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Development and validation of a LC-ESI-MS/MS method for simultaneous whole blood analysis of 51 new psychoactive substances

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In recent years, there has been a great increase in seizures and forensic analysis of new psychotropic substances (NPS) in the state of Rio Grande do Sul, Brazil. The analysis of these compounds needs to be performed in biological samples in cases of violent deaths. A sensitive and reliable liquid chromatography-tandem mass spectrometry with electrospray ionization interface (LC-ESI-MS/MS) method was developed and validated for qualitative analysis of 51 NPS in whole blood forensic samples. Synthetic cathinones, phenethylamines, opioids, tryptamines, synthetic cannabinoids, and other hallucinogens and stimulants were included in the method. The validation parameters assessed were specificity, limit of detection, retention time precision, and matrix effect. Drug free pools (n=6) were used for validation, including *post mortem* samples as well as from living individuals. Adulterants, pharmaceuticals, metabolites, and other illicit drugs, totalling 39 compounds, were analyzed and no interference was noticed. The detection limits obtained were suitable for evaluation at recreational and non-fatal levels of consumption, mostly. The results revealed an appropriate matrix effect in 24 out of 51 substances tested, indicating the potential for future quantitative analysis with this method for these drugs. The developed and validated method is easy to implement, fast, with low cost, and suitable for use in routine forensic toxicology laboratory analysis.

Keywords: Bioanalytical method; Forensic toxicology; Whole blood; Tandem mass spectrometry

Abbreviations

25B-NBOH	2-([2-(4-bromo-2,5-dimetoxiphenyl)etylamino]metyl)phenol
25B-NBOMe	2-(4-bromo-2,5-dimethoxyphenyl)-n,n-bis(2-methoxybenzyl)ethanamine
25C-NBOH	2-(((4-chloro-2,5-dimethoxyphenethyl)amino)methyl)phenol
25C-NBOMe	2-(4-chloro-2,5-dimethoxyphenyl)-n-(2-methoxybenzyl)ethanamine
25E-NBOH	2-[[2-(4-ethyl-2,5-dimethoxy-phenyl)ethylamino]methyl]phenol
25I-NBOH	2-({[2-(4-iodine-2,5-dimethoxy-phenyl)ethyl]amino}methyl)phenol
25I-NBOMe	2-(4-iodine-2,5-dimethoxyphenyl)-n-(2-methoxybenzyl)ethanamine
2C-B	4-bromo-2,5-dimethoxyphenetylamphetamine
2C-E	4-ethyl-2,5-dimethoxyphenetylamine
5-MeO-MIPT	5-methoxy-n-isopropyl-n-methyltryptamine
ADB-Fubinaca	n-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1Hindazole-3-carboxamide
Alpha-PVP	α-pyrrolidinopentiophenone
bk-DMBDB	dibutylone
bk-MDEA	ethylone
bk-MDMA	methylone
CP47-497	rel-2[(1S,3R)-3-hydroxycyclohexyl]-5-(2-methyloctan-2-yl)phenol
CP47-497-C8	rel-2-[(1S,3R)-3-hydroxycyclohexyl]-5- (2-methylnonan-2-yl)phenol
DMAA	1,3-dimethylpentylamine
DMT	n,n-dimethyltryptamine
DOB	4-bromo-2,5-dimethoxyamphetamine
DOC	4-chloro-2,5-dimethoxyamphetamine
DOI	4-iodine-2,5-dimethoxyamphetamine
HU-210	1,1-dimethylheptyl-11-hydroxytetrahydrocannabinol
JWH-018	(1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone
JWH-073	(1-butyl-1H-indol-3-yl)(naphthalen-1-yl)methanone
JWH-1503	[1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone
JWH-200	[1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl]-1-naphthalenyl-methanone
JWH-250	1-(1-pentyl-1H-indol-3-yl)-2-(2-methoxyphenyl)-ethanone
LSD	lysergic acid diethylamide
MBDB	n-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine
mCPP	1-(3-chlorophenyl)piperazine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-n-ethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
PCP	phencyclidine
TFMPP	1-(3-trifluoromethylphenyl)piperazine
THC	tetrahydrocannabinol
TH-PVP	2-pyrrolidin-1-yl-1-tetralin-6-yl-pentan-1-one

Introduction

The use of psychoactive substances is a worldwide phenomenon with incalculable consequences on society. The World Drug Report 2019, by the United Nations Office on Drugs and Crime (UNODC), reported that 35 million people around the world suffer from drug use disorders, revealing that the adverse health consequences of drug use are more severe and widespread than previously thought (1).

Older drugs, such as cocaine and *Cannabis sativa*, remain dominant, but the drug market is now peppered with a vast array of synthetic psychoactive substances. Clandestine laboratories manufacture synthetic drugs in different countries all the time showing the need for broader international cooperation to promote balanced and integrated criminal health and justice responses to supply and demand. New psychoactive substances (NPS), which induce stimulant and hallucinogenic effects even in small amounts, are designed to mimic established substances with similar properties that are under international control, such as amphetamine, methamphetamine, dimethoxyamphetamines, piperazines, cathinones, and several other drugs. These drugs are sold on the internet and they appear specially on the *dance scene* in the form of various tablets or blotters paper with questionable composition and potential risk for intoxications and death (1).

