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Identification and quantification of psychoactive drugs in whole blood using dried blood spot (DBS) by ultra-performance liquid chromatography tandem mass spectrometry

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Graphical Abstract

Highlights

- Psychoactive drugs were investigated in dried blood spot (DBS) samples to assess consumption;
- UHPLC-MS/MS assay was developed and validated for DBS;
- Fast and simple DBS extraction was applied;
- DBS analysis was successfully applied to recent drug consumption assessment.

Abstract

A procedure based on ultra-high-pressure liquid chromatography tandem mass spectrometry has been developed for the determination of twenty three psychoactive drugs and metabolites in whole blood using dried blood spot (DBS). Chromatographic separation was achieved at ambient temperature using a reverse-phase column and a linear gradient elution with two solvents: 0.1% formic acid in acetonitrile and 5 mM ammonium formate at pH 3. The mass spectrometer was operated in positive ion mode, using multiple reaction monitoring via positive electro-spray ionization. The method was linear from the limit of quantification (5 ng/ml for all the analytes apart from 15 ng/ml for Δ -9-tetrahydrocannabinol and metabolites) to 500 ng/ml, and showed good correlation coefficients ($r^2 = 0.990$) for all substances. Analytical recovery of analytes under

investigation was always higher than 75% and intra-assay and inter-assay precision and accuracy always better than 15%.

Using the validated method, ten DBS samples, collected at the hospital emergency department in cases of acute drug intoxication, were found positive to one or more psychoactive drugs. Our data support the potential of DBS sampling for non invasive monitoring of exposure/intoxication to psychoactive drugs.

Keywords: Ultra-high-pressure liquid chromatography tandem mass spectrometry, dried blood spots, DBS, psychoactive drugs.

Introduction

Psychoactive drugs are substances with the ability to act on normal brain mechanisms and change an individual's consciousness, mood or thinking processes. Many psychoactive drugs are legal and used as medications (i.e. benzodiazepines, antidepressant and sedatives), others, illegal, for recreational purpose (i.e. opiates, cannabis, hallucinogens, cocaine) [1]. The detection of psychoactive drugs in biological conventional (urine and blood) and non conventional (e.g. oral fluid, hair) matrices is of great importance in clinical and forensic toxicology such as workplace drug testing, roadside testing, therapeutic drug monitoring, rehabilitation programs and post-mortem cases. [2].

Blood is the specimen of choice to detect and quantify a drug if there is suspicion of current drug-related impairment or intoxication. However, its collection requires skilled professional figures, patients can undergo discomfort during collection, which can be not risk-free. The dried blood spot (DBS) use allows the collection of blood samples from a small finger prick with an automatic lancet and offers many benefits in comparison with conventional venipuncture: simple and non-invasive collection which can be executed by non skilled personnel; no need of anticoagulant or plasma separation, easy sample shipment and storage [3]. Most of the pathogenic agents, which can be present in blood, are deactivated on the filter paper [3].

Dried blood spots (DBS) technique was firstly developed in 1963 to be used in neonatal screening for phenylketonuria and for both qualitative and quantitative screening of metabolic disorders [4], but in the more recent years DBS has gained interest as an alternative haematic matrix [3,5,6] for therapeutic drug monitoring [7], pharmaco-toxicokinetic studies [8] and for drug testing in sports [9].

Several publications suggest also the potential of DBS for detecting exposure to psychoactive drugs. Analytes measured include both legal drugs (scheduled drugs available on prescription) and

psychoactive illicit drugs including benzodiazepines, amphetamines type substances, cocaine, cannabinoids, opiates, gamma-hydroxybutyric acid (GHB) and ethylglucuronide (EtG) [10-23].

A substantial number of these reports describe DBS application for only one class of drugs and their respective metabolites [11-13, 15-17, 19, 20, 23] and the vast majority of available publications describe the analysis of DBS obtained by pipetting patients blood samples onto the card [11, 12, 17-19, 22].

