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A fast and simple approach for the quantification of 40 illicit drugs, medicines, and pesticides in blood and urine samples by UHPLC-MS/MS

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Abstract

A fast and simple approach to overcome challenges in emergency toxicological analysis, using ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) has been developed, for the detection of analytes in blood and urine samples from the following drug classes: analgesics, benzodiazepines, antidepressants, anticonvulsants, drugs of abuse, and pesticides. These substances are relevant in the context of emergency toxicology in Brazil. The sample preparation procedure was relatively easy and fast to perform. The method was fully validated giving limits of in the range of 0.5 and 20 ng mL^{-1} for blood and urine samples. The intraday and interday precision and accuracy were considered adequate for all analytes once the relative standard deviation (RSD) (%) was lower than 20% for quality control (QC) low and lower than 15% for CQ medium and high. The developed method was successfully applied to 320 real samples collected at the Poison Control Center of São Paulo, and 89.1% have shown to be positive for some of the analytes. This confirms its applicability and importance to emergency toxicological analysis, and it could be very useful in both fields of clinical and forensic toxicology.

KEYWORDS

analytical toxicology, emergency, multi-analyte, UHPLC-MS/MS

1 | INTRODUCTION

Intoxications are considered an important cause of mortality, and it has increased over the last years in Brazil, accounting for a considerable proportion of all emergency care.[1] The clinical prognosis of a patient can be influenced by the rapid identification of the exposure and the treatment given in the first few hours of the emergency admission.[2-4] According to data collected from the Information System on Diseases of Compulsory Declaration (a governmental system responsible for poisoning notifications in Brazil), 499.986 human exposures to xenobiotic were reported during the period of 2013-2017. The most detected substance classes were medicines, drugs of abuse, and pesticides.[1]

Generally, blood represents the matrix of choice in clinical toxicological analyses, since pharmacological effects may be correlated to its concentration. Therapeutic and toxic concentration values of several compounds are well established.[5] However, in blood, analyte levels rapidly fall below the limit of quantification, requiring the use of a secondary matrix. In this context, urine analysis can be appropriate, allowing for the disclosure of substances and/or biotransformation products with a

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TABLE 1 SRM transitions and experimental conditions for all compounds and internal standards detection

Compound Name	RT, min	Precursor Ion, *m/z*	Product Ion, *m/z*	CE, V	Polarity[a]	I.S.
6-Acetylmorphine	1.26	327.90	165.20 211.10	38 27	+	1
Acetaminophen	0.92	152.10	110.05 65.05	18 31	+	15
AEME	0.84	182.10	91.10 118.10	27 21	+	15
Aldicarb	1.90	270.90	166.10 89.10	7 18	+	19
Alprazolam	2.66	309.25	281.05 205.10	26 41	+	16
Aminoclonazepam	1.74	286.70	121.10 122.10	29 24	+	2
Amitriptyline	2.47	278.10	278.10 278.10	18 24	+	3
Amphetamine	1.06	233.20	233.20 233.20	19 14	+	4
Benzoylecgonine	1.50	290.30	168.15 105.05	19 30	+	5
Bromazepam	2.87	315.9/317.9	182.05 290.05	32 26	+	19
Carbamazepine	2.26	236.95	194.10 192.15	20 22	+	19
Carbofuran	2.30	222.20	123.05 165.10	11 11	+	19
Clonazepam	2.47	316.20	270.05 214.05	27 37	+	16
Clordiazepoxide	1.91	300.70	282.15 227.15	23 15	+	16
Cocaethylene	1.99	317.90	196.20 82.15	20 31	+	6
Cocaine	1.79	304.30	182.20 105.00	21 32	+	7
Codeine	1.18	300.40	215.10 152.10	25 42	+	8
Desipramine	2.33	267.00	72.10 44.05	16 40	+	9
Diazepam	2.79	285.20	193.05 154.05	32 27	+	16
Flunitrazepam	2.64	314.00	268.10 239.10	25 34	+	16
Fluoxetine	2.37	309.95	43.95 184.40	12 09	+	10
Imipramine	2.40	281.00	86.10 58.10	17 42	+	11
MDA	1.16	180.20	105.05 163.20	12 21	+	12
MDMA	1.30	194.20	163.15 105.10	14 24	+	13
Metamphetamine	1.20	150.20	91.05 65.05	20 41	+	14

(Continues)

TABLE 1 (Continued)