The number of stimulant NPS identified over the period 2009–2017 increased more than fourfold, from 48 substances in 2009 to a peak of 206 in 2015, a number that has remained stable since then. In most years, stimulant NPS have been the largest group of NPS identified and reported by Member States, followed by synthetic cannabinoids. Over a third of all NPS identified since 2009 are stimulants, including 39 per cent of all NPS identified in 2017. Most of the new stimulant NPS identified on the markets and reported to UNODC in 2017 were cathinones or phenethylamines (1).

Although the mortality associated with NPS is still not comparable to that of opiate-related deaths, for example, there is uncertainty about the number of undetected cases, and this issue is becoming a more significant challenge for *post mortem* forensic toxicology. The measurement of NPS in biological forensic samples has become an extended topic over the present decade for monitoring trends of use and deaths among young people. According to the *post mortem* toxicology Technical Report in Europe, there is an urgent need to increase the screening capacity of many toxicology laboratories to determine certain NPS groups in biological samples, especially new multi-target methods (2).

Although stimulants amphetamine derivatives are analyzed by gas chromatography (GC), generally equipped with nitrogen–phosphorus or mass detectors, liquid chromatography (LC) has some advantages in comparison to GC. LC does not promote thermal degradation of the analyte and it does not require samples to be volatilized. To improve GC properties, some additional steps such as selective derivatization, are mandatory. While giving excellent results, the use of some derivatizing agents in GC increase cost and time of analysis and can be pernicious for column lifetime due to decomposition of the stationary phase making the application of LC analysis advantageous (3). The hyphenation of LC to high resolution mass spectrometry based on triple quadrupole for target analysis has advantages for identification of NPS in low levels in biological samples due to its increased resolving power and sensibility.

The simultaneous determination of a broad number of compounds in one injection, with a corresponding reduction of time and costs, without additional materials needed for sample preparation, is very important in the forensic routines of underdeveloped countries. The aim of the presented work was to establish a broad analytical tool for the analysis of different types of NPS in human whole blood *post mortem* samples, by liquid chromatography-tandem mass spectrometry with electrospray ionization interface (LC-ESI-MS/MS) method, according to the reality of the drugs seized in recent years in the state of Rio Grande do Sul (RS), Brazil (4-6). The study project was approved by the Research Ethics Committee of the Federal University of Rio Grande do Sul prior to the initiation of the study, under number 2.532.550.

Experimental

Materials, chemicals, and reagents

Certified reference standards for 25B-NBOMe (90.2%), 25C-NBOMe (89.6%), 25I-NBOMe (90.5%), 2C-B (87.0%), bk-MDMA (84.7%), heroine (90.0%), JWH-1503 (0.1 mg mL^{-1}), LSD (0.025 mg mL^{-1}), MBDB (84.1%), and mephedrone (82.6%) compounds were purchased from Lipomed, Inc. (Cambridge, MA, USA); for DOB (1.0 mg mL^{-1}), DOI (1.0 mg mL^{-1}), DMAA (1.0 mg mL^{-1}), Nordiazepam-d5 (1.0 mg mL^{-1}, standard internal), and TFMPP (1.0 mg mL^{-1}) were purchased from Cerilliant Corporation (Round Rock, TX, USA); for mCPP (99.7%) was purchased from LGC GmbH (Luckenwalde, BR, Germany); for diethylpropion (98.8%) and fenproporex (98.7%) were obtained from Aché Pharmaceutical Laboratories (Guarulhos, SP, Brazil); for CP47-497 (100.0 µg mL^{-1}), CP47-497-C8 (100.1 µg mL^{-1}), HU-210 (100.0 µg mL^{-1}), JWH-200 (100.0 µg mL^{-1}), JWH-250 (100.0 µg mL^{-1}), JWH-018 (100.0 µg mL^{-1}), JWH-073 (100.0 µg mL^{-1}), MDA (100.1 µg mL^{-1}), MDEA (99.9 µg mL^{-1}), MDMA (99.9 µg mL^{-1}), methamphetamine (100.3 µg mL^{-1}), methylphenidate (100.3 µg mL^{-1}), PCP (100.2 µg mL^{-1}), phentermine (100.0 µg mL^{-1}), cannabinol (100.4 µg mL^{-1}), fentanyl (50.0 µg mL^{-1}), and alfentanyl (50.0 µg mL^{-1}) were purchased from Agilent Technologies (Santa Clara, CA, USA); and for bupropion (100.7%) and sibutramine (100.0%) were obtained from Spengler Compounding Pharmacy (Porto Alegre, RS, Brazil).

Reference standard including 25B-NBOH, 25C-NBOH, 25E-NBOH, 25I-NBOH, 2C-E, 5-MeO-MIPT, ADB-Fubinaca, alpha-PVP, clobenzorex, bk-DMBDB, DOC, bk-MDEA, DMT, N-ethylpentylone, and TH-PVP were obtained from materials seized by Civil Police of RS, Brazil and were identified by, at least, five different analytical technics including A category according SWGDRUG guidelines (7), in cooperation studies among the Laboratory of Criminal Investigation (DPL-IGP/RS), Federal Laboratory of Agricultural Defense (Lanagro-RS), Federal University of Health Sciences of Porto Alegre (UFCSPA), and Federal University of RS (UFRGS) all located in Porto Alegre, RS, Brazil.

Methanol (HPLC grade) was purchased from J.T. Baker (Center Valley, MA, USA), sodium hydroxide (ACS grade) was purchased from Neon (Suzano, SP, Brazil), sodium nitrite (ACS grade) was purchased from Ecibra (Santo

Amaro, SP, Brazil), dichloromethane (HPLC grade) was purchased from Honeywell (Charlotte, NC, USA), and butyl chloride (HPLC grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained by purification of distilled water in-house using Purelab (Elga, UK). Formic acid and ammonium formate were purchased from Agilent Technologies (Santa Clara, CA, USA).