We here present the development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous determination of a panel of psychoactive drugs in DBS. The method has been applied in real cases of individuals admitted to the hospital emergency rooms for acute intoxication related to the consumption of principal psychoactive drugs.

2. Experimental

2.1. Chemicals and reagents

Morphine, codeine, 6-monoacetylmorphine (6-MAM), methadone, EDDP, cocaine, benzoylecgonine, Δ -9-tetrahydrocannabinol (THC), 11-hydroxy- Δ -9-tetrahydrocannabinol (THC-OH), 11-nor-9-carboxy- Δ -9-tetrahydrocannabinol (THC-COOH), amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-propyl-amphetamine (MDPA) and nalorphine used as internal standards (ISs) were supplied by LGC Standards (Milan, Italy).

Alprazolam, alprazolam-d₅ used as internal standard (IS), clonazepam, diazepam, diazepam-d₅ (as IS), nor-diazepam, lorazepam, lorazepam-d₄ (as IS), lormetazepam, medazepam, oxazepam, oxazepam-d₅ (as IS) and midazolam were obtained from Cerilliant (Austin, TX, USA).

Ultrapure water and all other reagents of UHPLC-MS/MS grade were obtained from Sigma-Aldrich (Milan, Italy).

Whatman (Maidstone, UK) 903 Protein Saver cards were used for DBS collection.

2.2 DBS samples

Ten individuals admitted to the Emergency Department of Hospital del Mar, Barcelona, Spain with symptoms of psychoactive drug intoxication underwent a clinical examination and symptomatic treatment to overcome the intoxication effects (eg. unconsciousness, agitation, high pressure, sweating, slurred speech, urinary incontinence). Urine samples were collected and immediately analysed for eventual presence of drugs of abuse by immunoassay. At the same time, DBSs were collected by patients finger prick on a pre-marked card (a blood drop filling a pre-marked circle

corresponded to approximately 30 μ l blood) and stored at ambient temperature. The study was approved by the Institutional Ethical Committee (IMIM-IMAS Hospital del Mar Ethical Committee) and conducted in accordance with the Declaration of Helsinki. Signed informed consent was obtained from each individual.

2.3 Calibration standards and quality control samples

Stock standard solutions (1 mg/ml) of each analyte were prepared in methyl alcohol and stored at -20°C. From stock solutions, working solutions of 10, 1 and 0.1 μ g/ml of the analytes under investigation were made in water and used for the preparation of calibration curves and quality control (QC) samples. ISs were diluted in water to give a working solution at a concentration of 1 μ g/ml and stored at -20°C.

Calibration standards containing 5, 10, 50, 100, 200 and 500 ng of analytes under investigation per ml of whole blood were prepared by adding suitable amounts of water working solutions to 1 ml of pre-checked whole blood pool samples. In case of Δ -9-tetrahydrocannabinol and metabolites, the first point of calibration curve was set at 15 ng/ml (0.45 ng/DBS). Then, by using a glass microsyringe, 30 μ L blood was applied to the card, filling the pre-marked circle (calibration ranges: 0.15, 0.3, 1.5, 3 and 15 ng/DBS). QC samples of 6 (0.18) and 20 (0.6) in the case of Δ -9-tetrahydrocannabinol and metabolites (low control), 150 (4.5) (medium control) and 400 (12) (high control) ng analytes/ml (ng analytes/DBS) whole blood and samples at the limit of quantification (LOQ) were also prepared as above reported for the calibration standards. QC samples were included in each analytical batch to check method linearity, intra- and inter- assay inaccuracy and imprecision, matrix effects, recovery and process efficiency.

2.4 DBS preparation

Pre-marked cards spotted with 30 μ L blank blood, calibrators, QC and real samples were cut, once dried, at the edges and placed into a tube with 10 μ l ISs working solution (1 μ g/mL) and 990 μ l methyl alcohol. The tube was then sonicated for 15 min and centrifuged at 3500 $\times$ g for 5 min. The supernatant was brought to dryness under vacuum, re-dissolved with 100 μ l UHPLC mobile phase (solvents A/B, 80/20, v/v) and 10 μ l injected into the UHPLC–MS/MS system.