Compound Name	RT, min	Precursor Ion, *m/z*	Product Ion, *m/z*	CE, V	Polarity[a]	I.S.
Midazolam	2.17	326.20	291.10 223.15	26 38	+	16
Morphine	0.84	285.95	152.15 201.20	55 25	+	15
Nitrazepam	2.39	281.90	236.10 180.05	24 36	+	16
Norcocaine	1.77	290.00	168.15 136.10	16 23	+	7
Nordiazepam	2.49	271.20	140.05 165.05	28 28	+	16
Norfluoxetine	2.31	296.30	134.15 30.15	07 24	+	17
Nortriptyline	2.40	264.40	233.20 91.05	14 21	+	18
Oxazepam	2.31	287.20	241.20 269.05	23 15	+	19
Paroxetine	2.30	329.90	192.25 70.10	21 32	+	19
Phenobarbital	1.85	231.10	41.95 187.95	22 10	–	20
Phenytoin	2.22	250.90	208.15 102.10	30 16	–	20
Sertraline	2.53	307.70	161.10 277.10	26 13	+	19
Temazepam	2.57	301.20	255.15 283.05	21 13	+	16
THC-COOH	3.10	343.20	299.30 191.15	21 29	–	21
Valproic acid	2.30	143.00	143.00	15	–	20
6-Acetylmorphine-d_3	1.26	331.00	165.15	39	+	–
Aminclonazepam-d_4	1.74	290.25	121.10	30	+	–
Amitriptyline-d_5	2.47	281.10	233.20	18	+	–
Amphetamine-d5	1.06	141.15	96.05	17	+	–
Benzoylecgonine-d_3	1.50	292.95	171.28	20	+	–
Cocaethylene-d_3	1.99	321.10	199.15	21	+	–
Cocaine-d_3	1.79	307.15	185.10	21	+	–
Codeine-d_3	1.18	303.05	251.25	25	+	–
Desipramine-d_3	2.33	270.20	75.10	17	+	–
Fluoxetine-d_6	2.37	315.95	44.00	13	+	–
Imipramine-d_3	2.40	284.15	89.20	17	+	–
MDA-d_5	1.16	184.95	168.25	12	+	–
MDMA-d_5	1.30	199.15	165.15	14	+	–
Methamphetamine-d_5	1.20	155.05		92.10	22	+
Morphine-d_3	0.84	289.15	152.15	55	+	–
Nordiazepam-d_5	2.49	276.25	140.05	27	+	–
Norfluoxetine-d_6	2.31	302.30	140.40	13	+	–
Nortriptyline-d_3	2.40	267.10	233.20	15	+	–

(Continues)

TABLE 1 (Continued)

Compound Name	RT, min	Precursor Ion, *m/z*	Product Ion, *m/z*	CE, V	Polarity[a]	I.S.
Oxazepam-d_5	2.31	292.25	246.10	24	+	–
Phenobarbital-d_5	1.85	236.20	41.95	22	–	–
THC-COOH-d_3	3.10	346.20	299.30	21	–	–

Abbreviations: CE, collision energy; I.S., the corresponding number under "Internal standards (I.S.)"; RT, retention time.

[a]Positive (+) or negative (−) polarity.

longer time interval between exposure and laboratory analysis. In fact, urine can be considered a biological matrix to be used in parallel with blood in the emergency analyses.[6-11]

Several articles have been published using different work-up procedures designed for the detection of different analytes of interest, and a wide variety of substances can be detected in blood or also in urine in a single analysis.[12-18] In some cases, there is no previous knowledge about the poisoning history of the patient. Then the preliminary screening procedures are necessary for the identification of the possible substance(s) involved. Therefore, the analytical strategy in toxicological analysis should focus on developing features such as simultaneous monitoring of a large number of toxic agents, simplicity, and rapid sample preparation.[9,10,12,19,20] In the last years, ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) has proven to be a very useful analytical tool that provides a satisfactory identification and quantitation of substances in the emergency toxicology field.[16,21-24] In addition, some classical techniques have been reported for multi-analyte approach aiming the quantitation of drugs and poisons in biosamples. The main techniques used for this purpose involve liquid-liquid extraction (LLE)[25-28] or solid-phase extraction (SPE).[7,22,29-31] However, the major limitation of both techniques is the consumption of large volumes of organic solvents, which can be toxic to the analysts and hazardous to the environment.

The newly trend in area include the use of a simple dilute and shoot approach. This technique combines the use of small volumes of solvents, besides being very simple and rapid, and it requires only a little amount of blood and urine sample. Therefore, the purpose of this study was to develop a fast and simple screening technique for the determination of different class of toxic agents, including pesticides, in blood and urine samples using UHPLC-MS/MS. Furthermore, the method was successfully applied in 320 real samples of Poison Control Center of Sao Paulo helping in the triage process and improving emergency healthcare.

2 | MATERIAL AND METHODS

2.1 | Chemical and reagents

Methanol and acetonitrile HPLC grade were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Billerica, Massachusetts). Formic acid (98%-100% grade) was purchased from Merck (Darmstadt, DE), and ammonium formate was obtained from Fluka (Buchs, Switzerland) (≥97% purity). All reference standards were ≥98% purity. Water (18 MΩ) was purified using a Milli-Q purification system (Millipore Corp, Bedford, Massachusetts).

2.2 | Standards and solutions

Drugs of abuse and metabolites (6-acetylmorphine, AEME, amphetamine, benzoylecgonine, cocaethylene, cocaine, codeine, MDA, MDMA, methamphetamine, morphine, norcocaine, and THC-COOH), benzodiazepines (alprazolam, aminoclonazepam, bromazepam, clonazepam, clordiazepoxide, diazepam, flunitrazepam, midazolam, nitrazepam, nordiazepam, oxazepam, and temazepam), antidepressants (amitriptyline, desipramine, fluoxetine, imipramine, norfluoxetine, nortriptyline, paroxetine, and sertraline), anticonvulsants (carbamazepine, phenobarbital, phenytoin, and valproic acid), and acetaminophen at a concentration of 1.0 mg mL^{-1} were obtained from Cerilliant Analytical Reference Standards (Round Rock, Texas). Internal standards (6-acetylmorphine-d_3, aminoclonazepam-d_4, amitriptyline-d_5, amphetamine-d_5, benzoylecgonine-d_3, cocaethylene-d_3, cocaine-d_3, codeine-d_3, desipramine-d_3, phenobarbital-d_5, fluoxetine-d_6, imipramine-d_3, MDA-d_5, MDMA-d_5, morphine-d_3, nordiazepam-d_5, norfluoxetine-d_6, nortriptyline-d_3, oxazepam-d_5, methamphetamine-d_5, and THC-COOH-d_3) at a concentration of 100 μg mL^{-1} were obtained from Cerilliant Analytical Reference Standards (Round Rock, Texas). Pesticides' standards (aldicarb and carbofuran) were obtained from Sigma-Aldrich (>99%; St. Louis, EUA). Stock solutions of each analyte were prepared in methanol and appropriately refrigerated (2°C-8°C), when not in use. Cocaine, norcocaine, cocaethylene, AEME, 6-acetylmorphine, and pesticides were prepared using acetonitrile.