All related substances used in specificity tests (Table 1), as well as all consumables, were provided by the Toxicology Division of DPL-IGP/RS, through authorization number 083/2017 present in the process number 17/1205-0001343-1.

Table 1 Substances analyzed by LC-ESI-MS/MS in whole blood at therapeutic or recreational concentrations [11,12], to evaluate the specificity of the developed method.

Substance	Description	Concentrations evaluated (ng mL^{-1})
11-hydroxy-THC	THC metabolite	1 and 100
11-nor-9-carboxy-THC	THC metabolite	1
Acetaminophen	analgesic	2,500 and 9,000
Alprazolam	anxiolytic	5
Amitriptyline	antidepressant	14 and 50
Amphetamine	stimulant	14 and 20
Atropine	muscarinic antagonist used in hospital care	2 and 14
Benzoylecgonine	cocaine metabolite	14 and 50
Bromazepam	anxiolytic	50 and 80
Caffeine	stimulant and cocaine adulterant	14, 2,000 and 2,500
Carbamazepine	anticonvulsant	2,000
Carisoprodol	muscle relaxant	14 and 1,500
Chlorpheniramine	antihistamine	1 and 3
Citalopram	antidepressant	14, 20 and 50
Cocaethylene	cocaine metabolite in the presence of ethanol	10 and 14
Cocaine	*Erythroxylum coca* active stimulant	14 and 50
Cotinine	present in tobacco and nicotine metabolite	14 and 30
Dextromethorphan	antitussive	10 and 14
Diazepam	anxiolytic	14, 20 and 100
Dipyrone	analgesic	10,000
Fluoxetine	antidepressant	100 and 120
Ibuprofen	analgesic	15,000
Ketamine	anesthetic	100 and 1,000
Lamotrigine	anticonvulsant and mood stabilizer	1,000
Levamisole	anthelmintic and cocaine adulterant	100
Lidocaine	anesthetic and cocaine adulterant	14 and 1,000
Mazindol	anorectic	2
Mirtazapine	antidepressant	30
Nicotine	present in tobacco	1 and 14
Olanzapine	antipsychotic	20
Omeprazole	antacid	50
Ritalinic acid	methylphenidate metabolite	5 and 20
Sertraline	antidepressant	10, 14 and 50
Sildenafil	erectile dysfunction	50
Tadalafil	erectile dysfunction	90
Tetrahydrocannabinol	*Cannabis sativa* active substance	1 and 100
Theobromine	present in chocolate	1,000
Venlafaxine	antidepressant	100
Zolpidem	hypnotic	80

Instrumentation

Nitrogen was generated using a Genius 1050 nitrogen generator by Peak Scientific (Billerica, MA, USA). An Agilent Technologies 6420 *Triple Quad* (Santa Clara, CA, USA) equipped with auto sampler thermostatically controlled (4°C) was used to analyze samples. Separation was achieved using an Agilent Technologies Series 1260 *Infinity* II LC system equipped with Agilent C_{18} Zorbax Eclipse Plus (2.1 x 100 mm; 1.8 µm particle size) column and an Agilent Eclipse XDB-C8 guard column (4.6 x 12.5 mm, 5 µm particle size) in a thermostatically controlled column compartment (50ºC). The mobile phase consisted of 0.1% formic acid and 5 mM ammonium formate in ultrapure water (A) and 0.1% formic acid and 5 mM ammonium formate in methanol (B). A flow rate of 0.4 mL/min was maintained using the gradient elution profile as follows: 95% A and 5% B; 50% A and 50% B (2.8 min); 100% B (13.6-18.8); 95% A and 5% B (18.9 min). The total acquisition time was 18.9 min with post time 6 min for re-equilibration. The LC triple quadrupole was equipped with an electrospray ionization interface source (ESI), under the following conditions: drying gas was N_2 12 L min^{-1}, drying gas temperature 320ºC, nebulizer 30 psi, and capillary voltage 3500 V (positive mode) and 3000 V (negative mode). Analytical conditions for each substance are given in Table 2. Acquisition and qualitative analysis were performed under dynamic multiple reaction monitoring mode (dMRM) in Agilent MassHunter® software.

Table 2 Substances analyzed by LC-ESI-MS/MS in whole blood, with their retention times, monitored transitions, analytical conditions, and detection limits obtained.