2.5 Ultra-high-pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS)

Analytes under investigation in DBS were detected using an ultra-high performance liquid chromatography system (Waters Acquity UPLC, Waters Corporation, Milan, Italy) coupled with a

triple quadrupole mass spectrometer (Waters Xevo TQ, Waters Corporation). Chromatography was carried out in reversed phase Acquity UPLC HSS C18 column (2.1 mm × 150 mm, 1.8 µm, Waters Acquity UPLC, Waters Corporation, Milan, Italy) using a linear gradient elution with two solvents: 0.1% formic acid in acetonitrile (solvent A) and 5 mM ammonium formate pH 3.0 (solvent B). Solvent A was maintained at 5% for the first 0.50 min. It was increased to 55% from 0.50 to 10.00 min, then increased to 90% from 10.00 to 10.75 min, held at 90% from 10.75 to 12.85 min, and then decreased back to 5% from 12.85 to 13.00 min and held at 5% from 13.00 to 16.50 min for re-equilibration. The flow rate was kept constant at 0.40 mL/min during the analysis. The separated analytes were detected with a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode via positive electrospray ionization (ESI). The applied ESI conditions were the following: capillary voltage 1.3 kV, desolvation temperature 600 ° C, source temperature 150 ° C, cone gas flow rate 20 L/h, desolvation gas flow rate 1000 L/h and collision gas flow rate 0.13 mL/min. Cone energy voltages, MRM transitions, and collision energy voltages were established for each analyte and the values are listed in Table 1.

2.6 Validation Protocol

Validation protocol applied in the present study included linearity, limits of detection (LOD) and quantification (LOQ), imprecision, inaccuracy, selectivity, carryover, matrix effect, recovery process efficiency and stability as previously reported [24,25]. Validation parameters were calculated using five different daily replicates of QC samples (low, medium, and high quality control) along five subsequent working days. Linearity was determined by least-squares regression with $1/X^2$ weighting. Acceptable linearity was achieved when the coefficient of determination was at least 0.990 and the calibrators were quantified within $\pm 20\%$ at the LOQ and $\pm 15\%$ at other concentrations. The LOD and LOQ were evaluated with decreasing analyte concentrations in DBS. LOD was defined as the lowest analyte concentration that can be detected and identified with a given degree of certainty. Standard deviation (SD) of the mean noise level over the retention time window of each analyte was used to determine LOD. A minimum requirement for signal to noise of 3 is widely accepted. LOQ was the lowest concentration that met LOD criteria and a signal-to-noise ratio of at least 10.

Imprecision and inaccuracy were determined at the three QC samples concentrations by analyzing five replicates on three different days (n=20). Imprecision and inaccuracy, expressed as the coefficient of variation (%) of the measured values and error (%) respectively, were expected to be less than 20%. Over-the-curve samples (drug free samples spiked with concentration of drugs of

abuse five or 10 times higher than the highest calibration point) were tested for calibration curve fitting, imprecision and inaccuracy once they were properly diluted.

Matrix effects, recovery and process efficiency were determined using the experimental design proposed by Matuszewski et al. [26]. Set 1 was five replicates of quality control material prepared in the mobile phase. Sets 2 and 3 were five replicates of blank DBS samples fortified with quality control material after and before extraction, respectively. Matrix effects were determined by dividing mean peak areas of set 2 by set 1 multiplied by 100. Recovery was determined by comparing the mean peak areas of analytes under investigation obtained in set 3 to those in set 2 multiplied by 100. Process efficiency expressed as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of the same analyte standards (set 1) multiplied by 100.

A mid-term stability experiment was performed using low, medium and high concentrations QC DBS samples stored at room temperature and analyzed at moment of preparation and after one, two and three month period. Six replicates were determined for each QC concentration.

