanol, isopropanol, formic acid and ammonium formate were purchased from Fisher Scientific (Hampton, New Hampshire, USA). Acetonitrile (ACN) was purchased from VWR (Radnor, Pennsylvania, USA). All water utilized was purified to deionized water (dH_2O) using a Millipore Milli-Q Ultrapure Water System from Millipore Sigma (Burlington, Massachusetts, USA). All nitrogen gas utilized was produced in-house to 1–5 ppm purity by a Peak i-Flow system (Inchinnan, Scotland, UK).

Specimens

Calibration, control and validation studies were performed using blank porcine blood (Del Monte; San Francisco, California, USA) and blank human urine samples containing 0.01% sodium azide (UTAK; Valencia, California, USA). Porcine blood was subsequently prepared in-house with 10 g/L of sodium fluoride and 6 g/L potassium oxalate. Applicability studies were performed using blood and urine samples from proficiency test and authentic cases. Results were compared to those obtained previously from other in-house methods. Proficiency samples were received from the College of American Pathologists and interagency comparison samples from the California Association of Toxicologists from 2018 to 2020. Authentic specimens were obtained by the Forensic Laboratory Division of the San Francisco Office of the Chief Medical Examiner during routine forensic investigations throughout a 6 month period starting from April 2020.

Analyte grouping

Target analytes were grouped into 13 concentration groups (i.e., AAA, B, BB, BBB, C, CC, CCC, D, DD, DDD, E, EE and EEE) depending upon blood concentration ranges found in typical DUID casework and recommended cut-offs (6, 7). The limit of detection (LOD), lower limit of quantitation (LLOQ), and upper limit of quantitation (ULOQ) concentrations are described in Table I. Quality control (QC) and individual blood calibration (CAL) concentrations of each analyte group are described in Supplementary Table SI.

Preparation of calibrator and control solutions

For effective reporting cut-off limits, two qualitative LOD stocks were prepared, one for blood and the other for urine (Table I). The blood LOD stock excluded tetrahydrocannabinol (THC), 11-hydroxy THC, cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN) and gamma-hydroxybutyric acid (GHB), in which case, CAL 1 served as both the effective LOD and LLOQ. The urine LOD stock included all target analytes in the method.

CAL/LOD and QC stocks were prepared separately and where feasible, from certified reference standards of differing manufactures. The concentrations in Cal 7 represented the ULOQ. Calibrators 1–6 were created by diluting each from CAL 7, with CAL 1 representing the LLOQ. The QC stock was designated as QC high (QCH), with two subsequent dilutions, QC medium (QCM) and QC low (QCL) created from QCH. In blood, the following target analytes were analyzed qualitatively only and thus prepared only in the LOD, not included in the CAL: amobarbital/pentobarbital, aprobarbital, barbital, butabarbital, butalbital/talbutal, hexobarbital, mephobarbital, phenobarbital, secobarbital, thiopental, cannabidivarin (CBDV), tetrahydrocannabivarin (THCV), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), tetrahydrocannabinol acid (THCA) and tetrahydrocannabivarinic acid (THCVA).

An ISTD stock was prepared with concentrations set at 25% of the ULOQ of the respective non-deuterated target analyte, except for GHB and all barbiturates ISTD concentrations that were set at the respective LLOQ concentration (Supplementary Table SIII).

Prepared blood LOD, urine LOD, CAL and QC stocks were all prepared with methanol, aliquoted into vials for short-term or single use and stored at −80°C until use.

Extraction procedure

Blood or urine sample (150 µL) were placed into eXtremeIFV® PTFE 0.2 µm filter vials (Thomson Instrument Company, New Jersey, USA). Control samples CAL, QC and LOD were each fortified with 15 µL of the appropriate solution. Extraction was achieved via the addition of 400 µL of cold ISTD-fortified ACN solution (prepared at least 30 min earlier and stored at −20°C) to each sample. Filter vials were capped with filter pistons and pulse vortexed at 2,500 RPM for 5 minutes. The samples were then subsequently filtered by slowly compressing the filter pistons. Four hundred microliters of filtrate from each sample were then transferred into a Verex™ V–Vial (Phenomenex; Torrance, California, USA) and allowed to evaporate

Table I. LOD (ng/mL), Blood Quantitation Group and Range (ng/mL), Retention Time (RT) and MS/MS Conditions for 127 Target Analytes