Spiking solutions in methanol or acetonitrile of the six drug classes were prepared separately to obtain the corresponding low-quality control (QC low), medium-quality control (QC medium), and high-quality control (QC high) concentrations of each analyte.

2.3 | Instrumentation

LC system was a Nexera X2 UHPLC, which consisted of a degasser, a binary pump, and an autosampler coupled to an LC-MS 8050 mass spectrometer (Shimadzu, Japan) with an electrospray source operating in the positive (ESI+) and negative (ESI−) ion modes, in two separate chromatographic runs. The chromatographic separation was achieved on a Raptor Biphenyl column (50 mm × 3 mm, 2.7 μm; Restek, USA) eluted with flow rate of 600 μL min^{-1} and 45°C.

Chromatographic conditions were evaluated in order to obtain a satisfactory chromatographic separation for all compounds. Based on electrospray ionization (ESI) mode of each substance, two chromatographic methods were developed in parallel.

2.3.1 | Positive ESI method

The mobile phase consisted of 2mM ammonium formate with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The gradient was programmed as follows: 0 to 4 minutes, 8% to 98%, B and 4 to 4.1 minutes, 98% to 8% B. The total run time was 6.0 minutes, including re-equilibration at the initial conditions: nebulizing gas flow, 2 L min^{-1}; heating gas flow, 10 L min^{-1}; drying gas flow, 10 L min^{-1}; interface temperature, 300°C; heat block temperature, 400°C; DL temperature, 250°C.

2.3.2 | Negative ESI method

The mobile phase consisted of 0.2% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B). The gradient was programmed as follows: 0 to 1.5 minutes, 35% to 95% B; 1 to 1.5 minutes, 95% B; and 1.7 to 1.8 minutes, 95% to 35% B. The total run time was 4.0 minutes: nebulizing gas flow, 2 L min^{-1}; heating gas flow, 10 L min^{-1}; drying gas flow, 10 L min^{-1}; interface temperature, 300°C; heat block temperature, 400°C; DL temperature, 250°C.

The selected reaction monitoring (SRM) transitions of all analytes are listed in Table 1. For data evaluation, LabSolutions software was used to obtain peak areas. Statistical analysis was performed on Microsoft Excel 2013.

2.4 | Preparation of samples

2.4.1 | Blood sample analysis

An aliquot of 800 µL of an acetonitrile/methanol mixture (80:20, *v*/v) was added to the blood samples (100 µL) spiked with 20 µL of the internal standards mix (I.S. mix; 0.5 µg mL^{-1}), and the mixture was shaken for 30 seconds. After centrifugation for 6 minutes at 9000 × g, a 3-µL aliquot was directly injected into the UHPLC-MS/MS system.

2.4.2 | Urine sample analysis

An aliquot of 100 µL of urine samples with 20 µL of I.S. mix (0.5 µg mL^{-1}) was mixed with 75 µL of ammonium acetate buffer 0.2M (pH 4.8) and 5 µL of β-glucuronidase enzyme (500 U) and incubated at 55°C for 1 hour. The sample was diluted with 800 µL of methanol and water (60:40, *v*/v). Afterwards, the sample tube was centrifuged at 9,000 × g for 6 minutes. An aliquot of 15 µL was injected into the UHPLC-MS/MS system.

TABLE 2 Quality controls (QC) used in method validation

	Quality Control			
	Blood	Urine	Blood and Urine	
Analyte	QC Low, ng mL^{-1}	QC Low, ng mL^{-1}	QC Medium, ng mL^{-1}	QC High, ng mL^{-1}
6-Acetylmorphine	3	3	40	80
Acetaminophen	60	60	750	1500
AEME	60	30	750	1500
Aldicarb	60	150	1,500	3000
Alprazolam	3	3	750	1500
Aminoclonazepam	3	60	750	1500
Amitriptyline	3	3	400	800
Amphetamine	3	15	750	1500
Benzoylecgonine	1.5	3	750	1500
Bromazepam	30	60	750	1500
Carbamazepine	3	1.5	750	1500
Carbofuran	1.5	3	750	1500
Clonazepam	30	15	750	1500
Clordiazepoxide	30	30	750	1500
Cocaethylene	1.5	3	750	1500
Cocaine	1.5	1.5	750	1500
Codeine	15	30	750	1500
Desipramine	3	3	400	800
Diazepam	15	3	750	1500
Flunitrazepam	15	15	750	1500
Fluoxetine	3	1.5	400	800
Imipramine	1.5	1.5	400	800
MDA	15	30	750	1500
MDMA	3	15	750	1500
Methamphetamine	1.5	1.5	750	1500
Midazolam	15	3	750	1500
Morphine	30	30	750	1500
Nitrazepam	15	15	750	1500
Norcocaine	15	3	750	1500
Nordiazepam	15	3	750	1500
Norfluoxetine	15	15	400	800
Nortriptyline	3	1.5	400	800
Oxazepam	15	30	750	1500
Paroxetine	15	15	400	800
Phenobarbital	30	60	900	1800
Phenytoin	30	60	900	1800
Sertraline	3	3	400	800
Temazepam	15	3	750	1500
THC-COOH	-	15	500	1000
Valproic acid	60	60	900	1800

2.5 | Validation

The validation was performed according to international guidelines[32,33] and recommendations,[34,35] and the parameters evaluated were the lower limit of quantitation (LLOQ), selectivity, linearity, precision, accuracy, matrix effect, and carryover.

