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Simultaneous determination of cocaine and opiates in dried blood spots by electrospray ionization tandem mass spectrometry

Ángel Antelo-Domínguez [a], José Ángel Cocho [b], María Jesús Tabernero [c], Ana María Bermejo [c], Pilar Bermejo-Barrera [a], Antonio Moreda-Piñeiro [a,*]

[a] Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Chemistry, University of Santiago de Compostela, Avenida das Ciencias, s/n, 15782 Santiago de Compostela, Spain
[b] Laboratory of Metabolic and Nutritional Disorders, University Clinical Hospital, 15782 Santiago de Compostela, Spain
[c] Department of Pathologic Anatomy and Forensic Sciences, Faculty of Medicine, University of Santiago de Compostela, Rúa de San Francisco, s/n, 15782 Santiago de Compostela, Spain

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ABSTRACT

A sample pre-treatment method based on blood spot collection filter cards was optimized as a means of using small volume samples for the screening and confirmation of cocaine and opiates abuse. Dried blood spots (DBSs) were prepared by dispersing 20 μL of whole blood specimens previously mixed with the internal standards (deuterated analogs of each target), and subjecting the whole DBS to extraction with 5 mL of methanol under orbital-horizontal shaking (180 rpm) for 10 min. Determinations were based on direct electrospray ionization tandem mass spectrometry (ESI-MS/MS) by injecting the re-dissolved methanol extract with the delivery solution (acetonitrile–water–formic acid, 80:19.875:0.125) at a flow rate of 60 μL min^{-1}, and using multiple reaction monitoring (MRM) mode with the *m/z* (precursor ion)→*m/z* (product ion) transitions for acquisition. Matrix effect has been found to be statistically significant (Multiple Range Test) when assessing cocaine, BZE, codeine and morphine, and the use of the standard addition method (dispersion of whole blood previously mixed with standards onto the filter papers) was needed for accurate determinations. The developed DBS-ESI-MS/MS procedure offered good intra-day and inter-day precisions (lower than 10% and 12%, respectively), as well as good intra-day and inter-day accuracies (inter-day absolute recoveries, expressed as the mean analytical recovery over three target concentration levels, of 103%, 100%, 101%, 98% and 100% for cocaine, BZE, codeine, morphine and 6-MAM, respectively). The high sensitivity inherent to MS/MS determinations combined with the minimal dilution of sample allowed low limits of quantification for all targets, and the developed method results therefore adequate for cocaine and opiates screening and confirmation purposes. The procedure was finally applied to DBSs prepared from whole blood from polydrug abusers, and results were compared with those obtained after a conventional sample pretreatment method based on solid phase extraction for plasma specimens and gas chromatography–mass spectrometry.

1. Introduction

Since the first report by Guthrie and Susi in 1963 describing the collection of blood on absorbent papers for determining phenylalanine in the detection of phenylketonuria in newborns [1], the dried blood spot (DBS) technique has become the method of choice in clinical laboratories worldwide for the screening of several metabolic disorders in newborns [2]. DBS consists of blood diffusion onto standardized filter cards followed by an air-drying stage, and target extraction using a 3.2 mm diameter punch from each DBS. The main advantage of this simple method is the retention of the major blood components on the filter card, whereas targets can be extracted to a solution for a further analysis. The method is therefore simple, and clean extracts are commonly obtained. DBS in combination with tandem mass spectrometry is today the basis of the well established Expanded Newborn Screening (ENBS) methodologies for the simultaneous assay of various newborn diseases [3].

The small sample volume required (typically 20 μL) as well as the ease of shipping/storage, and analyte stability [4], are some of the advantages of the DBS based methods. As reviewed by Tanna and Lawson [5], other advantages of the DBS extraction process are the possibility of automation, and also the potential of this technique for direct analysis of the sample without prior extraction. Although

* Corresponding author. Tel.: +34 881 814375; fax: +34 981 547141.
E-mail address: antonio.moreda@usc.es (A. Moreda-Piñeiro).

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plasma and serum can be loaded onto the filter cards, DBS is usually performed by dispersing whole blood. Methods based on whole blood are preferable in forensic-toxicological studies due to the difficulty of obtaining serum/plasma from whole blood in cases of death.