Target analyte[a]	LOD blood	CAL group blood	CAL range blood	LOD urine	RT (min)[b]	Q1 mass (Da)	Q3 mass (Da)	DP (Volts)[c]	CE (Volts)[d]	CXP (Volts)[e]
Cannabinoids										
11-hydroxy THC	CAL 1	B	1–50	2.5	5.9	331.23	313.3	44	20	21
							193.2	44	34	16
CBD	CAL 1	B	1–50	2.5	6.6	315.2	193.2	45	31	15
							123.1	45	44	14
CBG	CAL 1	B	1–50	2.5	6.6	317.143	193.2	76	19	8
							123	76	41	14
CBN	CAL 1	B	1–50	2.5	6.9	311.064	223.1	60	27	10
							195.3	60	35	10
Carboxy THC	2.5	BBB	5–250	5	6.0	345.15	299.2	36	27	26
							193	36	35	22
THC	CAL 1	B	1–50	2.5	7.0	315.25	193.1	50	31	21
							123.1	50	40	14
CBDV	5	–	–	5	6.1	287.2	165.16	44	31	14
							123.1	44	42	14
THCV	1	–	–	5	6.5	287.2	165.15	38	32	14
							135.1	38	26	15
CBDA	1	–	–	1	6.4	356.871	244.9	–50	–40	–35
							310.8	–50	–32	–33
CBGA	1	–	–	1	6.5	359.027	191	–40	–40	–21
							135.9	–40	–40	–19
THCA	1	–	–	1	7.1	357.074	313.2	–5	–32	–17
							245.2	–5	–42	–7
THCVA	1	–	–	1	6.6	329.035	285.1	–20	–34	–17
							217	–20	–36	–9
Amphetamines										
3,4-Methylenedioxy amphetamine (MDA)	2.5	C	10–500	10	2.0	180.079	133	60	25	14
							135	60	25	14
3,4- MDMA	2.5	C	10–500	10	2.2	194.017	163	60	17	16
							105	60	31	12
Amphetamine	10	CC	20–1,000	50	1.8	136.1	119.1	30	11	7
							91	30	39	10
Ephedrine/ Pseudoephedrine	2.5	C	10–500	10	1.6	166.15	117	19	25	12
							115	19	33	12
Mazindol	1	BB	2–100	10	3.2	285.1	44	40	57	5
							42	40	112	19
Methamphetamine	10	CC	20–1,000	50	2.1	150.058	119.101	21	5	12
							32.1	21	33	14
Methylphenidate	1	BBB	5–250	2.5	2.8	234.117	84	60	65	10
							56.1	60	87	26
Norephedrine/ Norpseudoephedrine	2.5	BBB	5–250	10	1.2	151.998	116.9	56	23	14
							115	56	31	14
Phenmetrazine	5	C	10–500	25	2.0	178.1	115	50	36	6
							133.1	50	24	7
Phentermine	25	CCC	50–2,500	50	2.1	150.15	133.1	12	14	10
							105.05	12	25	11
Phencyclidine (PCP)	1	BB	2–100	10	3.5	244.143	86	60	20	10
							159	60	14	18
Cocaine and metabolites										
Anhydroecgonine methyl ester	5	C	10–500	25	1.0	182.024	118	16	31	14
							122	16	27	14

(Continued)

Table I. Continued

Target analyte[a]	LOD blood	CAL group blood	CAL range blood	LOD urine	RT (min)[b]	Q1 mass (Da)	Q3 mass (Da)	DP (Volts)[c]	CE (Volts)[d]	CXP (Volts)[e]
Benzoylecgonine	10	CCC	50–2,500	10	2.4	289.764	168.2	80	45	18
							105.1	80	25	12
Cocaethylene	1	BBB	5–250	2.5	3.4	317.9	82	60	63	10
							196.1	60	47	22
Cocaine	1	BBB	5–250	2.5	3.1	303.772	82.1	156	33	10
							105.1	156	35	12
Meta-/para-hydroxy cocaine	1	BB	2–100	1	2.6	319.962	81.9	60	31	12
							182	60	29	0
Norcocaine	2.5	BBB	5–250	5	3.1	289.97	136	101	33	14
							107.9	101	37	22
Benzodiazepines										
7-amino clonazepam	1	BB	2–100	2.5	3.1	285.926	222.1	111	33	12
							250.1	111	27	16
7-amino flunitrazepam	1	BB	2–100	2.5	3.3	283.978	135	60	35	12
							227	60	33	22
7-amino nitrazepam	1	BB	2–100	5	2.7	252.006	120.9	46	35	14
							146.1	46	37	16
Alpha-hydroxy alprazolam	1	BB	2–100	2.5	4.0	325.1	297.1	60	35	21
							216.1	60	53	17
Alpha-hydroxy midazolam	1	BB	2–100	1	3.9	341.919	323.9	91	29	14
							202.9	91	37	22
Alpha-hydroxy triazolam	2.5	BBB	5–250	5	4.0	359.05	331.05	60	37	30
							261.05	60	39	21
Alprazolam	1	BB	2–100	1	4.3	308.957	280.9	60	35	30
							205	60	55	18
Bromazepam	2.5	C	10–500	5	3.7	315.837	182	61	43	20
							209	61	35	22
Chlordiazepoxide	1	CCC	50–2,500	10	3.6	299.953	227	56	31	26
							282.6	56	19	28
Clobazam	5	C	10–500	5	4.6	300.949	258.9	91	14.5	22
							224	91	65	24
Clonazepam	2.5	BBB	5–250	5	4.4	316.05	270.05	55	35	19
							151.05	55	84	16
Clotiazepam	1	BBB	5–250	1	5.1	318.887	217.9	166	35	24
							290.9	166	22.6	24
Delorazepam	2.5	BBB	5–250	5	4.6	305.05	241.1	40	38	18
							140.1	40	20	12
Desalkyl flurazepam	1	BB	2–100	1	4.4	288.87	140	60	41	16
							226	60	39	22
Diazepam	1	CC	20–1,000	1	4.9	284.938	193	131	41	6
							154	131	35	8
Diclazepam	1	BBB	5–250	2.5	5.0	318.878	226.9	116	35	24
							154	116	33.5	18
Etizolam	0.5	BB	2–100	1	4.5	342.958	313.9	111	35	34
							258.8	111	45	20
Flualprazolam	0.5	BB	2–100	1	4.2	326.897	292	6	37	30
							223	6	52	22
Flubromazepam	1	BB	2–100	1	4.5	332.881	225.9	96	39	24
							184.1	96	41	24
Flubromazolam	0.5	BB	2–100	1	4.3	370.888	291.9	21	39	30
							342.9	21	39	36
Flunitrazepam	0.5	BB	2–100	1	4.6	313.919	268	101	35	30
							238.9	101	47	26
Flurazepam	1	BB	2–100	1	3.7	387.966	314.8	81	13.5	36
							316.8	81	10	20