The LLOQ values were determined by empirical (experimental) method, which consists of analyzing a series of samples containing increasingly lower concentrations of the analyte.

To evaluate selectivity, 10 different drug-free biosamples were used. The samples were extracted and analyzed according to the previously described method. Additionally, 10 blank urine and blood samples fortified with lidocaine, nicotine, atenolol, diclofenac, diphenhydramine, caffeine, and acetylsalicylic acid were submitted to the method for the evaluation of potential interfering substances. Peaks at the retention time of interest were compared with those from urine and blood samples spiked with the analytes at the LLOQ.

The study of linearity was examined by the analyses of blood and urine samples spiked with standards at six different concentrations in six replicates at each concentration.

The precision and accuracy were evaluated using samples containing three quality control concentrations (Table 2). The study was performed with analysis of six replicates on each of 3 days, and the precision data (within and between-day) were calculated using one-way ANOVA with day as a grouping variable to adequately account and combine for within and between day effects. The results were expressed as percent relative standard deviation (%RSD). Accuracy was expressed as a percentage of the known concentration, ie, the mean measured concentration/nominal concentration × 100, or percent bias. Precision and accuracy should be within ±15%.[32]

Dilution integrity was estimated in urine samples, which are beyond the upper limit of the standard curve and need to be diluted. The samples were diluted 10 times with water and analyzed by

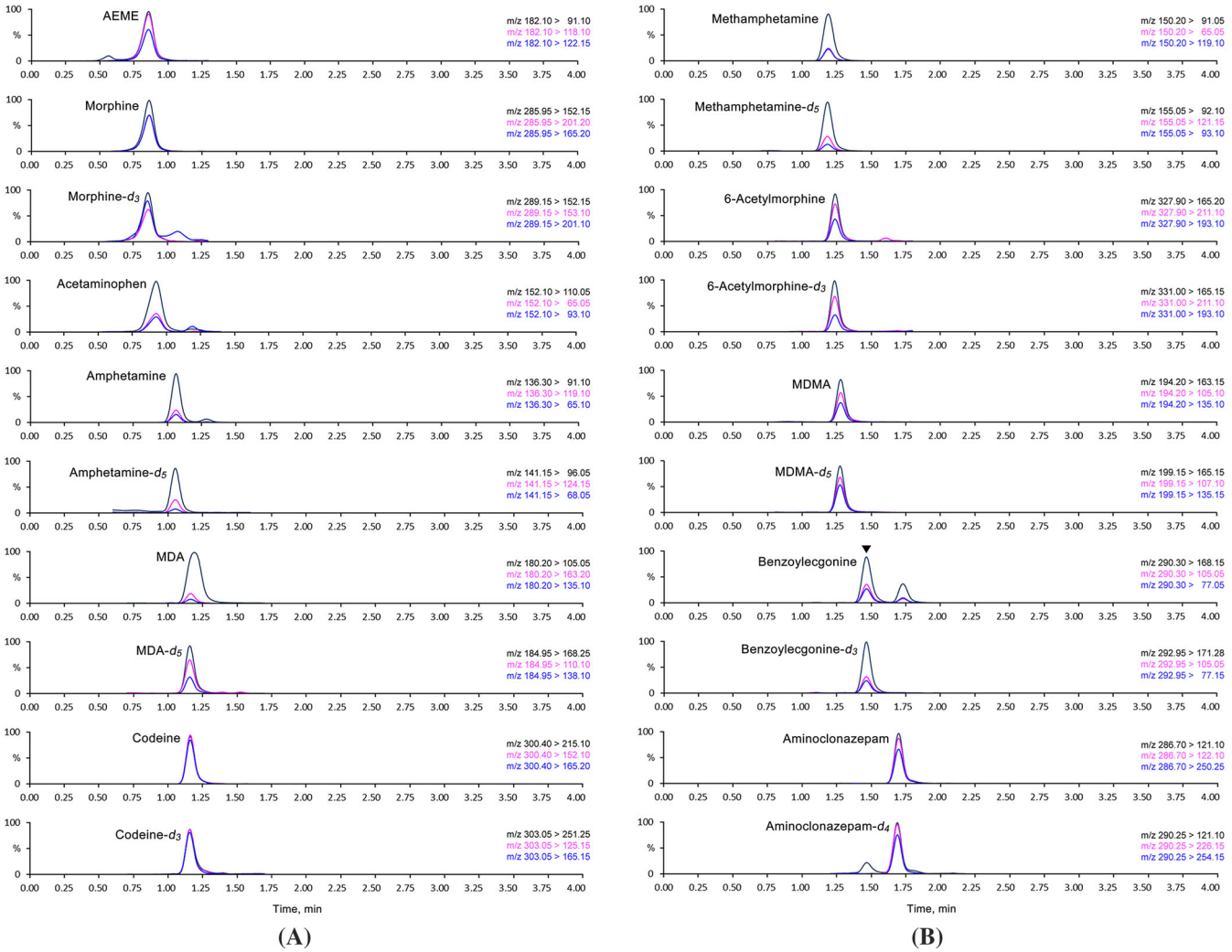

FIGURE 1 Chromatogram obtained by LC-MS/MS from a urine sample spiked with drugs of abuse, benzodiazepines, antidepressants, anticonvulsants, and pesticides, which have been submitted to the developed method [Colour figure can be viewed at wileyonlinelibrary.com]