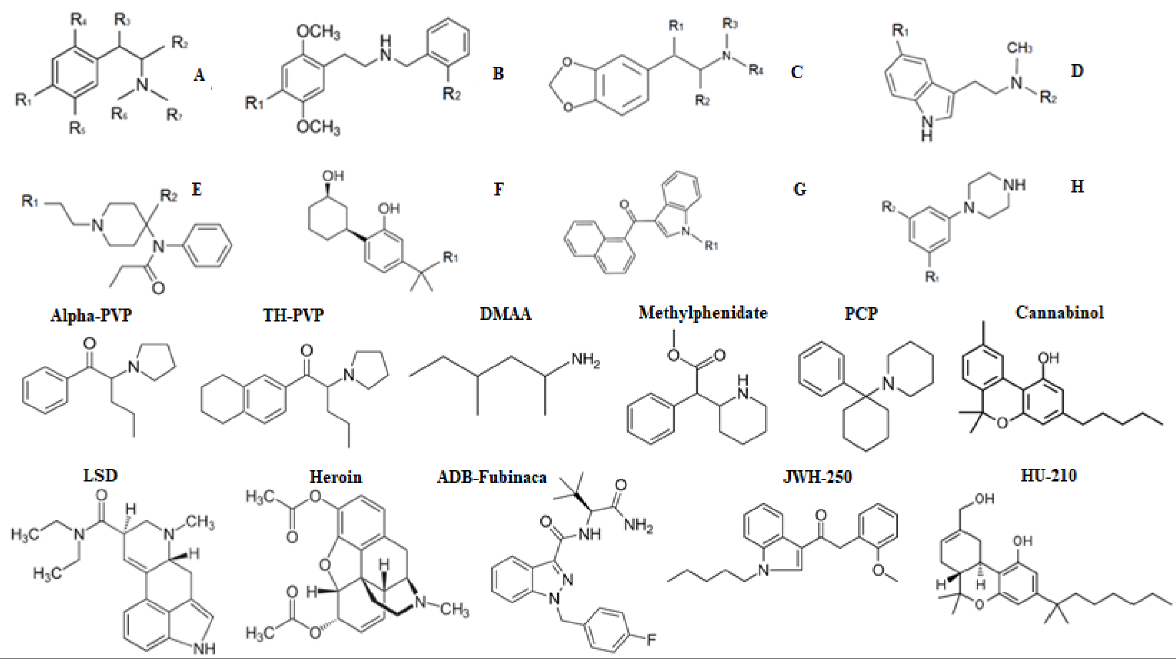
Substance	RT[a] (min)	Precursor (m/z)	Fragments (m/z)	FE[b] (V)	CE[c] (V)	Polarity	LD (ng mL^{-1})
25B-NBOH	7.5	366.0	105.0, 91.0 and 77.0	120	75, 55 and 40	positive	*
25B-NBOMe	8.1	380.1	121.1 and 91.1	120	18 and 58	positive	0.8
25C-NBOH	7.3	322.1	199.0 and 107.0	120	25 and 35	positive	*
25C-NBOMe	7.8	336.1	121.1 and 91.1	120	18 and 50	positive	0.7
25E-NBOH	8.6	316.2	193.0, 178.0 and 107.0	120	25, 30 and 40	positive	*
25I-NBOH	7.9	414.1	291.1 and 107.1	120	22 and 38	positive	*
25I-NBOMe	8.5	428.1	121.1 and 91.1	93	20 and 48	positive	0.5
2C-B	5.9	260.0	243.1 and 228.1	100	10 and 18	positive	15
2C-E	6.7	210.1	193.2 and 178.2	80	10 and 18	positive	*
5-MeO-MIPT	5.0	247.2	174.1 and 86.1	65	16 and 10	positive	*
ADB-Fubinaca	11.5	383.2	253.3 and 109.0	133	33 and 57	positive	*
Alfentanyl	6.9	417.3	268.1, 197.0 and 165.0	128	16, 24 and 36	positive	0.7
Alpha-PVP	5.6	232.2	91.1 and 77.1	130	26 and 54	positive	*
bk-DMBDB	4.9	236.1	191.1, 161.1 and 65.0	75	10, 16 and 48	positive	*
bk-MDEA	4.6	222.1	204.2, 174.2 and 91.1	100	10, 18 and 46	positive	*
bk-MDMA	4.3	208.1	190.2 and 160.2	100	10 and 14	positive	35
Bupropion	6.1	240.1	184.1, 166.0 and 131.1	150	10, 20 and 40	positive	425[d]
Cannabinol	15.0	311.2	293.2 and 223.0	126	16 and 20	positive	35
Clobenzorex	7.1	260.1	125.0 and 91.1	150	20 and 20	positive	*
CP47-497	15.0	317.2	299.2 and 245.2	232	24 and 32	negative	120[d]
CP47-497-C8	15.5	331.3	313.2, 259.2 and 159.0	247	24, 32 and 60	negative	150[d]
Diethylpropion	4.7	206.2	105.1 and 77.2	80	20 and 48	positive	20
DMAA	5.1	116.1	57.1 and 41.0	55	10 and 24	positive	360
DMT	4.4	189.1	144.1 and 58.1	55	12 and 8	positive	*
DOB	6.2	274.0	229.0, 178.1 and 105.1	60	16, 20 and 44	positive	15
DOC	6.0	230.1	213.1 and 185.0	150	10 and 20	positive	*
DOI	6.7	322.0	305.0 and 105.0	36	17 and 57	positive	15
Fenproporex	4.5	189.1	119.1 and 91.1	150	5 and 10	positive	30[d]
Fentanyl	6.5	337.2	216.1, 132.0 and 103.0	146	20, 32 and 60	positive	0.8
Heroin	5.4	370.2	211.1 and 58.1	150	40 and 40	positive	15[d]
HU-210	15.4	387.3	71.1 and 43.1	103	28 and 52	positive	50[d]
JWH-018	14.3	342.2	155.0 and 127.1	95	24 and 56	positive	20[d]
JWH-073	13.6	328.2	200.1, 155.0 and 127.1	133	24, 24 and 52	positive	10[d]
JWH-1503	12.8	360.2	155.0 and 127.1	138	24 and 48	positive	0.8
JWH-200	9.2	385.2	155.0 and 114.1	113	20 and 32	positive	10[d]
JWH-250	13.6	336.2	121.1 and 91.1	98	16 and 48	positive	10[d]
LSD	6.0	324.2	223.1 and 207.1	150	20 and 40	positive	5
MBDB	5.3	208.1	177.1 and 147.1	150	10 and 10	positive	15
mCPP	5.5	197.1	154.0 and 118.1	103	20 and 36	positive	25
MDA	4.6	180.1	163.1 and 105.1	80	4 and 24	positive	2
MDEA	5.0	208.1	163.1 and 133.1	98	8 and 16	positive	2
MDMA	4.7	194.1	163.1 and 105.1	80	8 and 24	positive	0.3
Mephedrone	5.1	178.1	160.2 and 145.2	100	10 and 22	positive	80[d]
Methamphetamine	4.6	150.1	119.1 and 91.1	75	8 and 20	positive	2
Methylphenidate	5.6	234.1	84.1 and 56.1	123	20 and 56	positive	1
N-ethylpentylone	5.7	250.1	232.3 and 202.2	120	10 and 18	positive	*
Nordiazepam-d5 (IS)	9.5	276.1	213.1 and 140.0	150	40 and 40	positive	not applicable
PCP	6.3	244.2	91.1 and 86.2	75	36 and 8	positive	0.3
Phentermine	5.1	150.1	133.0 and 105.0	75	8 and 16	positive	30
Sibutramine	8.9	280.2	139.0 and 125.0	150	10 and 20	positive	45[d]
TFMPP	6.1	231.1	188.1 and 44.0	123	20 and 20	positive	15[d]
TH-PVP	8.6	286.2	215.2 and 145.2	150	18 and 30	positive	*