3. Results and discussion

3.1. Ultra-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and validation results

Representative chromatogram obtained following the extraction of DBS from real case is shown in Figure 1. Linear calibration curves were obtained for the compounds of interest with correlation coefficients (r^2) of at least 0.990 in all cases and LODs and LOQs values resulted adequate for the purpose of the present study (Table 2). The intra and inter-assay imprecision (measured as coefficient of variation, CV) and inaccuracy (measured as % error) values were always lower than 15% (Table 3). Over-curve samples, tested for imprecision and accuracy after diluting 10 times, gave values always lower than 15% relative standard deviation (RSD) and 15% error. No additional peaks due to endogenous substances that could have interfered with the detection of the compounds of interest were observed in drug-free samples (figure 2). No drugs interfered with the assay and with the accurate quantification of the low QC samples. Blank samples injected after the highest point of the calibration curve did not present any traces of carryover. The mean absolute matrix effect ranged from 84.9% to 104.2%, recovery from 78.3% to 97.1%, process efficiency from 77.5% to 95.7 % (Table 4).). Over-curve samples, tested for imprecision and accuracy after diluting 10 times, gave values always lower than 20% relative standard deviation (RSD) and % error.

No relevant instability was observed after maintaining the DBS (spotted with whole blood fortified at the concentration of 50 ng/ml drugs of abuse) at room temperature for two weeks, with

differences in the initial concentration less than 10% for all the compounds under investigation. In reference to mid-term stability, no relevant degradation was observed for any of the analytes under investigation with differences to the initial concentration always lower than 10%.

3.2. Analysis of DBS samples

Drugs detected and quantified in DBS of individuals attending Emergency Department of Hospital del Mar, Barcelona, Spain for acute intoxication are reported in Table 5, together with individuals' self report of drug consumption and results obtained by urine immunoassay.

When analytes concentrations resulted higher than those in the calibration curve range, samples were re-injected opportunely diluted.

Most of our findings (i.e. case 1, 4, 5, 7, 8, 9 and 10) are in accordance with both the self-reported drugs and the presumptive results obtained from the urine immunoassay.

In samples 2 and 4 neither THC nor its metabolites could be quantified since only traces under the LOQ and above the LOD could be observed in the chromatograms. However, urine immunoassay revealed presumptive positive results for THC. It should be taken into account that the detection of cannabinoids in urine merely indicates that the individual had used or has been exposed to cannabis at some point prior to the collection [27]. Furthermore, cannabinoids are among the most challenging analytes in DBS, since once consumed, they disappear rapidly from blood. Indeed, THC was detectable in plasma at a limit of detection: 0.8 ng/mL (or 24 pg/DBS) for 3.5–5.5 hours after smoking two cannabis cigarettes containing a total of ca. 10 mg of THC [28], and with a limit of detection: 0.02 ng/mL (or 0.6 pg/DBS) up to 13 days in the blood of chronic cannabis users, who smoked four cigarettes containing deuterium-labelled THC [29]. In case 10, although THC was under LOQ, both THC-OH and THC-COOH were quantified at the concentration of 15.0 (0.45) and 23.8 (0.69) ng/mL (ng/DBS), respectively. The detection of all three cannabinoids, and in particular of THC and THC-OH, indicates recent intake (within about 2 h) before DBS sampling [20].

Regarding case 3, the consumer correctly declared the use of MDMA, which was quantified in DBS at the concentration of 354.9 ng/mL (10.65 ng/DBS) together with its metabolite, MDA, at the concentration of 48.4 ng/mL (1.45 ng/DBS). However, in the same case, methamphetamine was determined in DBS instead of amphetamine that was self-reported. In case 6, methadone and its metabolite EDDP were detected and quantified, although not reported during the interview.