In addition to the wide application of DBS techniques in newborn screening [2], some developments describing the use of DBS for assessing therapeutic drugs have been reported [4,6]. These applications have focused on determining antibiotics [7,8], non-steroidal anti-inflammatory drugs [9], antidepressants [10], and various other pharmaceuticals compounds in blood [11–18]. The technique has also been applied for assessing benzoylecgonine (BZE), cocaine's main metabolite, in newborns and childbearing women [19–21]; and more recently, to assess morphine and 6-monoacetylmorphine (6-MAM) [22], and morphine and its active metabolites morphine 3β-glucuronide (M3G) and morphine 6β-glucuronide (M6G) [23]. In these developments, radioimmunoassay (RIA) has been used for screening purposes [19,20]; whereas, methods based on high performance liquid chromatography–mass spectrometry (HPLC–MS) [20] and high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) [21–23] have been used for confirmatory studies.

Reported DBS treatments consist of BZE extraction from a 1/4-inch punch with 200 μL of buffer containing phosphate-buffered saline (PBS) under continuous stirring for 18 h [19,20]. On other occasions, the 1/4-inch punches were pre-treated with 200 μL of 2 mM aqueous ammonium acetate overnight, followed by vortex mixing with 1 mL of methanol and centrifugation for removing the precipitated protein [21]. Similarly, morphine and metabolites are extracted from DBSs with 100 μL of HPLC grade water, followed by protein precipitation with 600 μL of methanol/0.2 M $ZnSO_4$ (7:3, v/v) [23].

The objective of the current work has been the development of a DBS methodology as a simple and low cost sample pre-treatment method for the simultaneous isolation of cocaine, BZE, morphine, codeine and 6-MAM from whole blood. Efforts have been made for performing fast and efficient target isolation from DBSs. Different organic solvents (methanol, acetonitrile and methyl tert-butyl ether) were directly tested as extractants for achieving selective target extraction and avoiding protein solubilization from the DBSs. Direct electrospray ionization (ESI) tandem mass spectrometry (MS/MS) has been proposed in the current application for both screening and confirmatory studies. As previously mentioned, direct tandem MS/MS determinations are commonly performed in newborn screening trials after DBS [2,3]. Some direct MS/MS developments have also been reported when assessing therapeutic drugs in serum [24,25], amphetamines, opiates, cannabinoids and benzodiazepines in urine [26], and cocaine, opiates and metabolites in hair [27] after efficient target isolation procedures. The short acquisition time when performing direct MS/MS measurements (approximately 90 s for each run) leads to fast determinations, which allows for increasing the number of samples to be screened/confirmed per time unit, and reduces hence the time needed by the toxicological-forensic laboratory.

2. Experimental section

2.1. Apparatus

An API 2000 LC/MS/MS system equipped with a Perkin Elmer Series 200 chromatographic pump and a Perkin Elmer Series 200 autosampler (PE Sciex, Concord, Canada) was used for direct ESI-MS/MS determinations. A Hewlett Packard Model 6890 gas chromatograph (Hewlett-Packard, Avondale, PA, USA), equipped with a HP-5 capillary column (30 m, 0.22 mm I.D., 0.33 μm film thickness of cross-linked 5% phenyl methyl silicone) and a HP 5973 mass spectrometer was used for GC–MS analysis. DBSs were prepared and allowed to air-dry in a Class-100 clean fume hood (Telstar S.A., Terrassa, Spain). A Boxcult incubator situated on a Rotabit orbital-rocking platform shaker from J.P. Selecta (Barcelona, Spain) was used for performing targets extraction from DBSs. Other laboratory devices were: a Visiprep TM DL vacuum manifold from Supelco (Bellefonte, USA); Univeba and Digiterm 3000542 thermostatic baths (Selecta, Barcelona, Spain); an Orion 720A plus pH-meter with a glass–calomel electrode (Orion, Cambridge, UK); a Reax 2000 mechanical stirrer (Heidolph, Kelheim, Germany); and a VLM EC1 metal block thermostat and N_2 sample concentrator from VLM (Leopoldshöhe-Greste, Germany).

