Journal of Pharmaceutical and Biomedical Analysis 228 (2023) 115315

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Development and validation of a fast UPLC-MS/MS screening method for the detection of 68 psychoactive drugs and metabolites in whole blood and application to post-mortem cases

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ARTICLE INFO

Keywords:
Forensic toxicology
Psychoactive substances
LC-MS/MS
Blood
Post-mortem
Validation

ABSTRACT

We report a rapid and sensitive LC-MS/MS method that allows the simultaneous detection of 68 commonly prescribed antidepressants, benzodiazepines, neuroleptics, and metabolites in whole blood with a small sample volume after a rapid protein precipitation. The method was also tested on post-mortem blood from 85 forensic autopsies. Three sets of commercial serum calibrators containing a mix of prescription drugs of increasing concentration were spiked with red blood cells (RBC) to obtain 6 calibrators (3 "serum calibrators" and 3 "blood calibrators"). Curves obtained from serum calibrators and from blood calibrators were compared using a Spearman correlation test and by analyzing slopes and intercepts, to assess if the points from six calibrators could be plotted together in a single calibration model. The validation plan included interference studies, calibration model, carry-over, bias, within-run and between-run precision, limit of detection (LOD), limit of quantification (LOQ), matrix effect and dilution integrity. Four deuterated Internal Standards (Nordiazepam-D5, Citalopram-D6, Ketamine-D4 and Amphetamine-D5) and two different dilutions were assessed. Analyses were performed using an Acquity UPLC® System coupled with triple quadrupole detector Xevo TQD®. The degree of agreement with a previously validated method was calculated on whole blood samples of 85 post-mortem cases, by performing a Spearman correlation test with a Bland-Altman plot. Percentage error between the two methods was evaluated. Slopes and intercepts of curves obtained from serum calibrators and from blood calibrators showed a good correlation, and the calibration model was built plotting all points together. No interferences were found. The calibration curve appeared to provide a better fit of the data using an unweighted linear model. Negligible carry-over was observed, and very good linearity, precision, bias, matrix effect and dilution integrity were achieved. The LOD and the LOQ were at the lower limits of the therapeutic range for the tested drugs. In a series of 85 forensic cases, 11 antidepressants, 11 benzodiazepines and 8 neuroleptics were detected. For all analytes, a very good agreement between the new method and the validated method was demonstrated. The innovation of our method consists in the use of commercial calibrators, readily available to most forensic toxicology laboratories, for the validation of a fast, inexpensive, wide-panel LC-MS/MS method that can be used as a reliable and accurate screening for psychotropic drug in postmortem samples. As observed in the implementation on real cases, this method could be profitably applied in forensic cases.

1. Introduction

The trend of prescribing psychoactive drugs, such as antidepressants, benzodiazepines and antipsychotics, has seen a rise in recent years, as has their illicit non-medical use [1], especially in patients with concomitant depressive and psychotic symptoms and during the COVID-19 pandemic [2]. As a consequence, these drugs are frequently encountered in cases of forensic interest, namely fatal intoxications, accidental deaths, drug facilitated crimes and sexual assaults, investigation of Driving Under the Influence of drugs, and are often involved in deaths from poly-abuse intoxications and suicides [3–5]. In these cases, the broad systematic search for psychotropic drugs in cases of forensic

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https://doi.org/10.1016/j.jpba.2023.115315
Received 11 January 2023; Received in revised form 22 February 2023; Accepted 22 February 2023
Available online 24 February 2023

interest still represents one of the greatest challenges for forensic toxicology.

Recent studies have focused on the search for sensitive methods that could detect an ever-increasing number of classes of substances in reduced extraction volumes [6–8]. Blood and its derivatives (serum or plasma, that are generally preferred over blood in clinical cases) make up the sample of choice, because blood levels correlate best with clinical effects [9].

Liquid chromatography (LC) coupled with mass spectrometry (MS) has expanded the breadth of compounds which can be analyzed on different matrices of toxicological interest, with the required specificity and sensitivity, without the need for derivatization or removal of the aqueous phase [10–13]. Among the purification procedures, LLE is usually considered the cheapest and most straightforward. It is characterized by satisfactory recoveries for most applications, but in cases of complex biological matrices (i.e., post-mortem samples) it may lead to poor recovery or high matrix effect. SPE methods offer selective extraction of the target drugs, but in a multi-drugs approach or untargeted analysis, it will selectively remove compounds that do not match the chemistries utilized in the extraction [14]. Protein precipitation (PPt) is a sample pretreatment, easily applicable in routine cases, and has gained considerable popularity in recent years [15–17].

We developed a rapid and sensitive LC–MS/MS screening method for the simultaneous quantification of 68 psychoactive drugs and their metabolites in whole blood after a protein precipitation. This technique is based on the use of three sets of commercial serum calibrators, containing pre-defined concentrations of antidepressants, benzodiazepines and antipsychotics. The method was validated and tested on post-mortem blood samples collected from 85 autoptic cases of forensic interest.

2. Materials and methods

2.1. Chemicals and reagents

The ClinCal® Serum Calibrator Set for antidepressants, benzodiazepines and antipsychotics (Level 0 – 3), were purchased from RECIPE Chemicals + Instruments GmbH (Munich, Germany). Nordiazepam-d5, citalopram-d6, ketamine-d4 and amphetamine-d5 and ammonium formate were purchased from Sigma Aldrich® (Steinheim, Germany). Ultra-pure water was obtained by filtration with PURELAB® Chorus 1 Elga (United Kingdom). Formic acid, acetonitrile, 2-isopropanol and methanol were purchased from Merck® (Germany, Darmstadt).