[a]RT retention time; [b]FE fragmentor energy; [c]CE collision energy; [d]above the recreational or therapeutic concentration [11,12]; *reference chemical of no known purity.

Preparation of standards and reagents

Solutions containing structurally related substances or substances that could be present in the forensic biological samples, such as some metabolites, antidepressants, anxiolytics, illicit drugs, adulterants, and other pharmaceuticals were prepared in methanol and evaluated in the specificity test in recreational or therapeutic concentrations (Table 1).

Working standards solutions of each of the 51 compounds target (Figure 1) were prepared in methanol for the fortification of negative blood samples. The isotopically labelled internal standard solution was diluted in methanol at 1.0 µg mL^{-1}. Sodium hydroxide solution was prepared in ultrapure water at 0.2 M. Sodium nitrite solution was prepared in ultrapure water at 5 mg L^{-1}. The elution solvent was prepared daily. The extraction mixture solvent consisted of dichloromethane/butyl chloride 1:4 (v/v) and the resuspension solvent consisted of the mobile phase mixture A and B (1:1).



Structure	Substance	R_1	R_2	R_3	R_4	R_5	R_6	R_7
A	2C-B	Br	H	H	CH_3O	CH_3O	H	H
	2C-E	CH_3CH_2	H	H	CH_3O	CH_3O	H	H
	DOB	Br	CH_3	H	CH_3O	CH_3O	H	H
	DOC	Cl	CH_3	H	CH_3O	CH_3O	H	H
	DOI	I	CH_3	H	CH_3O	CH_3O	H	H
	Bupropion	H	CH_3	O	H	Cl	H	$(CH_3)_3C$
	Clobenzorex	H	CH_3	H	H	H	H	$C_6H_4ClCH_2$
	Diethylpropion	H	CH_3	O	H	H	CH_3CH_2	CH_3CH_2
	Fenproporex	H	CH_3	H	H	H	H	$CNCH_2CH_2$
	Mephedrone	CH_3	CH_3	O	H	H	H	CH_3
	Methamphetamine	H	CH_3	H	H	H	H	CH_3
	Phentermine	H	$(CH_3)_2$	H	H	H	H	H
	Sibutramine	Cl	$(CH_3)_2CHCH_2$	$(CH_2)_3$	H	H	CH_3	CH_3
B	25B-NBOH	Br	OH					
	25B-NBOMe	Br	CH_3O					
	25C-NBOH	Cl	OH					
	25C-NBOMe	Cl	CH_3O					
	25E-NBOH	CH_3CH_2	OH					
	25I-NBOH	I	OH					
	25I-NBOMe	I	CH_3O					
C	bk-DMBDB	O	CH_3CH_2	CH_3	CH_3			
	bk-MDEA	O	CH_3	H	CH_3CH_2			
	bk-MDMA	O	CH_3	H	CH_3			
	MBDB	H	CH_3CH_2	H	H			
	MDA	H	CH_3	H	H			
	MDEA	H	CH_3	H	CH_3CH_2			
	MDMA	H	CH_3	H	CH_3			
	N-ethylpentylone	O	$CH_3CH_2CH_2$	H	CH_3CH_2			
D	5-MeO-MIPT	CH_3O	$(CH_3)_2CH$					
	DMT	H	CH_3					
E	Alfentanyl	$CH_3CH_2N_4CO$	CH_3OCH_2					
	Fentanyl	C_6H_5	H					
F	CP47-497	C_6H_{13}						
	CP47-497-C8	C_7H_{15}						
G	JWH-018	C_5H_{11}						
	JWH-073	C_4H_9						
	JWH-1503	$C_5H_{10}F$						
	JWH-200	$(CH_2)_2N(CH_2)_4O$						
H	mCPP	Cl	H					
	TFMPP	H	CF_3					

Figure 1 Chemical structures of the 51 substances target analyzed by LC-ESI-MS/MS developed method.