2.2. Reagents and materials

Ultrapure water (resistivity, 18 MΩ cm) was obtained from a Milli-Q purification device (Millipore Co., Bedford, MA, USA). Drug stock standard solutions were prepared from cocaine (1000 mg L^{-1} dissolved in acetonitrile), BZE (1000 mg L^{-1} dissolved in methanol), codeine (1000 mg L^{-1} dissolved in methanol), morphine (1000 mg L^{-1} dissolved in methanol) and 6-MAM (1000 mg L^{-1} dissolved in acetonitrile) from Lipomed (Arlesheim, Switzerland). Deuterated drug stock standard solutions were prepared from cocaine-d_3 in acetonitrile (100 mg L^{-1}), BZE-d_3 in methanol (100 mg L^{-1}), codeine-d_3 in methanol (100 mg L^{-1}), morphine-d_3 in methanol (100 mg L^{-1}), and 6-MAM-d_3 in acetonitrile (100 mg L^{-1}) from Cerillant (Round Rock, TX, USA). The filter cards used for DBSs preparation were Whatman 2012-10 from Whatman (Dassel, Germany). Bond Elut Certify cartridges (130 mg sorbent weight, 3 mL volume) used for performing SPE were obtained from Varian (Lake Forest, CA, USA). Acetonitrile (supragradient HPLC grade) was from Scharlau (Barcelona, Spain). Methanol (HPLC grade) and formic acid (98–100%) were from Merck (Poole, U.K.). Other reagents were: methyl tert-butyl ether, hydrochloric acid 37% (m/m) and phosphoric acid 85% (m/m) from Panreac (Barcelona, Spain); ammonium hydroxide, chlorotrimethylsilane (TMCS) and N-methyl-tert-butylsilyltrifluoroacetamide (BSTFA), potassium dihydrogen phosphate, potassium dihydrogen phosphate, and disodium hydrogen phosphate from Merck.

Contamination was minimized after subjecting glassware and plastic ware to a washing procedure with a diluted soap solution, thoroughly rinsed with tap water, then three times with ultra-pure water, and finally kept in a bleach solution for 24 h. After this treatment, the ware was rinsed several times with ultra-pure water before use.

2.3. Whole blood samples.

Whole blood and plasma samples used in the current study were from polydrug abusers under control in an addiction research center in Santiago de Compostela. Drug-free whole blood samples used for method validation were obtained from the General Laboratory (Complexo Hospitalario Universitary de Santiago, CHUS) in Santiago de Compostela. For all cases, whole blood samples were kept at 4 °C, if necessary.

2.4. Dried blood spot sample preparation

1.5 cm × 1.5 cm pieces were previously cut from Whatman 2012-10 filter cards, and 20 μL of samples/solutions were dispersed into the center of the 2.25 cm^2 square pieces. Spotted samples (20 μL) consisted of whole blood (140 μL) mixed with 10 μL of a solution containing the internal standards (cocaine-d_3 and BZE-d_3 at 5 μg mL^{-1}, and morphine-d_3, codeine-d_3 and 6-MAM-d_3 at 25 μg mL^{-1}) and 100 μL of ultrapure water (final volume of 250 μL). When performing experiments with spiked whole blood samples (method validation), different volumes (0, 5, 25, 50 and 100 μL) of a mixture of standards (1 μg mL^{-1} of cocaine

and BZE, and 5 μg mL^{-1} of codeine, morphine and 6-MAM) were added to 140 μL of whole blood and 10 μL of an internal standards solution, and the volume was finally made up to 250 μL by adding ultrapure water (100, 95, 75, 50 and 0 μL, respectively). This offers spiked concentrations of 0, 20, 100, 200, 400 μg L^{-1} for cocaine and BZE, and 0, 100, 500, 1000, 2000 μg L^{-1} for codeine, morphine and 6-MAM.

For all cases, generated DBSs were allowed to air dry inside a clean fume hood for 4 h. After drying, the 2.25 cm^2 square pieces containing the whole spot were transferred to 25 mL Erlenmeyer flasks, and 5 mL of methanol was added. Targets extraction from the dried blood spots was assisted by orbital-horizontal shaking (150 u min^{-1}) for 10 min. The methanol extract was further evaporated to dryness (stream of N_2, 40 °C), and then re-dissolved with 40 μL of the delivery solution for ESI-MS/MS analysis (acetonitrile–water–formic acid, 80:19.875:0.125). The two-fold dilution implies spiked concentrations when performing the standard addition curve of 0, 10, 50, 100, 200 μg L^{-1} for cocaine and BZE, and 0, 50, 250, 500, 1000 μg L^{-1} for codeine, morphine and 6-MAM.