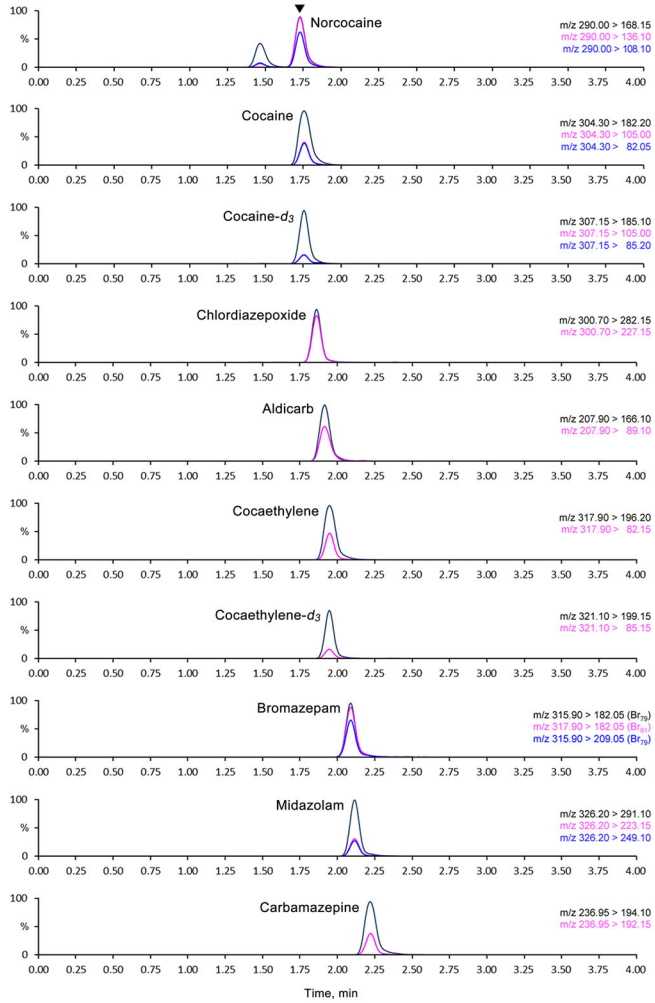

FIGURE 1 Continued.

calculation through the regression equation obtained. Accuracy and precision should be within the set criteria, ie, within ±15%.[33]

The matrix effect (ME) was calculated according Hegstad et al[21] and Matuszewski et al,[36] where six samples of different individuals added to the standard (low control concentration) were analyzed. The results were compared with the results in the solvent mixture (methanol/water for urine or acetonitrile/methanol for blood) as follows (Equation 1):

$$\mathrm{ME}\% = \left(\left(\frac{\text{Peak intensity matrix} \,/\, \text{Peak IS intensity matrix})_n}{\bar{x}\left(\text{Peak intensity solvent mixture} \,/\, \text{Peak IS intensity solvent mixture}\right)} \right) \times 100. \quad (1)$$

According the Scientific Working Group for Forensic Toxicology (SWGTOX), the carryover must be evaluated during method validation intended for confirmation and/or quantitation to verify potential contamination of the blank samples. The highest analyte concentration at which no analyte carryover is observe in the blank matrix sample is determined to be the concentration at which the method is free from carryover.[33]

2.6 | Proof of applicability

The developed method was applied to 320 real samples (blood and urine) collected from patients with suspected poisoning, subject to toxicological analysis, evaluated at the Poisoning Control Center-SP located in the Hospital Dr. Arthur Ribeiro de Saboya (HMARS), São Paulo, Brazil. Samples were collected between November 2014 and August 2016. All samples were analyzed immediately after collection and then were stored at −20°C. The protocol of study has been previously approved by the Bioethics Committee in Medicine of the Municipal Hospital "Arthur Ribeiro de Saboya" (Ethics Protocol Approval No.018/CEM/HMARS - 2014) and by the Research Ethics Committee of the School of Pharmaceutical Sciences of the University of São Paulo (Ethics Protocol Approval No. 902 088).

3 | RESULTS AND DISCUSSION

Considering limited sample volumes and the need for rapid results, a multi-analyte approach covering a broad range of potential toxic agents can be an appropriate choice for toxicology laboratories.