(Continued)

Table I. Continued

Target analyte[a]	LOD blood	CAL group blood	CAL range blood	LOD urine	RT (min)[b]	Q1 mass (Da)	Q3 mass (Da)	DP (Volts)[c]	CE (Volts)[d]	CXP (Volts)[e]
Lorazepam	5	C	10–500	10	4.2	321.05	229.1	35	42	16
							163.05	35	50	14
Lormetazepam	1	BB	2–100	2.5	4.6	335.01	289.06	42	28	15
							177.1	42	55	14
Midazolam	1	BB	2–100	1	3.7	325.941	291	121	37	16
							290.4	121	35	30
N-desmethyl flunitrazepam	1	BB	2–100	2.5	4.2	299.922	254	60	33	30
							197.9	60	53	20
Nimetazepam	1	BB	2–100	1	4.6	295.937	250	60	33	20
							220.9	60	45	24
Nitrazepam	1	BB	2–100	2.5	4.2	281.964	236	116	33	26
							179.9	116	47	20
Nordiazepam	1	C	10–500	1	4.5	270.914	139.9	91	39	16
							208	91	39	24
Oxazepam	10	CC	20–1,000	10	4.1	287.1	269.08	40	21	13
							241.12	40	40	10
Phenazepam	2.5	BBB	5–250	5	4.7	348.833	183.9	60	43	20
							241.9	60	39	26
Prazepam	0.5	BB	2–100	1	5.6	324.975	270.9	36	31	12
							139.8	36	49	16
Pyrazolam	2.5	BBB	5–250	5	3.6	353.851	325.6	60	37	48
							167	60	31	18
Temazepam	1	C	10–500	5	4.5	301.1	177.1	45	53	19
							193.1	45	46	16
Triazolam	0.5	BB	2–100	5	4.3	343.05	177.1	35	85	19
							308.1	35	24	12
Zolazepam	0.5	BB	2–100	1	2.9	286.962	138	60	39	16
							242.9	60	47	26
Z drugs										
Zaleplon	0.5	BB	2–100	1	4.1	305.971	264	116	29	14
							236	116	32	12
Zolpidem	0.75	BBB	5–250	1	3.2	308.083	235.1	60	82	26
							263	60	55	30
Zolpidem phenyl-4-carboxylic acid	0.5	BB	2–100	1	2.4	337.989	265	60	51	24
							292.9	60	35	26
Zopiclone/eszopiclone	1	BBB	5–250[f]	10	2.9	389.1	245.1	36	26	14
							345.1	36	13	18
Zopiclone-N-oxide	–	–	–	10	3.0	404.884	143	96	17	16
							244.9	96	33	26
Opioids										
6-monoacetyl morphine (6-MAM)	0.25	AAA	0.5–25	2.5	2.2	328.097	211	100	35	20
							165	100	39	18
Acetyl fentanyl	0.1	AAA	0.5–25	1	3.4	323.05	105	60	39	12
							188.1	60	9	22
Buprenorphine	0.5	B	1–50	1	3.8	468.3	414.3	60	47	35
							396.2	60	54	21
Codeine	1	BBB	5–250	5	2.0	300.259	152	80	83	14
							165.1	80	51	16
Dextromethorphan /levomethorphan	0.5	BB	2–100	5	3.6	273.052	216.2	36	33	24
							214.1	36	37	22
Dextrorphan/levorphanol	0.5	BB	2–100	1	2.7	258.049	157	51	49	16
							201	51	31	26
Dihydrocodeine	1	BB	2–100	5	1.9	302.089	201.1	101	39	22
							227.1	101	37	26

(Continued)