Blank samples

Drug free pools whole blood samples were obtained from two volunteers by the laboratory itself (n=1) and from fifty-five *post mortem* individuals (n=5), unidentified and all preserved with sodium fluoride and EDTA.
The same pools of negative samples have undergone modifications to simulate how the biological samples are eventually sent to the laboratory, which were: three heating cycles (40°C for 12 h) or dilution (20% in ultrapure water) or addition of nitrite (0.5 mg L^{-1}).

Blood extraction

The developed method was based on the work published by Marinetti and Antonides (8). Nordizepam-d5 internal standard solution (4 µL), sodium hydroxide solution (200 µL) and extraction mixture solvent (500 µL) were added to 500 µL whole blood sample, which was homogenized for 7 minutes at medium speed. After centrifugation at 10000 rpm for 7 min, the supernatant was transferred into vial. The aqueous residue was re-extracted with a second aliquot of 500 µL of extracting mixture solvent and the organic extracts were gathered in the same vial. After evaporation to dryness (at room temperature or up to 45 degrees), the residue was transferred to an insert with two aliquots of 25 µL each of mobile phase mixture A and B (1:1) and 18 µL were injected onto the LC-ESI-MS/MS for analysis.

Method Validation

The analytical method was validated in accordance with recommendations for qualitative analysis in biological specimens of UNODC (9), whose parameters were specificity, limits of detection, and precision under repeatability and reproducibility condition. The evaluation of the matrix effect was performed according to Brazilian Sanitary Surveillance Agency (10).

Specificity

Drug free pooled (n=6) whole blood samples were analyzed to verify the absence of interfering endogenous substances at the retention times of the analytes target and of the internal standard. Additionally, a blank whole blood sample from a living individual containing substances (n=39) that could be present in the forensic biological samples, in more than one concentration, recreational or therapeutic (11,12), were analyzed (Table 1). The specificity of the method was also evaluated after heating cycles, dilution and in the presence of sodium nitrite.

Limits of detection

Detection limits were obtained by extracting whole blood samples containing analytical standards from their respective therapeutic (or recreational) concentrations, increasing concentration or decreasing, until the results met the criteria of acceptability, which were: presence of all transitions at the same retention time, proportion of transitions within acceptable tolerance (13) and minimum abundance (3×10^2), whose definition occurred from blank assays.

Precision

Original drug free pooled matrix (n=6) and modified (n=3) containing substances target at their detection limits were analyzed on four days in duplicate. The repeatability of retentions time was evaluated through the coefficient of variation (CV%) values.

Matrix effect

Drug free pools matrices (n=6) were extracted and, posteriorly, added at concentrations in the respective detection limits. Ion suppression or enhancement was calculated by matrix factor normalized by PI (FMN), according to the Equation 1, below, evaluating the CV% values among matrices analyzed.

$$FMN = \frac{\text{peak area of drug in matrix} / \text{peak area of PI in matrix}}{\text{peak area of drug in mobile phase} / \text{peak area of PI in mobile phase}}$$

Eq. 1

Stability

Standard solutions, including internal standard, were prepared and used for method development and validation within a time period ranging from 16 to 20 months, always keeping refrigerated (4°C). The sample extracts were resuspended in mobile phase only on the day of injection in the equipment, remaining in the refrigerated sampler for a maximum of 24 h before analysis.

Results and Discussion

The necessary reference standards for the development and validation of forensic routine methods of emerging NPS are available from industrial sources, but only after a considerable time delay and at significant cost. To address this issue, the use of confiscated samples, after chemical purification processes, as a reference may solve the problem (2). This strategy has been adopted by DPL-IGP/RS in recent years.
Figure 2 shows the chromatogram obtained after the analysis of a whole blood sample containing the 51 substances target. Even though some analytes had very similar or equal retention times, there was no influence on the detection of each one, allowing the simultaneous analysis of all substances of interest.