2.2. Calibrators, quality controls and internal standards

The validation plan was performed using the ClinCal® Calibrator Serum 2021 set. Freeze-dried serum solutions at four increasing levels of concentration (S0, S1, S2, S3) were used. The method included: 19 antidepressants and metabolites, namely bupropion, citalopram, desmethylcitalopram, o-desmethylvenlafaxine, duloxetine, fluoxetine, fluvoxamine, methylphenidate, mianserin, milnacipran, mirtazapine, paroxetine, ritalinic acid, sertraline, tianeptine, tramadol, trazodone, venlafaxine, vortioxetine; 30 benzodiazepines and metabolites, namely alprazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, bromazepam, chlordiazepoxide, clobazam, clonazepam, demoxepam, desalkylflurazepam, diazepam, estazolam, flunitrazepam, flurazepam, 3-hydroxy-bromazepam, α-hydroxy-midazolam, lorazepam, lormetazepam, medazepam, midazolam, nitrazepam, nordiazepam, oxazepam, prazepam, temazepam, tetrazepam, triazolam, zaleplon, zolpidem, zopiclone; 19 antipsychotics and metabolites, namely amisulpride, aripiprazole, chlopromazine, clozapine, dehydroaripiprazole, desmethylolanzapine, haloperidol, levomepromazine, norclozapine, norquetiapine, olanzapine, paliperidone, pipamperone, promethazine, quetiapine, risperidone, sertindole, sulpiride, thioridazine. The quality controls (QC) used for precision, bias and matrix effect were ClinCal® Calibrator Serum belonging to a different set (2020) from those used for the calibration, stored in the freezer at − 20 °C.

The Internal Standards (ISs) mix tested in the experimental work consists of four ISs (nordiazepam-D5, citalopram-D6, ketamine-D4 and amphetamine-D5) chosen to cover the 15 min of the chromatographic run. The IS mix was prepared by appropriate dilution in methanol to reach a final concentration of 0.01 mg/ml for all ISs. All solutions were stored at − 20°C in glass vials.

2.3. Preparation of calibrators

Serum calibrators were prepared from the four levels of freeze-dried serum (S0, S1, S2, S3), rehydrated in 1 mL of distilled water, put on an agitator for 15 min and then at − 20°C for 30 min (according to the procedure described in ClinCal®). For each level, nine aliquots of 100 µL serum were obtained. The points of the calibration curve on serum were S1, S2 and S3, at the nominal concentrations of the analytes supplied by ClinCal® Calibrator Serum Set.

Blood calibrators were prepared from five aliquots of serum calibrators for each level, added with 100 µL of red blood cells (RBC), resulting from the centrifugation of a pool of fresh whole blood deprived of serum taken from a living donor, to obtain calibrator that could simulate whole blood. Points B1, B2, and B3 were obtained. The nominal concentrations of blood calibrators were the 50% of those of the corresponding serum calibrators. As a result, six calibration points were obtained at following increasing concentrations: B1, S1, B2, S2, B3, S3.

Comparison between set of calibrators. To test the possibility to plot together all points in the calibration model, the three-points serum curves (S1-S2-S3) and the three-points blood curves (B1-B2-B3) were compared. Five samples for each level were prepared and analyzed once per run in five separate runs.

A Spearman correlation test was performed (two tailed; CI 95%) for all analytes. The level of significance was set at $p < 0.05$. Moreover, slopes and intercepts were compared. A parametric paired t-test was carried out to evaluate the presence of significant differences between the slopes and intercepts for all analytes (level of significance 0.05). Statistical analyses were performed using the software Microsoft Excel and Prism – GraphPad v. 8.

2.4. Extraction procedure

In each polypropylene tube containing 100 µL of serum or blood, 10 µL ISs mix were added, followed by a protein precipitation step with 300 µL of acetonitrile (ACN) directly on vortex. The tube was capped, vortexed, kept for 30 min at − 20 °C and then centrifuged for 15 min at 14,000 rpm.

The supernatant was tested with two different dilutions: with ACN and mobile phase A (MPA) (1:2.5 v/v). Five µL were injected into the Ultra Performance Liquid Chromatography–tandem Mass Spectrometry (UPLC-MS/MS) instrument.

2.5. Instrumentation

Chromatography was performed by an ACQUITY UPLC® System (Waters Corporation, Milford, MA) equipped with an Acquity UPLC® HSS C18 column (2.1×150mm, 1.8 µm; Waters) using a MPA composed of aqueous solution 5 mM ammonium formate and mobile phase B composed of acetonitrile, both spiked with 0.1% formic acid. Analytes were eluted at a flow rate of 0.4 ml/min and the elution gradient was set as follows: mobile phase A starting concentration of 87% and mobile phase B starting concentration of 13%, kept constant for 0.5 min. From 0.5–10.0 min both mobile phases reached 50%; then, for 2.5 min, mobile phase A concentration decreased to 5% and mobile phase B concentration increased to 95%. In the last 2.5 min both mobile phases returned to the starting concentrations; total run time was 15 min. The column temperature was set to 50 °C.

The UPLC system was coupled with a Waters triple quadrupole detector Xevo TQD®, with electrospray ionization (ESI) in positive mode and acquisitions were carried out in Multiple Reaction Monitoring (MRM) mode. The source parameters were set as follows: capillary voltage 3.50 kV, desolvation gas temperature 400 °C; desolvation gas flow 800 L/h, cone gas flow 20 L/h, collision pressure 2.9×10^{-5} mbar. MRM transitions for each substance together with the retention time, the detection window, the cone voltage, and the collision energy, were supplied by the manufacturer [18]. For molecules not included in the panel supplied, specific MRM transitions and collision energies were determined by literature search, on substances tuned with the same MS-device, and a series of experiments performed on individual standards at a concentration of 1000 ng/ml. Two characteristic transitions were chosen for each analyte. Data acquisition and analysis was performed by MassLynx 4.2® software, whereas quantitation was performed by TargetLynx application.

2.6. Experimental design: validation plan and application on real cases

Interferences, carry-over, calibration model, bias (accuracy), precision, limit of detection (LOD), limit of quantification (LOQ) and stability were evaluated according to SWGTOX guidelines [19].

The approaches proposed by SWGTOX guidelines cannot be followed for the study of the matrix effect. The matrix effect was thus evaluated by analyzing 3 replicates of low- and high- Quality Control samples (QCs) added with RBC from 6 different sources, according to the International Guidelines of the European Medicines Agency [20].

The plan consisted in testing all IS (nordiazepam-D5, citalopram-D6, ketamine-D4 and amphetamine-D5) and two different dilutions (ACN and MPA) for all included molecules.