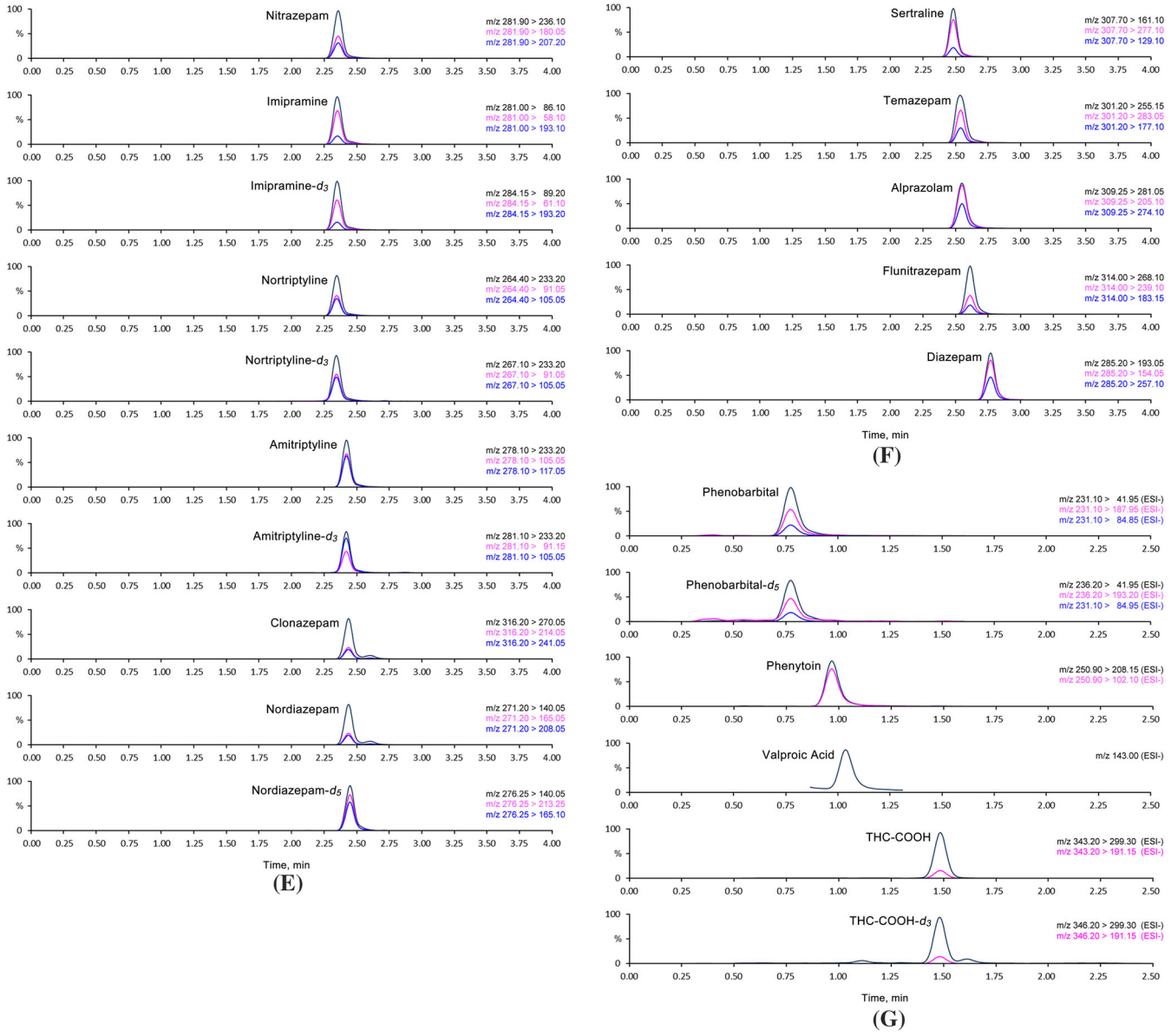

FIGURE 1 Continued.

Therefore, the "dilute and shoot" sample treatment is preferable to optimize and simplify the sample preparation. The developed procedure showed to be very simple and rapid, and only a little amount of blood and urine sample was necessary for the toxicological analyses. The newly developed method has been validated for limits of quantitation, linearity, selectivity, intraday and interday precision, accuracy, and matrix effects. Figure 1 shows the chromatogram obtained by LC-MS/MS from a urine sample spiked with drugs of abuse, benzodiazepines, antidepressants, anticonvulsants, and pesticides, which have been submitted to the developed method.

3.1 | Method validation

The selectivity study was performed under the specified test conditions, and no significant level of interfering endogenous or exogenous substances at the retention time of the analyte was observed in blood and urine analysis, thus confirming the selectivity of the method.

The limits found in the method in blood were considered satisfactory, since all present coefficients of variation within the acceptable range and are significantly below the toxic concentrations, which confirm the applicability of the method to the objective proposed in this work. The values were in the range of 0.5 and 20 ng mL^{-1}. These results were considered more significant than the values reported by Steuer et al[29] and Sempio et al.[37] In our study, we determined that LLOQs for whole blood ranged from 0.5 to 10 ng mL^{-1} for benzodiazepines and antidepressants, while Mut et al[38] reported values of 40 ng mL^{-1}. The LLOQs in urine analysis were considered adequate for all analytes once the RSD (%) was lower than 15%. The values were encountered in the range of 0.5 and 20 ng mL^{-1}. The values of LLOQ determined in our study are in agreement with the values