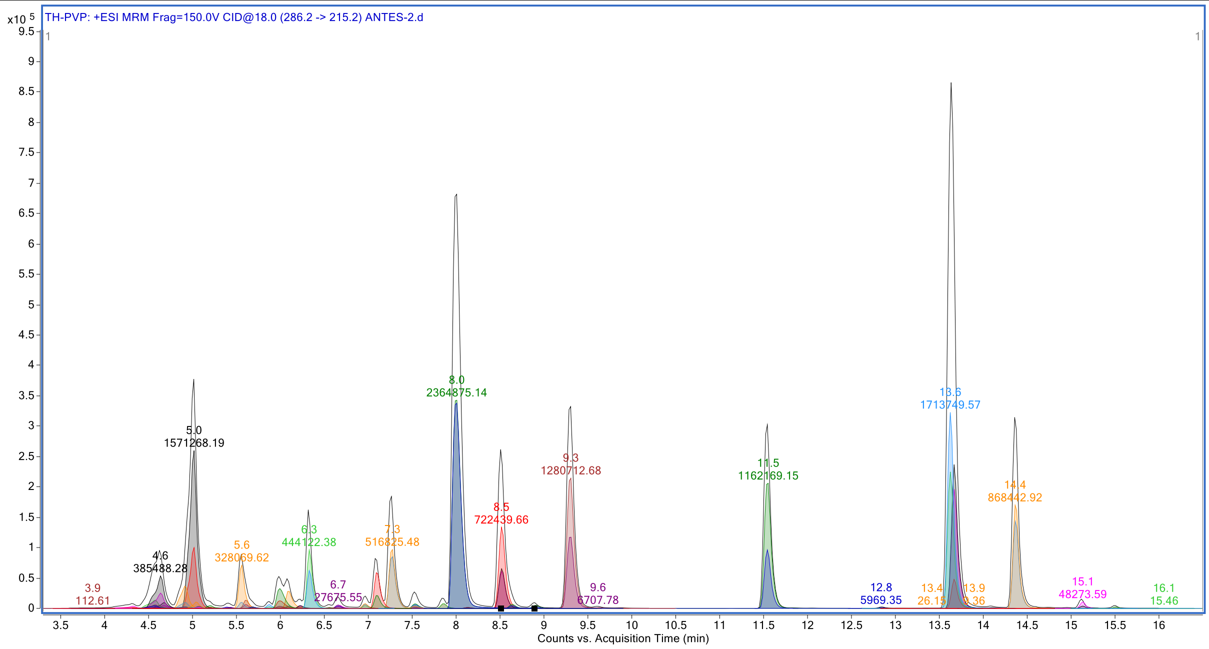


Figure 2 Chromatogram obtained by LC-ESI-MS/MS in whole blood containing the 51 substances target at the respective detection limits after extraction of the monitored transitions overlap.

Specificity

The tests with modified matrices aimed to verify if, an eventual, lack of refrigeration of the samples, collection of sites containing mixing of blood with other body fluids or the presence of sodium nitrite could harm the method developed by produce any unwanted interferences. In the state of RS there is only one forensic laboratory, located in Porto Alegre, where all the biological specimens from the interior are transported and some temperature problems may occur during this transport. Furthermore, depending on the state of putrefaction of the body, blood samples may be collected with other body fluids, diluting matrix components. Moreover, the developed method will also be used to analyse samples of suicide victims, which may be under the influence of psychotropic substances at the time of death. A common form of suicide in RS is the intake of sodium nitrite (*salitre*). This salt is used in the food industry to preserve the color of canned meat and sausages, as well as to prevent the spread of botulism-causing bacteria (14), but in a concentration higher than 0.5 mg L^{-1} can cause death (11). The evaluation of the specificity of the method in the presence of sodium nitrite occurred because the interface used in the equipment was ESI type, which may suffer interference due to salts in the sample. No original or modified matrix and no other illicit substance, drug, metabolite or adulterant interfered with retention time of analytes target or of internal standard (Figure 3), no false positive result was observed.

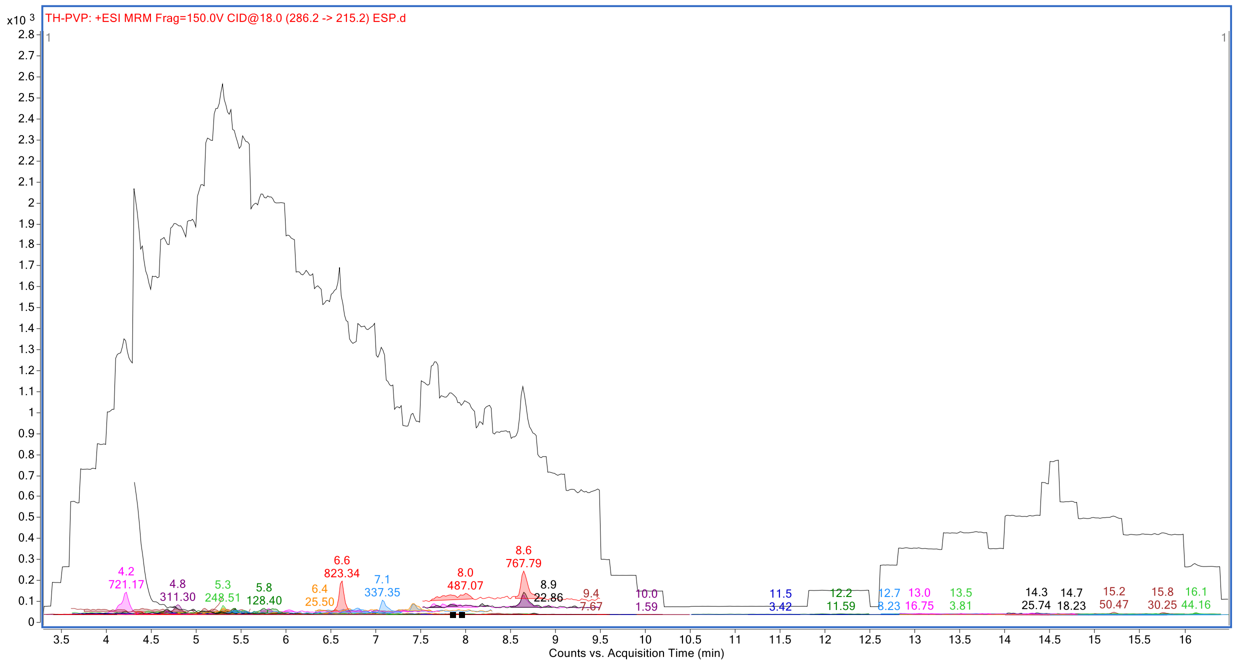


Figure 3 Chromatogram obtained by LC-ESI-MS/MS after extraction of the monitored transitions overlap in whole blood containing the substances listed in Table 1, at their highest concentrations, for evaluation of the specificity of the method

Limits of detection

To detect the use of NPS, metabolites and/or the parent molecule, is a particular analytical challenge in biofluids, being difficult because of the low concentrations encountered for the more potent substances and the lack of knowledge about many of them (15-20). Table 2 shows the analytical conditions defined for each substance with the respective detection limits obtained. For most of the analytes, fortunately, the values found were in the recreational or therapeutic range, making it possible to assess whether the victims were under the effect of the substances at the time of death.