The preparation that gave best results in terms of validation parameters and chromatographic condition was applied to 85 cases of suspected poisoning, analyzed at the Laboratory of Forensic Toxicology (University of Bologna) from 2019 to 2021. Samples were previously analyzed for forensic purposes with an internally validated multi-targeted LC-MS/MS method (herein reported as "Method 2″) with the same instrumentation, extraction procedures, validated according to SWGTOX guidelines with reference standards. After collection, the blood was stored at − 20 °C until analysis was repeated with the present method ("Method 1").

2.7. Interference studies

Ten blank sources of whole blood (5 drug-free living donors and 5 drug-free post-mortem whole blood samples taken during autopsy) were extracted without addition of internal standard and analyzed, in order to test co-elution of endogenous substances.

One randomly selected sample was added with exogenous substances (heroin 10 ng/ml, morphine 10 ng/ml, codeine 10 ng/ml, cocaine 10 ng/ml, benzoylecgonine 10 ng/ml, amphetamines 20 ng/ml, methamphetamines 20 ng/ml, methylenedioxymethamphetamine (MDMA) 20 ng/ml, methylenedioxyamphetamine (MDA) 20 ng/ml, methylenedioxyethylamphetamine (MDEA) 20 ng/ml, methadone 10 ng/ml), in order to test whether drugs of abuse commonly searched for forensic purposes could interfere with retention times of the molecules tested.

One randomly selected sample was also added with the four ISs (10 ng/ml), in order to test whether labeled ISs interfere with retention times of the molecules tested.

Two calibrators at the medium interval (S2 and B2) were extracted without the addition of ISs in order to test whether unlabeled analyte ions interfere with the signal for labeled ISs.

2.8. Calibration model and carry over

It was decided that the calibration model should be linear, in different calibration ranges for different analytes, as reported in the Supplementary material (Table 1). The calibration ranges suited the purpose of the present study, being within the pharmacological activity of each drug [21]. Four IS were tested for each molecule. The best IS for each molecule was chosen according to the results obtained from the correlation coefficient of the calibration model (r^2), precision and bias, and reported in Supplementary material (Table 1).

Each calibrator was analyzed once per run in five separate runs. After checking the correlation between the serum and blood curves, a six-point calibration curve (B1, S1, B2, S2, B3, S3) was evaluated for both ACN and MPA dilutions. Data from all runs were combined into a single calibration curve. Linearity was assessed by simple linear regression, accepting a correlation coefficient (r^2) greater than 0.990, and by residue plot analysis. Two extracted blank matrices were analyzed after the highest calibrator to evaluate carryover. Carry-over was considered negligible if the signal in the blank was lower than 10% of the method's LOQ.

2.9. Limit of quantification (LOQ) and limit of detection (LOD)

The LOQ was identified as the lowest non-zero calibrator that meets a signal-to-noise ratio of at least 10 and is able to reproducibly replicate + 20% bias and 20% CV % in three samples per run over three runs. To assess the LOD, the lowest calibrator was diluted with 100 μL of RBC, in order to obtain the 50% concentration of the lowest point. Three separate samples were analyzed in duplicate for at least three runs. The LOD was estimated as the value of the lowest non-zero calibrator (including the 50% dilution of the lowest calibrator) that: 1) yields a reproducible instrument response greater than or equal to three times the noise level of the background signal from the negative samples and 2) achieves acceptable retention time and peaks.

2.10. Precision and accuracy

Precision and accuracy were evaluated using QCs, selected among the ClinCal® Calibrator Serum 2020 set, spiked with different amounts of RBC, to obtain a low QC, with a concentration close to the LOQ and a high QC, with a concentration below the highest point of the curve. The concentrations of low and high QCs of each drug are reported in the Supplementary Material (Table 2). Each QC has been prepared according to the procedure described for the preparation of the calibrators.

Precision was expressed as the coefficient of variation (CV) provided by the percentage ratio of the R.S.D. (relative standard deviation%), and was measured using three different samples per concentration at two different concentration pools (QCs low and high), over five different runs. Within-run and between-run precision were assessed. Accuracy was measured using three separate samples per concentration at two different concentration pools (QCs low and high) over five different runs. The optimal bias and precision from the target concentration were ± 20%.

2.11. Matrix effect, dilution integrity and stability

Since commercial calibrators were used to reduce the need for a high number of standards in the validation procedures, the approach using the comparison between the peak area in the presence/absence of matrix proposed by SWGTOX guidelines [19] cannot be used.

The matrix effect was thus evaluated by analyzing 3 replicates of low and high QCs, added with RBC/blood from 6 different sources, according to the approach proposed by the International Guidelines of the European Medicines Agency [20]. Sources consisted in RBC obtained from 3 living donors and blood obtained from 6 post-mortem cases, taken during autopsy. A satisfactory matrix effect was defined if the individual accuracy and precision were within ± 30% of the nominal concentration.

Dilution integrity experiments were performed by preparing blood samples spiked with ad-hoc concentration of standards (2 substances for

Table 1
Analytes of interest. retention time (RT). cone voltage (CV). quantifier (*) and qualifier ion transitions and collision energies (Ce). N: number.