Table I. Continued

Target analyte[a]	LOD blood	CAL group blood	CAL range blood	LOD urine	RT (min)[b]	Q1 mass (Da)	Q3 mass (Da)	DP (Volts)[c]	CE (Volts)[d]	CXP (Volts)[e]
EDDP (Methadone metabolite)	5	C	10–500	10	4.0	277.789	234	60	61	26
							249	60	45	28
Fentanyl	0.1	AAA	0.5–25	1	3.7	337.2	188.2	65	31	9
							132	65	41	14
Hydrocodone	1	BB	2–100	5	2.3	300.1	128	156	71	14
							183	156	37	20
Hydromorphone	1	BB	2–100	5	1.6	285.8	185	60	39	20
							157.1	60	57	16
Methadone	1	C	10–500	1	4.3	310.127	105.001	60	18	12
							219	60	21	24
Morphine	1	BBB	5–250	5	1.2	286.17	153.1	45	55	16
							165.1	45	44	14
Nalbuphine	1	BBB	5–250	5	2.5	358.049	340.1	60	29	20
							296.2	60	41	4
Naloxone	1	BB	2–100	5	2.0	328	252.9	60	35	28
							211.9	60	41.5	24
Naltrexone	1	BB	2–100	5	2.2	342.041	323.9	51	29	12
							267	51	43	18
Norbuprenorphine	1	BB	2–100	5	3.2	414.3	152.1	45	120	19
							165.1	45	100	19
Norcodeine	5	C	10–500	5	1.8	285.962	165	61	53	18
							152	61	79	24
Norfentanyl	0.25	AAA	0.5–25	1	2.5	233.057	84.1	11	23	12
							56.1	11	41	6
Norhydrocodone	1	BB	2–100	10	2.2	285.936	198.9	60	37	20
							241	60	31	26
Noroxycodone	1	BB	2–100	10	2.1	302.17	198.12	30	61	20
							167.1	30	73	14
Noroxymorphone	5	C	10–500	50	1.0	287.963	213	131	39	20
							269.9	131	18.8	14
O-desmethyl-tramadol	1	BB	2–100	5	2.1	250.05	58	60	63	8
							42	60	115	18
Oxycodone	2.5	BBB	5–250	5	2.2	316.218	298	60	40	36
							241	60	52	26
Oxymorphone	1	BB	2–100	10	1.3	302.071	284	60	27	30
							227.1	60	39	24
Tapentadol	5	C	10–500	10	2.8	222.04	107.1	46	13.5	12
							134.9	46	10	14
Tramadol	1	BBB	5–250	10	2.8	264.131	58	60	89	26
							42.1	60	115	18
Anticonvulsants										
Gabapentin	10	D	100–5,000	100	1.3	172.17	95.05	25	31	10
							137.1	25	30	13
Lamotrigine	10	D	100–5,000	10	2.7	255.88	158.9	26	39	14
							144.9	26	43	18
Pregabalin	100	DDD	500–25,000	250	1.3	160.018	96.9	60	31	14
							83	60	11	10
Antihistamines (first generation)										
Benztropine	1	BB	2–100	1	4.3	308.039	166.9	60	27	26
							165	60	46	18
Chlorpheniramine	5	C	10–500	25	3.4	274.884	230	121	5	26
							201.1	121	30	20
Diphenhydramine	1	C	10–500	1	3.7	255.897	167	90	10	18
							165.1	90	38	16
Doxylamine	2.5	C	10–500	5	3.0	272.014	168	60	45	18
							182	60	21	20

(Continued)

Table I. Continued

Target analyte[a]	LOD blood	CAL group blood	CAL range blood	LOD urine	RT (min)[b]	Q1 mass (Da)	Q3 mass (Da)	DP (Volts)[c]	CE (Volts)[d]	CXP (Volts)[e]
Hydroxyzine	1	BB	2–100	2.5	4.2	375.269	201.1	156	23	10
							165.1	156	81	16
Promethazine	0.5	BB	2–100	1	3.9	285.2	86	40	14	10
							198.1	40	32	22
Muscle relaxants										
Carisoprodol	25	DDD	500–25,000	25	4.1	261.111	97	16	21	12
							158.1	16	20	18
Meprobamate	50	E	1,000–50,000	100	3.0	219.13	158.1	40	23	10
							97.1	40	31	10
Dissociatives and hallucinogens										
GHB	CAL 1	EEE	5,000–250,000	10,000	0.5	102.934	84.9	−15	−14	−9
							54.9	−15	−22	−25
Ketamine	5	CC	20–1,000	10	2.5	237.777	125.1	90	18	14
							220.1	90	33.5	24
Norketamine	1	C	10–500	10	2.4	223.765	207.1	90	17	24
							125	90	31	14
Deschloroketamine	2.5	C	10–500	2.5	2.3	204.031	173	51	27	20
							145	51	35	20
Psilocin	1	BB	2–100	5	1.7	205.2	160.1	25	24	8
							58	25	16	7
Psilocybin	2.5	–	–	50	0.8	284.932	205	60	23	12
							239.9	60	25	26
Miscellaneous										
Lidocaine	5	C	10–500	5	2.4	235.142	86	60	63	10
							58.1	60	51	26
Mitragynine	2.5	BBB	5–250	5	4.1	399.037	174	60	41	18
							226	60	33	24
Suvorexant	0.5	CC	20–1,000	1	5.8	450.994	185.9	11	19.5	20
							104	11	64	14
Barbiturates										
Amobarbital/ pentobarbital	10	–	–	25	3.8	225.003	42	−35	−46	−19
							182	−35	−18	−9
Aprobarbital	10	–	–	10	3.0	209.08	42	−10	−45	−11
							166.03	−10	−16	−14
Barbital	10	–	–	25	2.2	182.947	139.9	−40	−14	−19
							42	−40	−88	−5
Butabarbital	10	–	–	25	3.2	211.033	42	−15	−38	−19
							167.9	−15	−16	−17
Butalbital/Talbutal	10	–	–	10	3.4	223.008	41.9	−10	−50	−19
							180.1	−10	−18	−7
Hexobarbital	500	–	–	5,000	3.8	235.04	41.94	−16	−47	−19
							98.93	−16	−18	−12
Mephobarbital	500	–	–	2,500	4.0	244.965	41.9	−45	−78	−17
							99	−45	−18	−11
Phenobarbital	10	–	–	25	3.2	230.955	42	−20	−58	−11
							84.9	−20	−16	−11
Secobarbital	10	–	–	25	4.0	237.19	42.1	−5	−48	−19
							193.9	−5	−18	−23
Thiopental	10	–	–	25	4.4	240.915	101	−20	−18	−7
							41	−20	−64	−19