TABLE 3 Validation data on whole blood and urine samples

	Whole Blood						Urine					
Compound Name	LLOQ	L.R.	Intraday Precision (RSD), %	Interday Precision (RSD), %	Matrix Effect, %	Acc, %	LLOQ	L.R.	Intraday Precision (RSD) %	Interday Precision (RSD), %	Matrix Effect, %	Acc, %
6-Acetylmorphine	1	1-100	3.8-11.8	2.7-11.8	101.3	96-108	1	1-100	4.7-11.5	3.5-7.3	82.2	105-112
Acetaminophen	20	20-2000	7.5-8.0	6.8-9.6	98.1	100-106	20	20-2000	0.8-8.3	0.6-7.0	135.2	97-113
AEME	20	20-4000	7.4-13.7	6.7-13.5	115.2	99-101	10	10-4000	4.5-8.5	4.2-8.2	102.0	102-118
Aldicarb	20	20-4000	7.0-10.9	8.6-18.6	156.8	86-104	50	50-4000	8.9-18.6	7.4-13.7	109.7	90-112
Alprazolam	1	1-2000	7.1-13.5	6.4-10.4	98.0	86-100	1	1-2000	3.5-9.9	3.9-8.6	87.3	102-108
Aminoclonazepam	1	1-2000	4.9-11.8	23.0-7.2	81.4	90-111	20	20-2000	4.0-9.4	8.0-11.8	104.8	95-101
Amitriptyline	1	1-1000	4.7-16.3	3.3-13.3	109.1	82-94	1	1-1000	3.0-6.0	2.7-11.4	93.3	96-102
Amphetamine	1	1-4000	1.8-12.2	2.1-14.4	138.3	93-104	5	5-4000	7.5-8.6	7.0-10.9	74.0	96-110
Benzoylecgonine	0.5	0.5-2000	3.1-12.1	6.4-10.1	118.6	96-107	1	1-4000	2.7-10.4	7.8-18.0	102.0	92-103
Bromazepam	10	10-2000	8.5-15.8	6.7-12.9	152	96-98	20	20-2000	5.3-10.5	5.5-13.0	119.8	86-99
Carbamazepine	1	1-2000	7.8-17.2	6.1-12.5	114.6	97-108	0.5	0.5-2000	5.5-18.5	7.2-16.5	83.0	85-113
Carbofuran	0.5	0.5-2000	8.2-16.2	6.1-16.8	137.3	95-105	1	1-2000	5.8-7.8	4.3-15.3	69.2	90-110
Clonazepam	10	10-2000	7.2-13.9	9.8-10.3	114.8	99-115	5	5-2000	3.6-10.6	8.3-9.9	99.8	98-103
Clordiazepoxide	10	10-2000	8.6-9.3	7.9-10.9	110.3	97-105	10	10-2000	4.5-8.0	8.3-12.7	171.3	100-114
Cocaethylene	0.5	0.5-2000	2.9-15.7	2.5-9.9	131.9	89-103	1	1-4000	3.3-11.5	2.1-10.4	99.6	107-118
Cocaine	0.5	0.5-2000	2.6-15.8	2.6-9.1	110.1	95-99	0.5	0.5-4000	2.9-12.3	6.4-15.1	97.0	100-108
Codeine	5	5-2000	3.8-12.4	2.4-11.3	101.6	96-100	10	10-4000	3.0-4.9	4.5-7.1	98.6	101-113
Desipramine	1	1-1000	2.6-12.3	3.8-13.1	100.9	83-99	1	1-1000	6.9-10.5	8.9-12.7	107.3	89-111
Diazepam	5	5-2000	6.3-10.7	7.8-11.3	82.9	90-98	1	1-2000	7.3-14.2	4.4-15.6	87.9	83-105
Flunitrazepam	5	5-2000	6.4-10.3	6.3-9.7	91.3	89-98	5	5-2000	4.4-9.7	4.6-13.7	94.4	90-101
Fluoxetine	1	1-1000	3.9-9.9	3.7-8.2	132.3	98-99	0.5	0.5-1000	5.7-12.7	6.4-11.9	108.2	88-104
Imipramine	0.5	0.5-1000	2.7-13.6	4.6-14.0	82.5	95-100	0.5	0.5-1000	3.2-13.6	3.8-9.6	108.7	88-110
MDA	5	5-2000	1.8-9.1	2.3-11.9	113.0	95-99	10	10-4000	2.5-4.2	5.0-10.7	113.3	101-110
MDMA	1	1-2000	1.6-10.2	1.9-18.5	129.5	90-96	5	5-4000	2.0-4.5	4.5-6.6	99.1	100-105
Metamphetamine	0.5	0.5-2000	1.5-12.5	3.3-16.1	108.2	93-99	0.5	0.5-4000	2.6-16.7	3.4-18.2	97.6	96-109
Midazolam	5	5-2000	7.2-8.5	9.4-10.1	127.3	91-110	1	1-2000	3.6-11.0	14.1-17.5	99.8	93-104
Morphine	10	10-2000	6.8-11.5	7.6-17.5	100.3	97-106	10	10-4000	5.2-8.5	3.1-7.4	100.1	98-105
Nitrazepam	5	5-2000	6.9-12.1	6.0-14.0	134.2	92-96	5	5-2000	3.9-11.7	6.0-14.7	75.8	99-104
Norcocaine	5	5-2000	2.8-8.0	2.5-10.6	103.2	96-105	1	1-4000	2.3-7.5	1.9-5.3	140.9	100-104
Nordiazepam	5	5-2000	6.5-13.2	3.9-11.6	94.9	95-99	1	1-2000	3.5-12.4	3.5-10.5	87.4	105-111

(Continues)

TABLE 3 (Continued)

	Whole Blood					Urine					
Compound Name	LLOQ	L.R.	Intraday Precision (RSD), %	Interday Precision (RSD), %	Matrix Effect, %	Acc, %	LLOQ	L.R.	Intraday Precision (RSD) %	Interday Precision (RSD), %	Matrix Effect, %	Acc, %
Norfluoxetine	5	5-1000	12.6-14.4	12.4-14.3	122.4	94-99	10	10-1000	9.2-10.1	6.8-19.9	100.3	92-99
Nortriptyline	1	1-1000	3.4-12.1	4.2-6.0	99.7	88-99	0.5	0.5-1000	6.7-11.5	5.9-17.9	101.4	83-101
Oxazepam	5	5-2000	8.5-10.5	6.1-7.7	113.4	84-108	10	10-2000	4.7-8.2	3.4-8.3	87.3	97-100
Paroxetine	5	5-1000	9.5-9.6	9.2-17.9	125.8	94-103	5	5-1000	6.7-11.5	5.9-17.9	82.8	83-101
Phenobarbital	10	10-2000	9.3-19.8	6.9-13.9	84.0	93-110	20	20-4000	7.9-11.3	6.1-7.9	112.6	102-108
Phenytoin	10	10-2000	8.9-13.5	7.0-9.3	66.7	94-98	20	20-4000	7.8-10.0	8.3-11.5	91.7	89-108
Sertraline	1	1-1000	6.4-14.3	7.4-9.5	114.0	97-107	1	1-1000	4.7-6.6	6.0-14.4	60.8	88-98
Temazepam	5	5-2000	6.4-14.8	11.3-12.9	91.7	96-98	1	1-2000	4.1-17.1	7.2-15.2	85.6	98-113
THC-COOH	–	–	–	–		–	5	5-1200	3.4-8.6	13.6-15.6	127.9	96-110
Vaproic acid	20	20-4000	9.9-13.6	5.9-10.5	76.5	99-105	20	20-4000	10.9-13	9.2-14.4	40.15	94-113