Precision

Retention times presented CV% values ranging from 0.29 to 1.44, demonstrating a precision adequate, less than 2%. No false negative results were observed between the original and modified matrices.

Matrix effect

The potential impact of *post mortem* matrix-related effects on standard analytical methods and the interpretation of results are examples of issues relating to forensic toxicology (2), that is why, although the guide used as a reference for the validation of the qualitative method did not suggest the evaluation of the matrix effect, this parameter has been evaluated. Besides that, according to several authors (21-23), LC method coupled to the ESI ionization source suffers more influence of the matrix components.
Table 3 shows the FMN obtained for the substances that presented CV% around 15, considered satisfactory. These results indicate that, for these substances, there is a potential use of the method developed for quantitative analysis after the evaluation of the other validation parameters, however considering that FMN values lower than 1 had the signal suppressed in the matrix presence and those with values greater than 1, increment.

Table 3 FMN values obtained during matrix effect evaluation [10] with drug free pools whole blood samples by LC-ESI-MS/MS.

Substance	Alive	*Post mortem*					Mean	CV%
	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6		
25B-NBOH	0.9	0.9	0.8	0.9	0.9	0.8	0.9	4.8
25C-NBOH	0.9	0.7	0.8	0.9	0.9	0.8	0.9	9.7
25E-NBOH	0.8	0.9	0.9	0.9	0.9	0.8	0.9	5.1
25I-NBOH	1.0	0.9	0.8	0.9	0.9	0.8	0.9	6.2
5-MeO-MIPT	1.0	0.8	0.9	1.0	0.9	0.7	0.9	12.9
ADB-Fubinaca	0.6	0.5	0.5	0.5	0.6	0.4	0.5	14.8
Alfentanyl	1.4	1.3	1.3	1.3	1.3	1.3	1.3	3.3
Clobenzorex	1.2	0.8	1.0	1.0	1.0	0.7*	1.0	11.5
CP-047-497	0.7	0.7	0.6	0.7	0.8	0.4*	0.7	12.9
CP-047-497-C8	0.6	0.6	0.4	0.6	0.6	0.3*	0.6	15.8
DOB	1.1	1.2	1.0	1.2	1.1	1.0	1.1	8.6
DOC	0.9	1.0	0.9	0.8	0.8	0.2*	0.9	8.6
DOI	1.0	1.0	1.3	1.1	1.2	1.0	1.1	9.5
Fenproporex	1.0	1.0	1.0	0.7	1.0	0.3*	0.9	15.9
Fentanyl	1.3	1.3	1.3	1.2	1.3	1.4	1.3	6.1
HU-210	0.6	0.6	0.4	0.6	0.6	0.4*	0.6	14.4
JWH-018	0.3	0.3	0.3	0.3	0.3	0.2*	0.3	12.5
JWH-073	0.7	0.7	0.5	0.6	0.7	0.4*	0.6	9.1
JWH-1503	0.6	0.5	0.4	0.5	0.5	0.4	0.5	14.6
JWH-200	0.8	1.0	0.8	1.0	1.0	0.8	0.9	13.2
JWH-250	0.7	0.7	0.6	0.7	0.7	0.5	0.7	14.8
MBDB	1.3	1.5	1.1	1.6	1.4	2.9*	1.3	13.7
MDEA	1.0	1.0	0.8	0.9	1.0	0.8	0.9	10.2
Methylphenidate	1.9	2.7	2.3	2.4	2.6	3.2*	2.4	13.5

*value discarded

It is important to note, however, that there are often no defined concentration ranges associated with NPS that would correspond to degrees of toxicity and expected outcomes that indicates the likely role of a substance in contributing to or causing death (24).
On the other hand, the substances not listed in Table 3 showed great variability in the FMN factors obtained, demonstrating the application of the method developed for their qualitative analysis only, since all met the criteria for positivity.
For the epidemiological surveillance of NPS, the challenge is that at least qualitative results (detection and identification) are reported owing to the specific problems associated with the large and rapidly growing number of NPS. Indeed, there are special requirements in *post mortem* analysis and difficulties in their interpretation, which challenge forensic laboratories (2).
Post mortem blood samples are often hemolyzed and mixed with other biological fluids and tissues in decomposition, depending on the time between death and necropsy. The presence of large amounts of lipids in these samples is common and their interference in the analytical method was evaluated together with the other parameters.

Stability

Throughout the development of the method and validation, it was observed that the analyte areas varied slightly but remained sufficiently intense to meet all positivity criteria. One of the major limitations in forensic toxicology is the difficulty in obtaining reference standards as well as the small quantity of them, when available. Thus, stability is considered adequate as long as unambiguous identification of the substance is possible, even if there is some degradation.

Conclusion

The proposed LC-ESI-MS/MS method can be regarded as selective and validated for qualitative forensic analysis. Among the advantages of this method we have the ease of execution without the need for expensive consumables and a 25 min analytical run detecting 51 analytes simultaneously through a method accessible for medium laboratories of toxicology in countries such as Brazil, where the abuse of NPS compounds are increasing exponentially, and are considered as a public health problem.

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Interest conflicts

The authors declare no conflicts of interest.

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