N.B. Negative mode analytes are indicated with a negative DP, CE and CXP voltages.
[a]Qualifier MS/MS conditions are second row.
[b]Approxiate retention time.
[c]Declustering potential.
[d]Collision energy.
[e]Collision cell exit potential.
[f]Zopiclone/Eszopiclone degrades in methanol over time, thus is qualitative only.

to dryness under flowing house-made nitrogen at room temperature. After drying, samples were reconstituted with 50 µL of 80:20 mobile phase A (MPA):methanol (i.e., reconstitution mix). The vials were then capped and vortexed for 5 seconds and placed into the liquid chromatography-tandem mass spectrometry (LC–MS/MS) autosampler tray, prior to a 2 µL injection.

For qualitative urine casework, large concentrations of some analytes often saturated the detector (e.g., benzoylecgonine, cocaine, diphenhydramine). Any observed saturated samples were subsequently diluted 1/100 with reconstitution mix and 0.1 µL reinjected the following day, effectively a 1/2,000 dilution, to obtain accurate peak shapes and ion ratios.

Apparatus

The LC–MS/MS system consisted of a Sciex Nexera X2 LC-30 coupled with a Sciex QTRAP 6,500+ mass spectrometer utilizing an Ion Drive™ Turbo V electrospray ionization source (Redwood City, California, USA), operating in the positive and negative multiple reaction mode (MRM). Data acquisition and processing was performed with Sciex Analyst and MultiQuant software, respectively, with a custom built-in query created in-house that automated processing and enhanced quality assurance.

LC parameters

Liquid chromatographic separation of all analytes was achieved using a Kinetex 2.6 µm 100 Å phenyl-hexyl 100 × 2.1 mm column coupled with a SecurityGuard guard column containing two phenyl cartridges from Phenomenex (Torrance, California, USA). Eluents were degassed throughout use and consisted of aqueous MPA consisting of 5 mM ammonium formate in deionized water and pH adjusted to 4.5 using formic acid and, organic mobile phase B (MPB) consisting of ACN with 0.1% formic acid (v/v). Initial conditions of 6% MPB and 94% MPA were held until 0.5 minutes and then gradually increased to 98% MPB throughout 8 minutes. A 3 minute 98% MPB hold was performed for column cleansing, after which the initial conditions were instantaneously reinstated and held for 2 minutes to reach equilibrium. A combined flow rate of 0.6 mL/min was consistently maintained. The column oven temperature was set to 40°C and the autosampler at 6°C. A rinse cycle and needle wash consisted of 60% isopropanol, 20% methanol and 20% ACN.

Mass spectrometer parameters

Target analytes were identified via mass spectrometry in the scheduled MRM advanced mode with polarity switching between positive and negative. The Ion Drive™ Turbo V conditions were as follows: source temperature 600°C; voltage switching between 2,500 V and −2,500 V for positive and negative modes, respectively; nitrogen utilized for curtain gas, ion source gas 1 and ion source gas 2 and set at 40 (275.7), 60 (413.6) and 70 (482.6) PSI (kPa), respectively; and, nitrogen utilized for collision gas and set to high. All Q1 and Q3 resolutions were set to unit, except alpha-hydroxy alprazolam, delorazepam, lorazepam and pyrazolam (set to low), and zopiclone-N-oxide (set to high). All entry potential voltages were set to either 10 V or −10 V for positive or negative mode, respectively. Negative mode target analytes were all cannabinoid acids, barbiturates and GHB. The MRM detection window was 30 seconds, except for anhydroecgonine methyl ester, bromazepam, gabapentin, morphine, noroxymorphone, oxymorphone, psilocybin, and pyrazolam (45 second windows), and pregabalin (60 second window). The processing half windows were all set to 15 seconds, except 3,4-methylenedioxymethamphetamine (MDMA) (30 seconds), amphetamine (10 seconds), anhydroecgonine methyl ester (7.5 seconds) and alpha-hydroxy alprazolam, alprazolam, oxazepam (5 seconds). The minimum dwell time was 3 msec, the maximum dwell time was 250 msec, and the pause between mass ranges was 5.007 msec to mitigate any crosstalk. The target scan time was 0.4 seconds for the positive mode and 0.1 seconds for the negative mode. The MS run duration was 7.5 minutes, with 900 cycles and a cycle time of 0.5 seconds. A total of 335 transitions were selected for monitoring, where two MRM transitions were chosen for each analyte and one for each ISTD based on abundance, interferences and selectivity, as per drafted ASB standards (14). See Table I for the MS parameters for each target analyte transition, and Supplementary Table SIII for the MS parameters of ISTD.

Validation

All required validation studies were planned and assessed according to the national ANSI/ASB Standard Practices for Method Validation in Forensic Toxicology (15).