2.5. Conventional solid phase extraction procedure

An SPE procedure described by Fernández et al. for isolating opioids, cocaine and metabolites from human plasma [28] and blood [29] was used. Blood specimens (1 mL) previously mixed with 20 μL of a solution containing the internal standards (cocaine-d_3, BZE-d_3, morphine-d_3, codeine-d_3 and 6-MAM-d_3) at 1.0 μg mL^{-1}, were loaded through Bond Elut Certify cartridges, after solid support conditioning by passing 2 mL of methanol and 2 mL of phosphate buffer (pH 6). Cartridge washing was then performed by passing 3 mL of ultrapure water, 3 mL of 0.1 N HCl, 9 mL of methanol, and 3 mL of 0.3 M ammonia. After cartridge vacuum-drying (5 min), retained targets were eluted with 3 mL of 4:1(v/v) chloroform–isopropanol. The extract (approximately 2.5 mL) was evaporated to dryness in a thermostatic bath at 65 °C under a nitrogen stream, and the dried extract was re-dissolved with 40 μL of BSTFA-TMCS (99:1) and subjected to derivatization at 100 °C for 20 min before GC–MS analysis [29,30].

2.6. Electrospray ionization-tandem mass spectrometry measurement

ESI-MS/MS was operated in positive ionization mode, and the optimum operating conditions (Table 1) were established elsewhere [27]: ion spray voltage at 5000 kV, ion source temperature at 400 °C, nitrogen as the nebulizer gas at 20 psi, as the auxiliary gas at 60 psi, and as curtain gas at 40 psi. Samples aliquots of 20 μL were loaded via the autosampler, and were pumped to the electrospray source by the chromatographic pump using acetonitrile–water–formic acid (80:19.875:0.125) as a solvent (flow rate of 60 μL min^{-1}). Multiple reaction monitoring (MRM) with the *m/z* (precursor ion)→*m/z* (product ion) transitions (Table 1) was used for performing the determinations.

Deuterated cocaine and deuterated BZE at 100 μg L^{-1}, and deuterated codeine, deuterated morphine and deuterated 6-MAM at 500 μg L^{-}, concentrations referred to the extract (40 μL) after the DBS procedure (Section 2.4), were used as internal standards. As shown above (Section 2.4), the same sample volume (140 μL) and the same volume of the internal standards solution (10 μL) were used when assessing analytical performances (standard addition curves, intraday precision, inter-day precision and analytical recovery). In this cases the volume of ultrapure water was variable (from 100 to 0 μL) in accordance with the volume of standards solution added. After extraction and re-dissolution in 40 μL, target concentrations in the extracts were up to 200 μg L^{-} for cocaine and BZE, and up to 1000 μg L^{-1} for morphine, codeine and 6-MAM.

Table 1
Selected precursor (Q1 mass) and product (Q3 mass) ions and MS/MS parameters for analytes and internal standards.

Compound	Precursor ion, *m/z*	Product ion, *m/z*	Dwell time (ms)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
Cocaine	304.2	180.0	100	35.42	376	7.81	25.0	6.17
Cocaine-d_3	307.1	185.2	100	26.00	360	8.00	25.0	6.00
BZE	290.2	168.1	100	29.66	400	9.46	23.8	5.74
BZE-d_3	293.0	171.1	100	26.00	360	11.0	27.0	6.00
Codeine	300.2	165.2	100	52.06	368	10.5	52.0	3.98
Codeine-d_3	303.0	165.3	100	31.00	370	11.5	51.0	4.00
Morphine	286.0	165.2	100	52.44	371	9.17	55.0	4.75
Morphine-d_3	289.0	165.1	100	31.00	370	12.0	49.0	4.00
6-MAM	328.2	211.0	100	59.67	367	10.0	35.9	6.13
6-MAM-d_3	331.0	165.1	100	31.00	360	11.0	49.0	6.00

DP=declustering potential; FP=focusing potential; EP=entrance potential; CE=collision energy; and CXP=collision cell exit potential.

2.7. Gas chromatography–mass spectrometry measurement

GC–MS operating conditions are described elsewhere [29,30]. Measurements were performed by injecting 2 μL aliquots in the pre-heated injection port at 240 °C (splitless injection mode for 2 min). The GC program (helium, flow rate of 1 ml min^{-1}) consists of a first stage at 90 °C (1 min), a second step (temperature rate of 30 °C min^{-1} to reach 190 °C, plus 1 min at this temperature), a third step (temperature rate of 8 °C min^{-1} until 260 °C, plus 5 min at this temperature), and a final cleaning stage (290 °C, 5 min). The mass spectrometer operates with electron impact ionization at 70 eV and the ion source set at 300 °C. Targets were identified by using the retention time and the relative abundance of three confirming ions [30]. The selected ion monitoring (SIM) mode for each compound and the use of the internal standard method with deuterated analogs was performed for determinations.