N	Analytes	RT (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	CV (V)	Ce (V)
Antidepressants						
1	Bupropion	5.38	240.1	166.1	30	20
			240.1	184.1 *	30	10
2	Citalopram	6.79	325.2	58.2	40	24
			325.2	109.1 *	40	24
3	Desmethylcitalopram	6.59	311.3	108.8	30	25
			311.3	262.3 *	30	20
4	Duloxetine	8.13	298.2	44.2	18	12
			298.2	154.0 *	18	6
5	Fluoxetine	9.18	310.1	44.2	20	12
			310.1	148.2 *	20	8
6	Fluvoxamine	8.13	319.2	71.2	29	17
			319.2	200.2 *	29	15
7	Methylphenidate	4.05	234.2	56.1	31	40
			234.2	84.1 *	31	18
8	Mianserine	7.08	265.2	208.2	45	22
			265.2	222.3 *	45	22
9	Milnacipran	5.05	247.3	99.8	25	18
			247.3	230.3 *	25	12
10	Mirtazapine	4.03	266.2	72.2	40	18
			266.2	195.1 *	40	20
11	Paroxetine	7.59	330.2	70.1	40	29
			330.2	192.2 *	40	20
12	Ritalinic Acid	3.20	220.1	56.0	20	46
			220.1	84.1 *	20	20
13	Sertraline	9.36	306.1	159.0	30	40
			306.1	275.1 *	30	20
14	Tianeptine	7.07	437.1	228.1	20	38
			437.1	292.1 *	20	15
15	Tramadol	4.34	264.2	58.1 *	25	15
			264.2	264.2	25	5
16	Trazodone	5.51	372.2	148.1	47	35
			372.2	176.2 *	47	23
17	Venlafaxine	5.35	278.2	58.1	25	18
			278.2	121.2 *	25	18
18	Venlafaxine. O-Desmethyl	3.29	264.2	58.1	30	19
			264.2	246.2 *	30	12
19	Vortioxetine	9.50	299.2	109.1	50	42
			299.2	150.1 *	50	26
Benzodiazepines						
20	3-OH-Bromazepam	5.29	332.0	287.0	35	24
			332.0	303.0 *	35	24
			332.0	314.9	35	30
21	7-Aminoclonazepam	3.70	286.1	222.1	50	24
			286.1	250.1 *	50	20
22	7-Aminoflunitrazepam	4.52	284.2	135.0	45	26
			284.2	148.1 *	45	26
23	7-Aminonitrazepam	1.99	252.1	121.1	65	35
			252.1	146.1 *	65	30
24	Alpha-OH-Midazolam	7.07	342.0	203.0	35	25
			342.0	324.0 *	35	20
25	Alprazolam	8.59	309.2	205.2	50	43
			309.2	281.2 *	50	30
26	Bromazepam	6.45	316.1	182.2	43	32
			316.1	209.2 *	43	26
27	Chlordiazepoxide	5.67	300.1	227.1	40	25
			300.1	283.2 *	40	16
28	Clobazam	9.53	301.1	224.0	45	32
			301.1	259.0 *	45	22
29	Clonazepam	8.29	316.1	214.1	55	39
			316.1	270.1 *	55	24
30	Demoxepam	7.02	287.0	104.9	35	20
			287.0	180.0 *	35	20
31	Desalkylflurazepam	9.39	289.1	140.1	55	30
			289.1	226.1 *	55	28
32	Diazepam	10.56	285.1	154.1	50	28
			285.1	193.2 *	50	32
33	Estazolam	8.13	295.1	205.2	50	30
			295.1	267.1 *	50	25
34	Flunitrazepam	8.93	314.1	239.2	50	34
			314.1	268.2 *	50	32
35	Flurazepam	6.73	388.1	288.2	30	25
			388.1	315.2 *	30	20
36	Lorazepam	8.34	321.0	229.1	30	20
			321.0	275.1 *	30	20
37	Lormetazepam	10.13	335.1	177.1	40	40
			335.1	289.2 *	40	25
38	Medazepam	7.13	271.4	91.4	35	45
			271.4	207.4 *	35	38
39	Midazolam	6.55	326.1	244.2	55	26
			326.1	291.2 *	55	26
40	Nitrazepam	8.26	282.1	180.1	48	35
			282.1	236.2 *	48	25
41	Nordiazepam	9.08	271.1	140.0	50	35
			271.1	165.1 *	50	25
42	Oxazepam	8.02	287.1	241.2	35	20
			287.1	269.1 *	35	15
43	Prazepam	11.57	325.1	140.1	40	34
			325.1	271.2 *	40	22
44	Temazepam	9.29	301.1	177.1	35	40
			301.1	255.1 *	35	30
45	Tetrazepam	11.20	289.1	197.1	50	27
			289.1	225.2 *	50	27
46	Triazolam	9.19	343.1	308.2	56	26
			343.1	315.2 *	56	26
47	Zaleplon	7.25	306.1	236.1	40	25
			306.1	264.1 *	40	20
48	Zolpidem	5.24	308.2	235.2	55	34
			308.2	263.1 *	55	28
49	Zopiclone	3.58	389.1	217.1	25	30
			389.1	245.1 *	25	17
Antipsychotics						
50	Amisulpride	2.78	369.9	196.1	20	42
			369.9	242.2 *	20	30
51	Aripiprazole	8.14	448.2	98.1	35	38
			448.2	176.1	35	30
			448.2	285.1 *	35	25
52	Chlorpromazine	8.90	319.1	58.2	45	45
			319.1	86.1 *	45	30
53	Clozapine	6.14	327.1	192.2	35	40
			327.1	270.2 *	35	23
54	Dehydroaripiprazole	7.66	446.2	84.1	35	62
			446.2	98.1	35	42
			446.2	285.1 *	35	18
55	Desmethylolanzapine	1.89	299.1	198.0	35	38
			299.1	213.0	35	26
			299.1	256.0 *	35	22
56	Haloperidol	7.20	376.1	123.1	30	40
			376.1	165.1 *	30	20
57	Levomepromazine	8.39	329.2	58.1	38	25
			329.2	100.1 *	38	20
58	Norclozapine	5.60	313.2	192.0	46	48
			313.2	270.1 *	46	30
59	Norquetiapine	6.29	296.1	253.2	45	30
			296.2	210.1 *	45	45
60	Olanzapine	2.06	313.2	84.2	60	35
			313.2	256.1 *	60	35
61	Paliperidone	5.20	427.2	82.1	45	50
			427.2	110.0 *	45	40
62	Pipamperone	3.35	376.2	164.9	35	29
			376.2	123.0	35	50
			376.2	291.0 *	35	14
63	Prometazine	7.37	285.2	86.2	35	25
			285.2	198.1 *	35	20
64	Quetiapine	6.60	384.1	221.1	35	38
			384.1	253.0 *	35	24
65	Risperidone	5.28	411.2	110.1	50	75
			411.2	191.2 *	50	40
66	Sertindole	10.00	441.2	71.2	35	54
			441.2	113.1 *	35	30
67	Sulpiride	1.33	342.1	214.2	45	30
			342.1	112.2 *	45	30
68	Thioridazine	10.38	371.2	98.1	40	28
			371.2	126.2 *	40	22

(*continued on next page*)

Table 1 (*continued*)