Interferences and selectivity

Peak resolution was assessed chromatographically and by comparison of retention times of a given analyte, with those of preceding and succeeding analytes.

Interference and quadrupole crosstalk studies were carried out to assess the effect of the matrix, target drugs, utilized ISTD and other drugs possibly encountered during routine analysis. The lack of detectable drug responses in these samples indicated that the blank matrix did not interfere with the identification or quantitation of analytes in this method.

Blank matrix interferences were assessed with the extraction of 60 authentic sources (15 antemortem blood, 15 antemortem urine, 15 postmortem blood and 15 postmortem urine) without the addition of ISTD. In addition, the evaluation of the blank matrix used for routine analysis was assessed by the extraction of porcine blood and human urine without ISTD.

All 127 targeted analytes and 80 isotope-based ISTDs were evaluated by the extraction of each separately in both blood and urine, to confirm no interference with any other analytes above 10% of the blood and urine LOD or ISTD areas. Further, evaluation of any interferences from 376 analytes, consisting of other common pharmaceutical, recreational and deuterated drugs was achieved by analyzing fortified matrix samples in prevalent and relatively high concentrations, listed in Supplementary Table SIV.

Carryover

Carryover was assessed by analyzing blank matrix following a sample from 1 to 5 times the concentration of the ULOQ in blood, or 1 to 5 times the concentration of QCH in urine. Any carryover peaks were compared to their respective LOD response.

Ion suppression/enhancement

The effects of the matrix on ion suppression/enhancement of target analytes and ISTD were estimated with two different sample sets of QCL and QCH with ISTD using 20 different volunteer samples each (10 × blood, 10 × urine) (16). The neat standard (set 1) and blank matrix spiked with target analytes after extraction (set 2) were used to estimate ion suppression/enhancement by comparison of the peak area ratios of set 2 to those of set 1. All comparisons were made with percentages and coefficient of variation percentage (CV%). Positive values indicate ion enhancement, while negative values indicate

ion suppression. The targeted average range for each analyte was ≤ ±25% and the targeted CV% ≤ 20%.

LOD and LLOQ

The LOD for each analyte was established through parallel dilutions to the lowest concentrations that would still retain a signal-to-noise ratio (S:N) of at least 3:1 in blood and urine samples. The LOD was evaluated from spiked blank matrix samples from three different sources of blood and nine different sources of urine. The LLOQ (i.e., CAL 1) was assessed with an S:N of at least 10:1 in blood samples. In addition to S:N, retention times and ion ratios were assessed (14). The LLOQ was evaluated from spiked blank matrix samples from three different sources of blood, and accuracy and precision were tested using one-way ANOVA. Additionally, visual inspection was undertaken to ensure appropriate chromatography and acceptable integration for both LOD and LLOQ samples.

Calibration model

Linearity was evaluated through the analysis of all blood calibration points from CAL 1 (LLOQ) to CAL 7 (ULOQ) over five analyses on separate days at the same time as accuracy and precession studies. The residual plots for linear and quadratic (with none, 1/x or $1/x^2$ weighting) regression models were assessed for suitability for quantitative target analytes. The acceptability criteria for all quantitative blood calibration curves were set as a coefficient of determination (R^2) greater than 0.990 and ± 20% accuracy on any individual calibration point, and not more than one of seven calibrators was excluded.

Accuracy and precision

Accuracy (bias) and precision were evaluated in triplicate samples over five different days at the QCL, QCM and QCH concentrations fortified in blood matrix. Accuracy was calculated as the relative difference of the grand mean from the nominal value per analyte per concentration. The acceptability criterion for accuracy was ≤ ±20% for each target analyte and at each concentration. Precision was expressed as the CV. Two different types of precision studies were assessed during method validation: within-run precision (within-run CV) and between-run precision (between-run CV). Within-run and between-run CV were calculated using the one-way ANOVA approach. The acceptability criterion for within-run and between-run CV precision studies was ≤ ±20% for each target analyte and at each concentration.

Dilution integrity

Dilution integrity was assessed at 1/2 and 1/5 dilutions of double the concentration of QCH (i.e., QCHH) with the same above accuracy and precision criteria applied to QCL, QCM and QCH.

Processed sample stability

To assess processed sample stability under the conditions of the described method, extracted blood and urine samples were spiked with ISTD and either urine's LOD or QCH and suspended in the analytical method reconstitution matrix, pooled, redistributed into separate autosampler vials and subsequently analyzed. The autosampler tray was controlled at the same temperature as the analytical method. Injections occurred ∼every 6 hours for a total of 96 hours (4 days). Stability was accepted when ≥ 80% peak area ratio and S:N greater than 10:1 were maintained.

Applicability

Applicability experiments were carried out by testing proficiency samples received within recent years. Fifty blood and twenty-eight urine samples were analyzed using the described method and assessed with consideration for sample integrity, degradation over time, microbial putrefaction of sample and additional information from the proficiency sample providers.

A second applicability study was accomplished by comparing postmortem casework results of an established in-house method that contained 37 of the same target analytes in the described method and analyzed contemporaneously (17).