Gabapentin, olanzapine and mirtazapine have been also identified in sample 9 by specific fragmentation patterns (MRM transitions m/z 173.2 > 137.4; 173.2 > 154.0 for gabapentin, m/z 266.2 > 72.2; 266.2 > 199.1 for mirtazapine and MRM transitions m/z 312.2 > 197.8; 312.2 > 255.8 for olanzapine) in agreement with the spectrum spectra library available in our instrument. Anyway,

the quantification was not possible because at the time of analysis, the pure standards of the above reported substances were not available.

A limitation of the current study was the low sensitivity (relatively high LOQ (15 ng/mL or 0.45 ng/DBS) for cannabinoids. Therefore, the presented method is able to determine cannabinoids in DBS only if the collection had been performed shortly after the cannabis intake.

When a method has been solely designed for the determination of cannabinoids, as in case of Mercolini et al [20], a much greater sensitivity (LOQ values of 0.25 ng/mL (2.5 pg/DBS) for THC and 0.50 ng/mL (5 pg/DBS)), for THC-OH and THC-COOH) could be achieved, compared to methods that simultaneously determine a number of compounds, including cannabinoids, as it has been our method.

Similarly, Thomas et al. could achieve a LOQ for THC and THC-COOH 0.25 and 1 ng/ml in DBS investigated for several classes of prohibited drugs only performing a targeted higher collision dissociation (HCD) cell experiment with UHPLC coupled to benchtop quadrupole/Orbitrap mass spectrometer [30].

Furthermore, a comparison with analysis of blood samples from the same individuals providing DBS could have been useful to assess the adherence of the results obtained by means of conventional toxicological analyses to those obtained using this new approach. Unfortunately, no ethical permission was given to extract blood from patients at the emergency room, since urine samples could be collected as in the protocol for acute intoxication and dried blood spots gathering did not require supervision. Nonetheless, the results of urine immunoassay were in complete accordance with results of DBS.

Conclusion

An assay including a simple biological sample treatment and fast UHPLC-MS/MS method was developed for the determination of 23 drugs from different classes, including cocaine, opiates, benzodiazepines, amphetamines, THC and some of their metabolites in DBS. The assay was fully validated and the procedure was successfully applied to ten real cases coming from the emergency room after being admitted to due to the adverse effects of drugs of abuse.

The findings obtained from the analysis of 10 real samples with the here described method support the potential of DBS sampling for non-invasive monitoring of exposure/intoxication to psychoactive drugs. The major advantage of the here described method is the possibility to detect drugs even in 30 μ L blood, with a simple sample collection and an easy pre analytical treatment.

The current method could find application in both clinical and forensic toxicology analysis; in emergency rooms, roadside drug testing and workplace drug testing.

Conflict of interest

None

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Figure legend

Figure 1. UHPLC–MS/MS chromatogram of a DBS real sample.

Figure 2. UHPLC–MS/MS chromatogram of a DBS blank sample.

Fig. 1

Fig. 2

Table 1. UHPLC-MS/MS parameters for the multiple reaction monitoring (MRM) acquisition mode in DBS analysis for drugs of abuse.

Analyte	Retention time (min)	MRM transitions					
		Quantification			Confirmation		
		m/z	CV(V) ^a	CE(eV) ^b	m/z	CV(V) ^a	CE(eV) ^b
Amphetamine	3.66	136.1>119.1	15	8	136.1>91.1	15	23
Methamphetamine	3.96	150.2>91.1	20	12	150.2>119.1	20	10
MDMA	4.00	194.2>163.1	20	20	194.2>105.1	20	14
MDA	3.69	180.1>163.1	22	10	180.1>133.1	22	18
Morphine	2.04	286.3>152.9	46	38	286.3>181.1	46	34
Codeine	3.27	300.3>215.1	20	24	300.3>165.1	20	25
6-MAM	3.63	328.2>165.1	22	18	328.2>181.2	26	28
Methadone	8.74	310.3>265.0	30	15	310.3>105.1	30	28
EDDP	7.92	278.2>186.2	50	35	278.2>234.2	50	26
Cocaine	5.70	304.3>182.2	30	20	304.3>105.1	30	30
Benzoylcegonine	4.36	290.2>168.1	30	20	290.2>105.1	30	30
THC	12.75	315.3>193.2	30	28	315.3>135.2	30	26
THC-OH	11.79	331.2>193.2	20	34	331.2>105.0	20	44
TCH-COOH	11.80	345.3>193.1	24	40	345.3>299.2	24	26
Medazepam	7.48	271.2>207.1	30	26	271.2>91.1	30	26
Oxazepam	8.39	287.1>269.1	35	15	287.1>241.2	35	20
Lorazepam	8.58	321.0>275.1	30	20	321.0>229.1	30	20
Alprazolam	8.79	309.2>281.2	50	30	309.2>205.2	50	43