Determinations involved the use of 1 mL aliquots of plasma samples previously mixed with 20 μL of a solution containing each deuterated analog (1.0 μg mL^{-1}) before the conventional solid phase extraction (Section 2.5), which provide after re-dissolution of the dried extract with 40 μL of BSTFA-TMCS (99:1) a concentration of 500 ng mL^{-1} for each deuterated analog. Calibrations were performed by loading 1 mL of drug-free plasma samples also mixed with 20 μL of an internal standard solution (1.0 μg mL^{-1} of each deuterated analog) and with 20 μL of different solutions containing 0.1, 0.2, 0.5, 1.0, 1.5, and 2 μg mL^{-1} of each target. The LODs of the method were 10 ng mL^{-1} for cocaine, BZE, codeine, and 6-MAM; whereas, 40 ng mL^{-1} was obtained for morphine (LOQs of 30 ng mL^{-1} for cocaine, BZE, codeine, and 6-MAM; and 130 ng mL^{-1} for morphine).

3. Results and discussion

3.1. Preliminary studies.

Preliminary experiments consisted of dispersing directly 20 μL of a positive whole blood onto 1.5 cm × 1.5 cm pieces cut from Whatman 2012-10 filter cards. Good repeatability when measuring the weight of the dispersed sample was obtained (19.4 ± 0.503 mg, RSD of 3% for a series of five different spots). Experiments involving spiked whole blood samples and whole blood previously mixed with the deuterated internal standards offered bad repeatability due to the partial protein precipitation attributed to the

presence of methanol as a solvent in most of the target standards and deuterated analog solutions. Therefore, deuterated analogs and target solutions were prepared (diluted) in ultrapure water when adding the internal standards to samples, and also when performing spiking experiments. In these cases, good weight repeatability (mean mass of 19.7 ± 0.823 mg, RSD of 4%, for a series of five different spots) was observed. The DBS preparation procedure is therefore as shown in Section 2.4.

3.2. Selection of the extractant.

A comparative study of the use of different solvents for extracting cocaine and opiates from DBSs was performed. Hydrophilic organic solvents such as methanol and acetonitrile were tested. In addition, methyl tert-butyl ether, used by Suyagh et al. when extracting canrenone [15] was also evaluated. Experiments when testing each solvent were performed in duplicate with 140 μL of a drug-positive whole blood mixed with 10 μL of a solution containing the internal standards (25 μg mL^{-1} each one) plus 100 μL of a solution containing 0.5 μg mL^{-1} of each target (spiked experiments). Extraction was performed with 2 mL of solvent (methanol, acetonitrile and methyl tert-butyl ether) under orbital-horizontal shaking at 150 rpm for 15 min. The different extracts were evaporated to dryness, re-dissolved with 40 μL of acetonitrile–water–formic acid, 80/19.875/0.125 (delivery solution), and analyzed against external calibrations. Results plotted in Fig. 1A show that opiates (codeine, morphine and 6-MAM) are properly extracted when using the three solvents. However, higher cocaine concentrations are recovered when using methanol and acetonitrile (Fig. 1B); whereas, the highest BZE concentration is observed when using methanol (713 ± 10 μg L^{-1}), a value quite higher than those obtained when using methanol (386 ± 9 μg L^{-1}) and methyl tert-butyl ether (397 ± 7 μg L^{-1}). Therefore, methanol was chosen as a solvent to guarantee a proper extraction of all targets.

3.3. Multivariate optimization of DBS methanol extraction

An orthogonal 2^3+star central composite design (CCD) with 6 error degree of freedom, 2 centers, 2 replicates and 16 runs was used for the multivariate optimization of variables affecting the methanol extraction of cocaine and opiates from DBSs. The three factors under study were the methanol volume, *V* (2 and 5 mL as low and high cube values); the speed of the orbital-horizontal shaking, *S* (100 and 200 rpm as low and high cube values); and the extraction time, *T* (10 and 20 min as low and high cube values). The set of conditions derived from the CCD are shown in Table S1 (supplementary information). The different DBSs generated to perform the experiments listed in Table S1 were prepared from a positive whole blood sample for cocaine and opiates (volume of 560 μL) mixed with 40 μL of the internal standard solution (cocaine-d_3 and BZE-d_3 at 5 μg mL^{-1}; and morphine-d_3, codeine-d_3 and 6-MAM-d_3 at 25 μg mL^{-1}) plus 400 mL of ultrapure water (total volume of 1.0 mL). Each DBS was prepared by dispersing 20 μL of this mixture, and after subjecting the DBSs to each set of conditions, analyte concentrations (also listed in Table S1, supplementary section) were obtained using external calibrations.