Results

Validation

Interferences and selectivity

Chromatographic resolution (peak resolution ≥ 1.0 widths) was achieved for most target analytes, depicted by Supplementary Figure S1. The LC method produced baseline chromatographic resolution between isobaric analytes (codeine and hydrocodone; morphine and hydromorphone; 6-monoacetylmorphine and naloxone; methamphetamine and phentermine). The following isomers were not separated and thus reported as a group, with the specific reference material utilized to represent the group listed first; ephedrine/pseudoephedrine, norephedrine/norpseudoephedrine, meta-hydroxy cocaine/para-hydroxy cocaine, dextromethorphan/levomethorphan, dextrorphan/levorphanol, zopiclone/eszopiclone, amobarbital/pentobarbital and butalbital/talbutal. Chiral resolution was not established for this method and, thus, all identified analytes are reported racemic. All peaks except for psilocybin and GHB displayed a general Gaussian distribution.

No analytes were falsely identified when analyzed in the presence of over 376 common therapeutic and illicit drugs and poisons that may possibly be encountered in forensic casework (Supplementary Table SIV). No ISTD created an interference with target analytes greater than the LOD. Following the injection of 40 authentic samples, any observed peaks were either separable by retention time, separable by ion ratio and/or below LOD.

Carryover

No target analyte carryover response greater than the LOD area ratio was observed following the injection of blood samples at 1–5 times the ULOQ and urine samples at 1–5 times the QCH.

Ion suppression/enhancement

Ion suppression and enhancement results are listed in Supplementary Table SV. Blood had no outliers exceeding ±25% average ME or exceeding ±20% CV. Urine had some outliers namely with analytes that did not have a matched deuterated ISTD (CBD, carisoprodol, deschloroketamine, lamotrigine, nalbuphine, naloxone, norbuprenorphine, norcodeine, norephedrine/norpseudoephedrine, noroxymorphone, O-desmethyl tramadol, psilocin, tapentadol, tramadol, zolazopam and zolpidem phenyl-4-carboxylic acid). To determine any influence outliers had on the ability for analytes to be qualitatively detected in urine above the LOD, nine authentic urine samples were spiked with LOD over 3 days in addition to the LOD study below. All analytes were observed with acceptable identification criteria including an S:N greater than 3:1, ion ratios and retention times.

LOD and LLOQ

The concentrations for blood LOD and LLOQ are summarized in Table I. The area ratio of the blood LOD sample was used as the threshold for qualitative reporting below the blood LLOQ concentration for all analytes except THC, 11-hydroxy THC, CBD, CBG, CNB, and GHB that used the LLOQ as a threshold for qualitative and quantitative reporting. The LOD and LLOQ for analytes were administratively set at, or below, concentrations recommended in DUID and other forensic casework. Even so, significantly greater S:N than the required 3:1 (LOD) or 10:1 (LLOQ) were observed, often above 100:1, in addition to correct ion ratios and retention times. All analytes met accuracy and precision requirements at LLOQ, with the exceptions of alpha-hydroxy alprazolam, buprenorphine, CBG, deschloroketamine, GHB, nalbuphine, norbuprenorphine, promethazine, psilocin, suvorexant and zolpidem phenyl-4-carboxylic acid. These analytes still maintain their LOD cut-off and, however, are reported qualitatively less than the QCL following the demonstration of acceptable accuracy and precision at that concentration.

For urine analysis, the area ratio of the urine LOD sample was used for to produce a qualitative threshold for positive identification and qualitative reporting with all analytes demonstrating acceptable ion ratios, retention times and S:N of above 3:1, often above 100:1.

Calibration model

Random distribution was best observed with quadratic and weighted 1/x regression model for analytes and thus was deemed appropriate for the quantitation of the analytes in blood across the chosen analytical range. All quantitative data collected for accuracy and precession, and applicability studies contained calibrations with R^2 values >0.990, calibrator accuracies with ±20% the target and no more than one of seven calibrators excluded.

Accuracy and precision

Accuracy and precision data for each analyte in blood are summarized by averaging triplicate data repeated over 5 days, shown in Supplementary Table SII. All analytes produced acceptable results for accuracy (bias) at the QCL, QCM and QCH levels. All blood quantitated analytes produced acceptable precision results for within-run CV, between-run CV, except for anhydroecgonine methyl ester and carisoprodol at QCL and QCH concentrations.

Dilution integrity

Accuracy and precision data for each analyte with 1/2 and 1/5 dilutions of fortified matrix samples prepared at double the QCH concentrations (QCHH) providing quantitative results otherwise above of the analytical range (i.e., >ULOQ) were analyzed in triplicate over 5 days. Dilution integrity showed generally acceptable results for most target analytes. Eight analytes (benzoylecgonine, CBD, CBG, carisoprodol, lamotrigine, norbuprenorphine, norephedrine/norpseudoephedrine, and psilocin) produced some results beyond the targeted criteria.

Processed sample stability

In blood and urine samples, analytes exhibited >80% stability for at least 12 hours, enabling a large number of samples to be analyzed considering the 8 minute acquisition time. At the 24 hour period, psilocin, mazindol, zopiclone-N-oxide and CBGA showed some degradation in blood, and THCA and THCV in urine.

Applicability

The pre-implementation application study was performed on 50 blood and 28 urine previously analyzed proficiency test samples received over an ~3 year period. In blood samples, there were 118 expected results comprised of 51 unique targeted analytes that were within the scope of the described method. Of the expected 118 results, 116 were detected above the LOD. The "absent" two were indeed observed with acceptable identification criteria and, however, were below the administrative LOD. In both cases, the analyte was 11-hydroxy THC, which likely occurred due to the age of the samples (7 months for one, 1 year for the other), since 11-hydroxy THC is known to degrade in blood over time (18–20).