Clonazepam	8.60	316.1>270.1	55	24	316.1>214.1	55	39
Midazolam	7.31	326.1>291.1	55	26	326.1>244.2	55	26
Nor-diazepam	9.34	271.1>140.0	50	35	271.1>165.1	50	25
Lormetazepam	9.67	335.1>289.2	40	25	335.1>177.1	40	40
Diazepam	10.41	285.1>154.1	50	28	285.1>193.2	50	32
Nalorphine (IS)	3.06	312.2>152.9	42	42			
MDPA (IS)	5.09	222.2>163.1	22	14			
Oxazepam-d5 (IS)	8.30	292.1>274.1	35	15			
Lorazepam-d4 (IS)	8.54	325.2>279.1	34	24			
Alprazolam-d5 (IS)	8.72	314.2>210.1	34	42			
Diazepam-d5 (IS)	10.33	290.2>154.1	50	28			

a CV: cone voltage, b CE: collision energy

Table 2. Calibration results, limits of detection (LOD) and limits of quantification (LOQ) for UHPLC-MS/MS analysis of drugs of abuse in DBS

Analytes	Calibration curve parameters		LODs (ng/mL)	LOQs (ng/mL)
	Equation	Correlation coefficient (r ²)		
Amphetamine	$y = 0.002x - 0.005$	0.995	1.5	5
Methamphetamine	$y = 0.0004x + 0.0006$	0.994	1.5	5
MDMA	$y = 0.003x + 0.01$	0.995	1.5	5
MDA	$y = 0.001x + 0.007$	0.992	1.5	5
Morphine	$y = 0.0003x + 0.0008$	0.994	1.5	5
Codeine	$y = 0.004x - 0.012$	0.994	1.5	5
6-MAM	$y = 0.007x - 0.031$	0.994	1.5	5
Methadone	$y = 0.019x - 0.044$	0.996	1.5	5
EDDP	$y = 0.005x - 0.040$	0.993	1.5	5
Cocaine	$y = 0.084x + 0.104$	0.992	1.5	5
Benzoylcegonine	$y = 0.019x - 0.220$	0.991	1.5	5
THC	$y = 0.003x + 0.006$	0.990	4.5	15
THC-OH	$y = 0.002x + 0.003$	0.991	4.5	15
TCH-COOH	$y = 0.01x + 0.02$	0.990	4.5	15
Medazepam	$y = 0.021x + 0.071$	0.993	0.3	1
Oxazepam	$y = 0.015x + 0.164$	0.993	1.5	5
Lorazepam	$y = 0.001x + 0.042$	0.990	0.3	1
Alprazolam	$y = 0.004x + 0.246$	0.991	0.3	1
Clonazepam	$y = 0.001x - 0.015$	0.996	0.3	1
Midazolam	$y = 0.096x - 0.026$	0.994	0.3	1
Nor-diazepam	$y = 0.012x - 0.129$	0.995	0.3	1
Lormetazepam	$y = 0.101x + 0.051$	0.994	0.3	1
Diazepam	$y = 0.026x - 0.102$	0.994	0.3	1

Table 3. ntra-day (n = 5) and inter-day (n = 15) precision and accuracy for analytes under investigation in DBS