Statistical evaluation at a confidence interval of 95% showed some quadratic terms as statistically significant for opiates, mainly the two-order interaction between extracting time and methanol volume when extracting codeine; two-order interaction between methanol volume and shaking speed when extracting morphine; and two-order interaction between extracting time and shaking speed when extracting 6-MAM. This means that the three variables are dependent factors for extracting these compounds. However, no quadratic terms after statistical evaluation were statistically significant when extracting cocaine and BZE, which implies that the three variables under study are independent factors. When performing the statistical analysis by using the sum of all target concentrations as a response variable, the methanol volume is statistically significant (with a positive effect), as well as the two-order interaction methanol volume and shaking speed (Pareto chart given in Fig. 2).

The study of response surfaces when assuming the sum of target concentrations as a response variable (Fig. 3) allows the selection of compromise conditions for the simultaneous extraction of all targets. The chosen values were finally set at 5 mL of methanol, an orbital-horizontal shaking speed of 180 rpm, and an extraction time of 10 min. The chosen methanol volume is higher than those reported in other DBS applications, typically methanol volumes between 0.5 and 1 mL [19–21,23]. However, it must be said that in those applications only a small punch of the dried blood spot was treated.

3.4. Analytical performances

3.4.1. Study of matrix interferences

A comparison between external calibrations and standard addition calibrations (standard mixed with blood onto filter paper cards) was performed. External calibrations (EC) consisted of standard solutions containing the targets (10, 50, 100 and 200 μg L^{-1} for cocaine and BZE; and 50, 250, 500 and 1000 μg L^{-1} for codeine, morphine and 6-MAM) and the internal standards (100 μg L^{-1} for

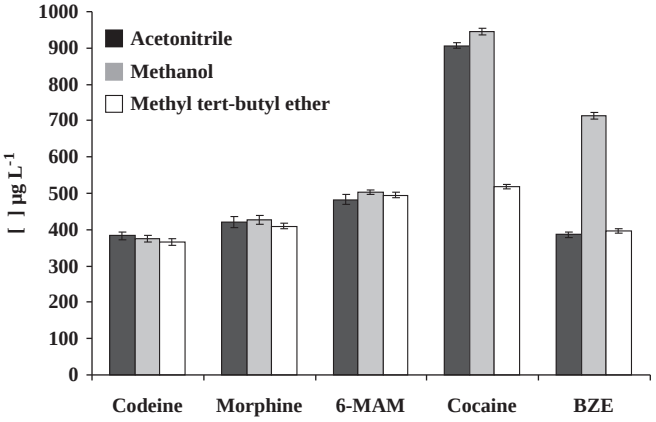

Fig. 1. Target concentrations ($n=3$) after DBSs extraction with acetonitrile, methanol, and methyl tert-butyl ether: DBSs were prepared by spiking a poly-drug positive whole blood with 200 ng mL^{-1} of each target.

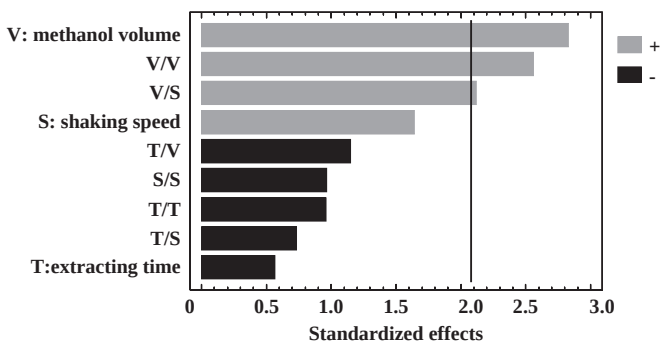

Fig. 2. Standardized main effect and two-order interactions Pareto chart after 2^3+star central composite design (sum of target concentrations as a response variable).