N	Analytes	RT (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	CV (V)	Ce (V)
Antidepressants						
	Internal Standards					
1	Nordiazepam D5	9.04	276.1	165.1	50	28
			276.1	213.0 *	50	28
2	Citalopram D6	6.76	331.2	109.1 *	40	25
3	Ketamine D4	3.18	242.2	129.1 *	20	25
			242.2	211.1	20	25
4	Amphetamine D5	2.19	141.0	93.0 *	38	22

each class were chosen: citalopram, trazodone, alprazolam, midazolam, citalopram, quetiapine, haloperidol, quetiapine) at 2x and 3x of the upper limit of quantification (ULOQ). Subsequently, these samples were 10-fold diluted using blank whole blood. Both dilution samples were analyzed in triplicate over five different runs, versus a fresh calibration curve. Bias and precision calculations were performed. The acceptance criteria for dilution integrity were concentrations calculated within ± 15% of the nominal value and RSD 15% within the replicates. Stability was assessed on QCs after 24 h storage at − 20 °C and calculating percent deviation on freshly prepared QCs.

2.12. Application on real samples and degree of agreement

After a qualitative comparison (positive/negative), the quantitative degree of agreement of the resulting method with the previously validated method ("Method 2″) was tested for each class of drug (antidepressants, benzodiazepines, antipsychotics).

A Spearman r correlation test (two tailed; Confidence Interval or CI 95%; level of significance set at $p < 0.05$) and a Bland-Altman plot (95% CI) were performed. Afterwards, mean, and median percentage errors were calculated as set out below (((Method 1 - Method 2) / Method 2) x100) for the three class of drugs separately, to assess the differences in terms of bias. A Wilcoxon matched pairs signed rank test was applied to the three groups and the level of significancy was set at $p < 0.05$. Statistical analyses were performed using the software Prism – GraphPad v. 8.

3. Results and discussion

3.1. Method optimization

An UPLC-MS/MS analytical method was developed for the determination of 68 psychotropic drugs in blood matrix, including 19 antidepressants, 30 benzodiazepines and 19 antipsychotics. The retention times, precursor ion, product ions (qualifier and quantifier), cone voltages (CV) and collision energies (Ce) are shown in Table 1. Target identification was performed on the basis of precursor ion, two/three diagnostic fragments ratio, retention time, and area ratio of quantifier and qualifier ions, fulfilling the EU Commission Decision 2002/657/EC confirmation criteria. The overlay of chromatograms is reported in the Supplementary Material **(Figures A-C)**.

3.2. Method validation

3.2.1. Interference studies

No interferences from endogenous substances nor other exogenous substances tested coeluting within the time frame of the method were observed, nor interferences between the analyte and the internal standards.

3.2.2. Comparison between set of calibrators

Correlation coefficients greater than 0.990 were observed at Spearman test for all analytes of interest ($p < 0.05$). The slope and the intercepts of the curves obtained with serum calibrators (S1, S2 and S3) and those obtained with blood calibrators (B1, B2, B3) did not show any statistical differences, both in ACN and MPA dilutions.

3.2.3. Calibration model

The calibration model was thus tested using the six-point curve. The simple linear regressions for all the analytes of interest, in both ACN and MPA dilutions, were characterized by r^2 greater than or equal to 0.990 as reported in Supplementary Material (Table 1). The calibration curve appeared to provide a better fit of the data using an unweighted linear model, as confirmed by the residual plot, which showed a random distribution around the zero line (Supplementary material**, Figures D-I)**. Carryover was not present for all drugs or the internal standard in any of the extracted blank matrix that followed the highest calibrator, and it was deemed acceptable.

Better results in term of bias and precision were observed for dilutions with MPA. *Limit of Detection* (LOD), *Limit of Quantification* (LOQ), within-run and between-run *precision* and *bias* are shown in Table 2.

The *Matrix effect* of analytes under investigation were always ± 25% for both QCs, except for the following molecules (low QC and high QC): clobazam (19.7% and 47.4%); flunitrazepam (34.9% and 38.2%), prazepam (21.1% and 35.2%); desmethylcitalopram (30.1% and 30.8%); ritalinic acid (61.1% and 64.7%), levomepromazine (49.6% and 53.7%), promethazine (27.9% and 40.7%); desmethylolanzapine (44.0%; 56.2%). *Dilution integrity* was satisfactory for the molecules tested. This provided proof of no detrimental impact when diluting the samples before extraction. Stability of the processed sample at 24 h was acceptable, with values comprised between 2% and 14% (Table 2).

Considering the dilutions tested (ACN and MPA), most of the analytes showed accuracy and precision within the requirements reported in the adopted guidelines [27], both for ACN and MPA dilutions. As expected, chromatography improved in case of dilution with MPA (Supplementary material**, Figure L)**.

3.3. Application on real samples and degree of agreement

Eighty-five real autoptic samples were analyzed with the extraction procedure using MPA dilution. Twenty-six samples were negative for substances tested; 59 samples were positive for at least 1 drug, most of which were positive for more than one drug. All positive single findings (120/120) were qualitatively confirmed with the new method, showing a 100% qualitative agreement.

The following drugs were found.

11 antidepressants (39/39 positive findings): bupropion (1), citalopram (5), desmethylcitalopram (2), fluoxetine (3), mirtazapine (4), paroxetine (5), sertraline (4), tramadol (4), trazodone (8), venlafaxine (2), vortioxetine (1).

11 benzodiazepines (62/62 positive findings): alprazolam (6), bromazepam (1), clonazepam (1), diazepam (17), flurazepam (2), lorazepam (5), midazolam (8), nordazepam (16), oxazepam (2), temazepam (3), zolpidem (1).

8 antipsychotics (19/19 positive findings) were found: aripriprazole (2); chlorpromazine (1), clozapine (1), haloperidol (3), levomepromazine (2), norquetiapine (2), olanzapine (3), quetiapine (5).

Some drugs were found out the calibration range and cannot be quantified: 6 antidepressants (2 results below the LOQ: paroxetine and trazodone); 6 benzodiazepines (1 below the LOQ: nordiazepam); 3 antipsychotics (1 below the LOQ: levomepromazine).