Analytes	Intra-day precision (RSD)			Intra-day accuracy (Error%)			Inter-day precision (RSD)			Inter-day accuracy (Error%)		
	Low QC ^a	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
Amphetamine	7.7	0.8	10.6	8.6	6.9	11.7	9.6	5.2	11.0	8.6	7.6	9.5
Methamphetamine	10.2	11.4	10.3	7.6	6.5	11.8	6.8	5.4	8.2	5.1	5.2	8.6
MDMA	11.9	7.3	11.7	8.2	5.1	9.8	11.9	6.3	8.7	8.4	8.2	9.3
MDA	3.1	5.7	6.9	5.2	4.0	9.5	4.3	4.6	5.7	6.7	3.9	2.1
Morphine	6.3	10.4	11.3	4.4	10.2	11.4	7.9	11.1	7.0	5.5	10.4	9.0
Codeine	8.7	10.6	8.8	8.7	10.1	6.5	7.0	8.1	7.0	6.2	9.0	9.2
6-MAM	7.3	1.3	9.5	5.0	10.7	10.0	8.0	3.0	7.1	5.7	10.4	9.3
Methadone	6.3	7.3	3.4	6.7	5.1	2.4	6.4	4.7	2.5	8.7	6.6	6.2
EDDP	1.0	4.1	10.5	2.0	10.0	7.3	1.3	4.1	7.7	1.7	10.2	6.1
Cocaine	10.7	5.6	6.3	7.7	10.9	4.4	6.9	8.5	7.4	4.9	9.3	5.1
Benzoylcegonine	10.7	1.9	2.2	8.9	2.3	8.9	10.7	6.1	5.6	8.2	4.9	9.5
THC	9.5	10.7	13.1	10.8	4.4	10.3	8.0	6.6	8.9	9.6	5.5	8.6
THC-OH	11.8	6.8	10.2	8.0	8.8	10.9	8.2	4.4	11.1	9.5	7.4	9.2
TCH-COOH	10.0	10.9	9.6	9.4	10.1	7.6	10.0	8.0	12.9	11.9	5.3	8.3
Medazepam	10.4	7.5	7.6	11.4	11.9	5.6	9-0	10.1	5.6	9.0	7.3	5.4
Oxazepam	6.4	1.1	10.9	5.6	12-0	10.4	3.4	10.0	10.4	11.1	13.0	12.6
Lorazepam	10.1	4.7	2.5	10.4	3.4	15.0	10.3	4.3	10.0	9.0	7.3	5.4
Alprazolam	12.1	3.4	12.6	11.0	10.3	4.7	10.5	11.5	4.7	13.9	3.7	10.8

Clonazepam	7.0	11.4	10.2	4.7	11.5	5.5	8.5	6.8	7.4	12.4	10.1	7.0
Midazolam	10.4	7.5	7.6	11.4	11.9	4.7	4.4	6.0	9.2	5.0	2.9	6.3
Nor-diazepam	6.4	1.1	10.9	5.6	12.0	4.5	10.6	10.3	8.9	4.5	12.6	12.3
Lormetazepam	8.4	6.8	10.7	6.3	4.8	8.0	10.4	2.8	9.6	2.9	8.9	7.7
Diazepam	8.1	6.3	8.5	4.8	2.0	5.3	10.0	9.3	6.5	7.3	3.9	8.0

^aQC: quality control samples.

Table 4. Mean absolute matrix effect, recovery and process efficiency for analytes under investigation in DBS.