Abbreviations: Acc, accuracy; LLOQ, lower limit of quantitation (ng mL^{-1}); L.R., linearity range (ng mL^{-1}); RSD, relative standard deviation (%).

reported in recent publications using the techniques of GC-MS and LC-MS.[25,29,30]

The calibration curves for each analytical in blood were determined taking into account, where possible, the therapeutic and toxic concentrations of each substance. The concentration ranges in blood determined in this study are in agreement with those reported by Dziadosz et al[39] that stipulated values between 2 and 50 ng mL^{-1}. Arora et al[26] used working concentrations between 7.8 and 250 ng mL^{-1}. The work ranges used in urine method were in accordance with those reported in the literature for the analysis of drugs. Shin et al[22] determined curve concentrations for drugs of abuse and benzodiazepines between 10 and 125 ng mL^{-1}. Hegstad et al[21] used working concentrations between 100 and 10 000 ng mL^{-1}. Thus, the working ranges used were described in Table 3. Coefficients of determination were in the range of 0.990 to 0.999 for blood and 0.990 to 1.0 for urine method.

Intraday and interday precision were evaluated in blood at three concentrations in six replicates each, on three consecutive days. The values obtained were analyzed by analysis of variance (ANOVA), and the results of intraday precision presented a variation of 1.8%, which corresponds to the average controls of amphetamine and MDA, to 19.8%, referring to the low control of phenobarbital. For the interday accuracy, the values found were within the range of 1.9% (high MDMA control) to 18.6% (low aldicarb control). Chen et al[40] reported similar accuracy values. Arora et al[27] found values between 0.1% and 19% in the precision assay and 82.1% to 119% in the accuracy of the LC-MS method. All the results obtained for the precision and accuracy of the method were considered satisfactory.

The intraday precision values obtained for urine presented a variation of 0.8% to 18.6%, which correspond to the values of the mean acetaminophen control and the low aldicarb control, respectively. All values obtained were within the recommended range. For the interday precision, the coefficients of variation found were 0.6% to 19.9%, being the mean controls of 0.6% acetaminophen and norfluoxetine low, respectively. The values reported for precision are in agreement with the values found in similar studies.[25,26] Table 3 presents the details of the precision and accuracy data at the tested concentrations for the two matrices.

A significant matrix effect of blood was observed for some substances (aldicarb and bromazepam [increase of signal] and phenytoin and valproic acid [suppression]). However, they are not considered as discrepant compared with those published in the literature with similar methodologies.[37,40] In addition, since the sensitivity of the method was considered satisfactory, the matrix effect did not negatively influence the application of the method. The matrix effect results for analytes were described in Table 3.

The study of dilution integrity was carried out by analyzing urine samples, in six replicates, at concentrations of 8000 and 30 000 ng mL^{-1}. The samples were diluted 10 and 20 times, respectively, with methanol and water (60:40, *v*/v) mixture and analyzed by calculation through the regression equation obtained. In the dilution integrity study, the results demonstrated acceptable bias and precision.

To evaluate the carryover, six blank matrix samples were analyzed immediately after the highest calibration point, and no carryover effects were detected.

3.2 | Application of the methods

The developed methods were successfully applied to 320 real samples collected at the Poison Control Center of São Paulo (PCC-SP) between December 2014 and December 2017. In most of the analyzed samples (blood and urine), at least, one analyte was detected. In some cases, multiple drugs were detected in the same sample. Samples with concentrations lower than the LLOQ were considered negative. Of the total samples, 285 samples have shown to be positive for some of the analytes. From these, 58 samples were positive only for blood, and 22 samples were positive only for urine, while the remaining 205 were positive for both of these matrices. Analyzing all the positive samples, drugs of abuse were presented in 67.0% of the samples, followed by benzodiazepines (52.9%), acetaminophen (42.4%), anticonvulsants (17.5%), antidepressants (14.0%), and pesticides (3.5%). The total number of positive samples in blood, 27 shown a concentration higher than the therapeutic or normal ranges, from which 14 cases have presented intoxication by antidepressants, followed by benzodiazepines (8) and anticonvulsants (5). From the latter, benzodiazepines were detected in 50% of the samples and, at least one analyte of cocaine has been detected in 23% of the samples. This extraction procedure was preferred over that described by Hegstad et al,[21] Sempio et al,[37] and Dziadosz et al[39] because the present study features an approach of sample preparation and the extensive variety of substances in multi-analyte screening, including the pesticides, that has not been reported in recently published papers.

4 | CONCLUSION

In this study, a simple protein precipitation and dilution was used for simultaneous monitoring of a large number of drugs and pesticides in blood and urine samples. The sample preparation technique is relatively cheap, easy, and fast to perform. The developed method was successfully applied to 320 real samples collected at the Poison Control Center of São Paulo, Brazil. This confirms its applicability and importance to emergency toxicological analysis, and it could be very useful in both fields of clinical and forensic toxicology.

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CONFLICT OF INTEREST

There are no financial or other relations that could lead to a conflict of interest.

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