Correlation coefficients (r^2) between Method 1 and Method 2 were 0.974 (0.946–0.987) for antidepressants, 0.988 (0.979–0.993) for benzodiazepines and 0.985 (0.953–0.995) for antipsychotics ($p < 0.001$). The Bland-Altman is reported in Fig. 1. All measures except for 2 results fell within 95% CI.

Mean (SD) and median percentage error between the two methods were as follows: antidepressants 12.9 (11.0) % and 9.1%; benzodiazepines 12.0 (11.1) % and 10.5%; antipsychotics 17.4 (14.3) % and 11.9%. The Wilcoxon matched pairs signed rank test did not show differences

Table 2
Within run and between-run precision (relative standard deviation or RSD). Bias (error%). Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ). * = precision or accuracy greater than 20%.

			Precision and Bias						Stability	
			QC low			QC high			QC low	**QC high**
Antidepressants										
	LOD **(ng/ml)**	LLOQ **(ng/ml)**	Precision RDS (%)		Bias (%)	Precision RDS (%)		Bias (%)	Deviation n. 5 (%)	
			Within run	**Between run**		**Within run**	**Between run**			
Bupropion	5.80	5.80	6.2	6.9	18.2	7.5	8.5	22.4 *	4	10
Citalopram	4.13	8.25	7.5	8.5	16.0	5.7	6.8	11.1	5	12
Desmethylcitalopram	4.63	9.25	8.2	8.4	5.9	4.4	6.1	15.9	10	9
Duloxetine	4.63	9.25	9.6	15.8	12.5	8.3	10.3	3.1	11	23
Fluoxetine	8.80	17.60	7.6	8.2	16.8	4.5	7.5	17.3	14	5
Fluvoxamine	8.73	17.45	13.0	13.0	8.6	7.6	8.6	19.4	5	6
Methylphenidate	0.95	1.90	4.3	5.4	9.4	4.5	6.8	8.0	7	8
Mianserine	5.15	10.30	6.8	7.6	21.5 *	7.5	6.8	19.7	9	11
Milnacipran	7.30	14.60	4.1	5.1	15.9	11.4	12.3	13.8	23	5
Mirtazapine	3.00	6.00	4.0	6.7	12.2	5.9	6.7	5.0	3	8
Paroxetine	4.75	9.50	8.3	8.7	2.3	5.3	5.5	11.8	12	9
Ritalinc Acid	6.15	12.30	7.7	5.6	5.9	12.4	12.9	13.6	8	4
Sertraline	2.31	2.31	5.2	5.6	8.0	6.2	8.9	10.6	3	7
Tianeptine	2.55	5.10	5.9	5.9	11.8	5.6	7.1	7.0	2	11
Tramadol	21.13	42.25	4.5	9.5	3.3	6.7	10.3	5.6	8	5
Trazodone	40.25	80.50	6.5	8.5	16.2	6.9	8.3	9.3	8	10
Venlafaxine	5.73	11.45	12.5	12.5	8.5	4.5	6.4	14.3	3	8
Venlafaxine. O-Desmethyl	9.35	18.70	3.2	6.6	6.2	8.4	8.3	4.7	10	3
Vortioxetine	2.24	4.48	4.3	5.8	12.8	6.1	7.1	8.8	10	5
Benzodiazepines										
3-OH-Bromazepam	8.30	16.60	8.2	10.2	19.7	9.4	12.8	28.7 *	10	12
7-Aminoclonazepam	2.51	2.51	10.1	11.8	29.7 *	6.8	8.5	19.0	4	3
7-Aminoflunitrazepam	2.68	2.68	16.8	17.2	12.6	8.3	9.1	19.7	13	10
7-Aminonitrazepam	5.40	10.80	6.1	7.0	30.7 *	4.3	6.7	15.5	3	7
Alpha-OH-Midazolam	2.65	5.30	8.7	9.6	8.0	4.4	7.8	3.0	12	5
Alprazolam	1.29	2.57	8.1	9.1	18.5	4.9	5.3	8.7	5	2
Bromazepam	5.33	10.65	25.9 *	26.1	19.5	8.1	12.5	14.8	3	10
Clordiazepoxide	65.00	130.00	4.8	6.3	3.5	2.2	4.4	1.8	8	12
Clobazam	6.93	13.85	2.7	3.1	2.8	4.5	6.4	7.0	5	13
Clonazepam	6.25	12.50	7.8	8.8	19.8	8.9	11.2	13.9		8
Demoxepam	57.75	115.50	11.2	12.3	8.9	6.1	7.4	4.2	3	7
Desalkylflurazepam	4.28	8.55	9.1	10.1	8.1	7.	8.3	5.6	7	5
Diazepam	32.00	64.00	4.3	5.8	3.8	6.4	8.3	0.4	9	2
Estazolam	11.15	22.30	4.7	5.8	6.7	3.7	10.3	3.5	2	5
Flunitrazepam	1.32	2.63	4.4	5.2	9.4	8.7	13.7	7.4	12	9
Flurazepam	2.60	5.20	5.9	6.5	27.9 *	9.4	12.6	6.2	4	13
Lorazepam	24.65	49.30	4.9	5.2	17.4	14.0	23.7 *	14.3	10	3
Lormetazepam	2.29	4.58	18.9	19.5	14.6	14.4	16.3	16.1	7	4
Medazepam	18.83	37.65	3.2	7.5	3.6	7.6	12.7	5.6	8	8
Midazolam	5.13	10.25	5.8	6.2	3.4	6.5	8.2	12.0	7	5
Nitrazepam	4.73	9.45	10.9	11.7	10.0	3.5	5.4	2.6	3	7
Nordiazepam	29.00	58.00	4.2	5.5	4.5	2.6	5.2	1.6	8	9
Oxazepam	29.25	58.50	2.4	3.5	10.0	4.8	5.7	14.9	7	14
Prazepam	22.35	44.70	10.5	11.4	9.4	6.4	7.4	8.5	4	12
Temazepam	28.75	57.50	3.8	5.1	3.1	4.7	6.8	5.1	9	5
Tetrazepam	11.25	22.50	8.4	9.2	5.8	12.8	13.5	19.5	9	14
Triazolam	1.73	1.73	16.3	16.3	14.7	10.7	12.3	8.1	9	4
Zaleplon	4.33	8.65	17.6	18.2	11.7	10.9	11.3	6.0	12	5
Zolpidem	9.15	18.30	3.0	4.5	19.8	14.0	14.0	8.9	8	12
Zopiclone	4.13	8.25	4.2	5.4	32.3 *	9.9	10.4	7.0	13	13
Antipsychotics										
Amisulpride	9.28	18.55	4.0	4.5	6.8	8.0	8.4	13.3	12	7
Aripiprazole	13.70	27.40	10.8	11.3	8.2	5.0	6.5	3.6	5	4
Chlorpromazine	4.63	9.25	4.2	6.5	6.7	7.0	7.3	36.9 *	5	7
Clozapine	14.28	28.55	2.7	7.3	8.0	8.7	9.3	15.3	9	9
Dehydroaripiprazole	2.63	5.25	4.6	8.3	9.7	9.9	10.4	2.5	4	12
Desmethylolanzapine	3.38	6.75	4.6	7.3	4.7	6.8	9.4	4.4	8	9
Haloperidol	0.32	0.32	5.5	5.5	38.3 *	6.6	8.5	17.1	5	10
Levomepromazine	2.14	4.27	3.3	6.3	4.1	8.5	10.5	9.2	10	5
Norclozapine	11.33	22.65	8.1	8.3	18.5	8.0	11.4	2.1	8	8
Norquetiapine	2.18	4.36	4.7	9.3	5.5	2.9	7.5	20.2	9	13
Olanzapine	3.57	7.14	11.1	14.4	15.5	4.1	7.4	19.9	4	9
Paliperidone	3.35	3.35	7.0	5.3	13.2	5.9	8.3	2.8	13	9
Pipamperone	7.73	15.45	4.5	4.6	0.8	6.5	6.8	10.1	5	14
Prometazine	5.20	10.40	4.8	5.5	23.8 *	4.9	6.6	27.5 *	5	5