Analytes	Matrix effect (%)	Recovery (%)	Process efficiency (%)
Amphetamine	93.6	97.1	84.2
Methamphetamine	93.7	89.5	77.5
MDMA	95.3	99.8	80.2
MDA	87.9	95.5	82.5
Morphine	90.2	83.6	77.9
Codeine	103.2	88.0	85.1
6-MAM	90.5	89.2	86.1
Methadone	89.3	81.7	80.7
EDDP	101.1	79.5	79.9
Cocaine	93.8	90.8	81.6
Benzoylcegonine	91.0	89.9	81.4

Δ -9-THC	90.3	81.1	85.1
THC-OH	104.2	79.0	81.5
THC-COOH	93.0	78.3	81.1
Medazepam	101.8	84.8	95.7
Oxazepam	87.9	81.5	88.6
Lorazepam	98.6	88.0	79.2
Alprazolam	84.9	89.6	85.3
Clonazepam	85.7	83.7	79.1
Midazolam	87.4	81.2	85.7
Nor-diazepam	89.9	88.4	86.6
Lormetazepam	101.7	88.6	78.7
Diazepam	87.9	89.3	77.7

Table 5. Intoxicated individuals' interview, urine analysis and psychoactive drugs in DBS sample from patients

Sample ID	Intoxicated individuals' Interview	Urine immunoassay determination	DBS drugs concentration (ng/ml)	DBS drugs concentration (pg/DBS)
1	Benzodiazepines	Benzodiazepines	Lorazepam: 623.8	Lorazepam: 18.7
2	Methamphetamine, cannabis, MDMA Midazolam (administered at emergency department admission)	Cocaine, THC, amphetamine, methamphetamine, MDMA	Amphetamine: 116.7 Metamphetamine: 30.2 MDA: 26.9 MDMA: 240.3 Cocaine: 55.5 Benzoyllecgonine: 220.8 Midazolam: 280.7 THC: traces* THC-OH: traces* THC-COOH: traces*	Amphetamine: 3.5 Metamphetamine: 0.9 MDA: 0.8 MDMA: 7.2 Cocaine: 1.7 Benzoyllecgonine: 6.6 Midazolam: 8.4 THC: traces* THC-OH: traces* THC-COOH: traces*
3	MDMA and amphetamine	MDMA and amphetamine	Methamphetamine: 73.1 MDA: 48.4 MDMA: 354.9	Methamphetamine: 2.2 MDA: 1.5 MDMA: 10.6
4	Cannabis	THC	THC: traces* THC-OH: traces* THC-COOH: traces*	THC: traces* THC-OH: traces* THC-COOH: traces*
5	Cocaine	Cocaine	Cocaine: 6003.9 Benzoyllecgonine: 748.8	Cocaine: 180.1 Benzoyllecgonine: 22.5
6	Cocaine Diazepam (administered at emergency department admission)	No sample	Cocaine: 686.0 Benzoyllecgonine: 617.5 Methadone: 1494.5 EDDP: 25.1 Diazepam: 16.9 Nor-diazepam: 27.6	Cocaine: 20.6 Benzoyllecgonine: 18.5 Methadone: 44.8 EDDP: 0.8 Diazepam: 0.5 Nor-diazepam: 0.8
7	Benzodiazepines	Benzodiazepines	Diazepam: 106.2 Nordazepam: 95.0	Diazepam: 3.2 Nordazepam: 2.9
8	Methamphetamine, cocaine and diazepam to control agitation	Amphetamine, methamphetamine, cocaine and benzodiazepines	Amphetamine: 30.9 Methamphetamine: 73.6 Cocaine: 53.0 Benzoyllecgonine: 79.0 Diazepam: 31.0	Amphetamine: 0.9 Methamphetamine: 2.2 Cocaine: 1.6 Benzoyllecgonine: 2.4 Diazepam: 0.9
9	Clonazepam, Olanzapine, gabapentin and mirtazapine	Benzodiazepines and cocaine	Cocaine: 32.1 Benzoyllecgonine: 44.9 Clonazepam: 176.7	Cocaine: 1.0 Benzoyllecgonine: 1.3 Clonazepam: 5.3
10	Cannabis	THC	THC: traces* THC-OH: 15.0 THC-COOH: 23.8	THC: traces* THC-OH: 0.5 THC-COOH: 1.2

*<LOQ and >LOD