In urine samples, there were 64 expected results comprised of 36 unique targeted analytes within the scope of the described method. Of the expected 64 results, 61 were detected above the LOD. The "absent" three urine results, like those in blood, were observed with acceptable identification criteria and, however, were below the administrative LOD. In all three cases, the expected concentrations of these analytes were simply less than the LOD.

Further, there were 60 blood and 16 urine analytes that were outside of the scope of this method and none interfered with target analytes within the scope of this method.

A second post-implementation application study was performed on authentic forensic casework over an ~6 month period on blood ($n = 940$) and urine ($n = 705$) samples using the described method and an existing published in-house analytical method (17). Matched target analytes in both methods correlated and were assessed to be acceptable at the time of reporting. The described method demonstrated the efficient ability to comprehensively detect drug and polysubstance trends apparent in current forensic casework.

Discussion

Multi-class methods currently in existence tend to be limited in scope to less than 50 target analytes (12, 13, 21, 22). Even with quantitative methods with more than 50 targets, many of the recommended Tier I drugs may be excluded and/or not meet ASB recommended sensitivity requirements for DUID toxicology testing (17, 23). Further, most methods require either liquid–liquid, solid-phase or supported liquid extraction techniques. Although these procedures can be streamlined, they typically require more involvement during the extraction and are more selective in nature, which can inhibit inclusion of all recommended drugs (8–13, 21, 22).

With a change in forensic toxicological regime ideology and considering improvements in available technologies, the development of a more inclusive methodology approach is feasible. Mass spectrometry continues to have faster MRM acquisition and polarity switching speeds, allowing for the acquisition of many transitions in one method in both positive and negative ionization modes without data loss and with acceptable quantitation as ample points across the peak are achieved. Additionally, the development of mass spectrometer detectors with larger dynamic response ranges and improved quadrupole electronics can increase the sensitivity of methods by enabling detection of both very low, and high, concentrations of target analytes within the same analysis.

Data processing software was customized in-house to include automatic calculations assessment, color-coding and flagging of relative retention time, QC acceptance of bracketed casework, ISTD response, ion ratios and analytical ranges, and improves on a previously developed processing query by the authors (17). This further allowed for the automated and efficient ability to identify results in

analyte-tailored reporting ranges of either quantitation or qualitative formats, even when both formats existed within a single testing batch.

The presented method demonstrated the sensitive, rapid and multi-class testing capability to comprehensively analyze the full scope of standardized and recommended DUID drugs, eliminating blind spots and reducing the number of tests typically required. Decreased time and resource requirements were achieved by the simple extraction of blood and urine by protein precipitation followed by size-exclusion filtration and a rapid 8 minute LC–MS/MS analysis. Screening and identification was achieved for 127 target drug and metabolite analytes utilizing 80 ISTDs. Target drug categories and the number of analytes within each group were cannabinoids (12), amphetamines (11), cocaine and metabolites (6), benzodiazepines (36), Z-drugs (5), opioids (27), anticonvulsants (3), first-generation antihistamines (6), muscle relaxants (2), dissociatives and hallucinogens (6), barbiturates (10) and miscellaneous (3). Zopiclone-n-oxide was not included in blood analysis. Quantitation in blood was performed for all analytes except: cannabinoid acids, by design; barbiturates, due to the infeasibility of incorporating their ULOQ concentrations into the main calibration mix using commercially available certified reference standards; zopiclone/eszopiclone, due to degradation in methanol over time; and psilocybin; for a total of 108 quantitated analytes. While the quantitation of cannabinoid acids may not be beneficial in typical DUID casework, barbiturate quantitation may be quantitated via a separate calibration preparation using the same extraction and LC–MS/MS conditions described herein.

Limits of Detection were designed to be more sensitive than the NSC-ADID recommendation and the drafted ANSI/ASB standard required for DUID analysis, in order to be utilized for other forensic casework also, such as drug-facilitated crime (DFC) (e.g., sexual assault DFC). Forensic toxicology laboratories may increase the limit of reporting of DUID casework if desired, while still enabling the same method to be utilized in other forensic casework requiring enhanced sensitivity testing, improving the overall laboratory testing efficiency. The successful application and efficient assessment of proficiency tests and authentic casework was observed using the described method. The described method serves as an example of the capability and effectiveness of considered use of newer technology towards the advancement of forensic toxicology laboratories and their ability to withstand the ever-increasing demands of both more casework and more complex toxicological profiles.

Conclusion

The described fully validated and applied LC–MS/MS method exceeds the national standardized and recommended scopes of DUID screening categories. By increasing the analytical scope of multiple drug-class categories via a single method, this technique detects drugs that may have previously gone undetected and improves laboratory efficiency by reducing the number of tests required. The described method is, to the authors' best knowledge, the only single procedure to meet all proposed ANSI/ASB standardized drugs, and NSC-ADID Tier 1 and traditional Tier 2 recommended drugs, for DUID testing while also meeting many required recommendations for DFC and postmortem testing.

Supplementary Data

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

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