(*continued on next page*)

Table 2 (*continued*)

Antidepressants			Precision and Bias						Stability	
			QC low			QC high			QC low	**QC high**
	LOD **(ng/ml)**	LLOQ **(ng/ml)**	Precision RDS (%)		Bias (%)	Precision RDS (%)		Bias (%)	Deviation n. 5 (%)	
Quetiapine	7.50	15.00	5.4	7.2	20.4	8.0	8.6	3.5	12	9
Risperidone	3.26	6.51	5.8	6.3	5.1	9.0	11.4	2.1	2	14
Sertindole	2.58	5.15	2.3	3.3	0.9	6.5	8.0	4.2	9	1
Sulpiride	13.68	27.35	3.5	5.5	7.6	9.8	10.7	3.4	13	12
Thioridazine	4.78	9.55	4.9	6.4	15.3	13.2	13.4	6.4	11	4

*For QC concentration see Supplementary material, Table 2

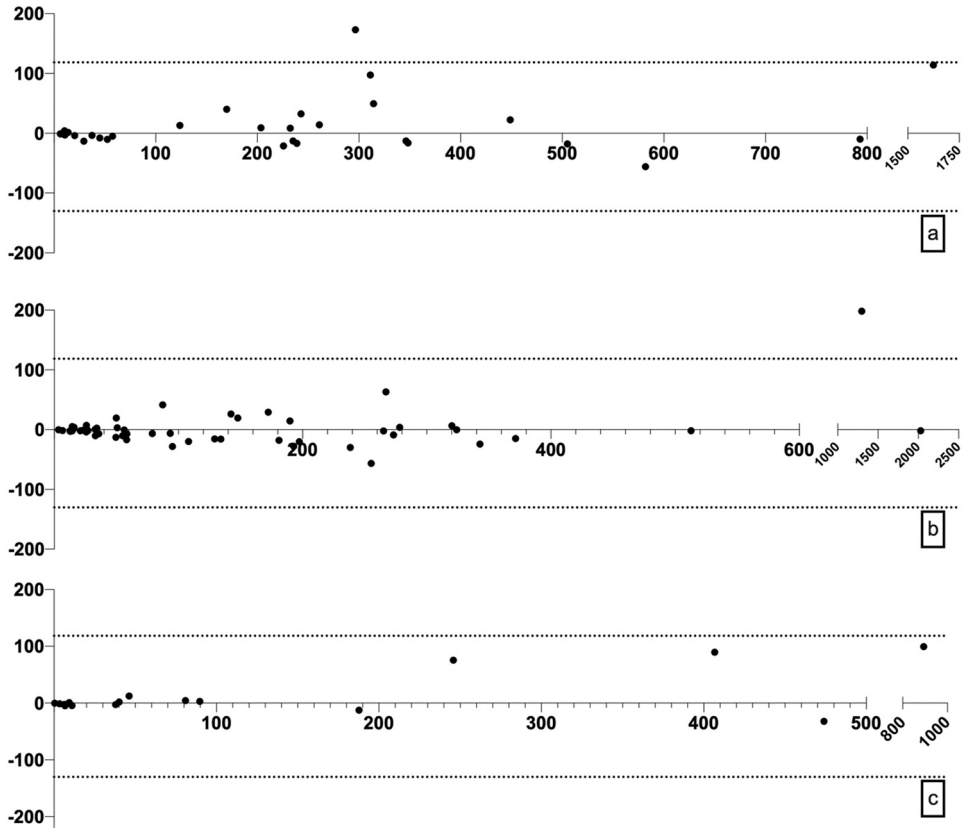

Fig. 1. The Bland-Altman plot between Method 1 and Method 2. The difference of the two paired measurements is plotted against the mean of the two measurements. The plotted lines represent the 95% CI (lower and upper). a: antidepressants; b: benzodiazepines; c: antipsychotics.

between measurements for any of the three classes of drugs ($p = 0.57$; $p = 0.13$; $p = 0.62$, respectively). A very good agreement between the new method and the internally validated method was demonstrated.

3.4. Innovations and future perspectives

The investigations of substances of abuse for medico-legal purposes are susceptible to continuous analytical improvement. An increasing number of new methodologies and instruments, as well as multiclass methods have been applied, aiming at the identification of new specific markers of abuse as well as the usability of alternative or complementary biological matrices to those of traditional use. These assessments, assuming the nature of judicial evidence, must possess the requisites of certainty and reliability [10–13]. Many of methods published in toxicological and forensic literature are limited to less than 50 target analytes or omit many drugs that may be forensically relevant [22,23]. Efficient and comprehensive targeted method for analysis and quantitation or semi-quantitation of drugs on small volumes of whole blood were also recently published [12,13], bringing significant improvements to forensic investigations. Farley et al. [17] observed that most methods require either LLE or SPE techniques. Although these procedures can be streamlined, they typically require more complex extraction processes, are more selective, and can hamper the inclusion of all the recommended drugs of interest [24–26]. The innovation of our method consists in the use of commercial calibrators, readily available to most forensic toxicology laboratories, that were used for validation procedures of many compounds of forensic interest, and of a limited number of IS. In fact, four IS were tested and, according to the results achieved, three of them gave satisfactory results to cover all analytes. We also tested a method to obtain six calibrators for the calibration model starting from 3 serum calibrators: three levels of lyophilized commercial serum calibrators were diluted with 50% of RBC, to simulate whole blood. The negligible effect of RBC allowed the use of the six points for the calibration model. Excellent linearity and carry-over and

acceptable precision, bias and matrix effect have been achieved. The LOD and the LOQ obtained were also at the lower limits of the therapeutic range described in the literature for all drugs [20], demonstrating wide applicability in forensic casework, both in fatal intoxication and in case of death occurring after drug exposure. To achieve better chromatography and validation parameters, acetonitrile dilution and MPA dilution were compared. Although the peak shape was better with MPA, both dilutions gave acceptable results.

One of the main strengths of this study is the versatility and the applicability of the method, in consideration of the fast sample pretreatment and the limited number of deuterated IS, which contribute to a routine applicability in the forensic laboratories. Although the use of commercial calibrators can be considered a pitfall, as it is not in full agreement with the guidance that requires the use of pure standards in matrices, validation parameters were accomplished. Nevertheless, better results could be expected by using specific deuterated internal standards, with more chemical similarity to the various classes of drugs. Moreover, the validation of the method on blood samples makes it particularly suitable for forensic applications where neither serum nor plasma are available or when post-mortem sampling is performed after a longer post-mortem interval PMI [27]. In fact, postmortem blood is characterized by a variable grade of hemolysis, sedimentation, clotting, loss of fluid portions and putrefaction, preventing serum or plasma separation. Whole blood may be the only matrix available, also in the living subject. When an intoxication is suspected or in cases of suspected driving under the influence (DUI) of drugs, blood and/or urine samples are often collected at hospitals. When the separation of serum is not performed directly in the hospital where the blood is taken, the vials are sometimes frozen and sent to a forensic laboratory, where separation of the hemolyzed material is no longer achievable [28].

The application of the method to a series of 85 deaths from suspected acute poisoning revealed the presence of 30 of the 68 molecules included in the method, namely 11 antidepressants, 11 benzodiazepines and 8 antipsychotics. This demonstrates that the proposed panel includes substances of forensic interest that may have an impact in cases of poly-abuse intoxication. The qualitative agreement with a previously validated method was 100%. A very good quantitative agreement between the new method and the internally validated method was demonstrated, since as all but two measures were within the 95% CI. In some cases, concentrations higher than the calibration range were observed, needing the confirmation with a method developed using reference standards in matrices. Nevertheless, the present method could be profitably applied as a quick and easy screening method for the substances included in the panel. Nevertheless, it must always be taken into account that, when a quantitative analysis is requested in real forensic casework, a multidisciplinary case-by-case evaluation, including an assessment of circumstantial, clinical, post-mortem, and toxicological data, is necessary. Moreover, for some target molecules not included in our study, additional steps may be required to allow the removal of lyso-phospholipids and phospholipids and an increase in matrix effect and recovery [29].

3.5. Limitations

Our study presents some limitations, mainly attributable to the use of commercial calibrators. The surrogate matrix that used is not identical to post-mortem blood, even resulting suitable for the scope of the study and for the analytes of interest. The choice of analytes is predetermined through the analytes being present in the standard mixture, resulting in a pre-defined panel of drugs, and should be adjusted in line with special needs with reference standards. The recovery was not tested, as commercial calibrators contain the analytes of interest. However, recovery may not be part of the validation model if LOQ, LOD, precision, bias and matrix effect are satisfactory [27]. Moreover, the study of LOD and LOQ, which was defined as the lower calibrator, was necessarily influenced by the pre-defined concentration of commercial calibrators. For this reason, our methods present higher LOD and LOQ for some molecules compared to published methods, but in line with the therapeutic ranges of each of them, fulfilling the requirements of a screening method. As observed in some of the real cases, the concentration in forensic cases, especially in fatal intoxications, can be higher and sometimes lower than the tested calibration range. In this case, a targeted method with a specific standard must be applied to increase accuracy at higher concentration. Some compounds have shown significant matrix effects, as also observed in methods involving protein precipitation, as this has been described to lead to large amounts of endogenous compounds in the injected sample, increasing or decreasing signals.

4. Conclusion

The developed method can be profitably applied in any situation where a multi-analyte screening is advantageous, by reducing time and costs of analysis, both in a clinical and forensic toxicology context, with a 15-minute run time and a broad panel of compounds. Even with some unavoidable limitations related to the use of calibrators for the validation procedure, this method could lead to an innovation in terms of simplicity of analysis and the availability of chemicals needed. Moreover, the requirements for validation have been met, and the presented method can be inserted into the routine work in the forensic laboratories, in the frame of acute poisoning, Drug Facilitated Crimes, Driving Under the Influence (DUI) of psychoactive drugs or other challenges that can arise from forensic casework. In a future perspective, the number of molecules to be analyzed in the panel can be increased as well as the forensic casework application.

Compliance with ethical standards

Not applicable.

CRediT authorship contribution statement

Rossella Barone: Writing – original draft; **Arianna Giorgetti**: Methodology; **Rachele Cardella**: Data curation; **Francesca Rossi**: Formal analysis; **Marco Garagnani**: Software; **Jennifer Paola Pascali**: Writing – review & editing; **Susan Mohamed**: Investigation; **Paolo Fais**: Conceptualization; **Guido Pelletti**: Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115315.

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