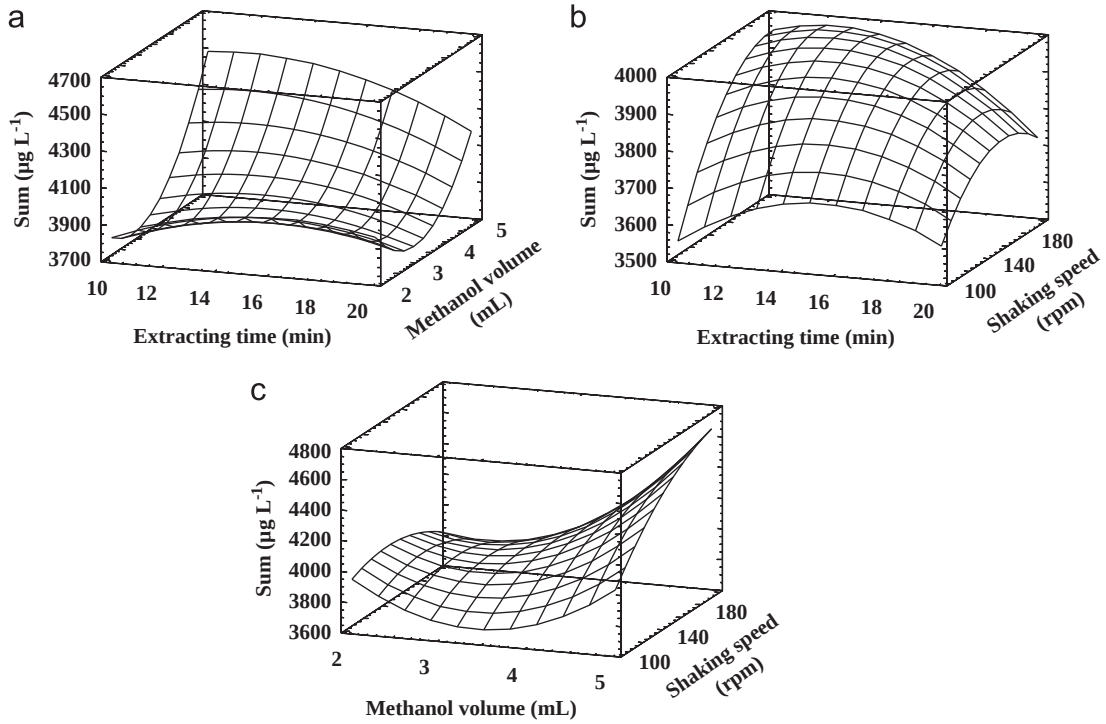

Fig. 3. Estimated response surfaces from the central composite design (of target concentrations as a response variable): Extraction time/methanol volume (A), extraction time/shaking speed (B), and methanol volume/shaking speed (C).

cocaine-d_3 and BZE-d_3, and 500 μg L^{-1} for codeine-d_3, morphine-d_3 and 6-MAM-d_3). A second calibration mode was established by dispersing whole blood mixed with standards onto the filter papers. In this case, the standard addition with whole blood (SAB) was prepared by mixing 140 μL of whole blood with 10 μL of a solution containing the internal standards (5 mg L^{-1} for cocaine-d_3 and BZE-d_3, and 25 mg L^{-1} for codeine-d_3, morphine-d_3 and 6-MAM-d_3), and with variable volumes (0, 5, 25, 50, and 100 μL) of a standard solution containing the targets (1 mg L^{-1} for cocaine and BZE, and 5 mg L^{-1} for codeine, morphine and 6-MAM). After dilution to 250 μL, internal standards were fixed at 200 μg L^{-1} for cocaine-d_3 and BZE-d_3, and 1000 μg L^{-1} for codeine-d_3, morphine-d_3 and 6-MAM-d_3; whereas, target concentrations were fixed at 20, 100, 200 and 400 μg L^{-1} for cocaine and BZE; and 100, 500, 1000 and 2000 μg L^{-1} for codeine, morphine and 6-MAM. Each spiked whole blood solution (20 μL) was dispersed onto filter papers in triplicate, and the prepared DBSs were subjected to the optimized extraction procedure described above (Section 2.7.). Standard solutions (EC) and extracts (SAB) were measured by ESI-MS/MS in three different days, and results expressed as mean ± standard deviations are listed in Table 2. A statistical comparison (95% confidence interval) of standard deviations of the slopes (Cochran'C and Barlett tests) proved that there are statistically significant differences between the standard deviations for all targets, except for 6-MAM. The application of the Multiple Range Test showed that there are statistically significant differences between the mean slopes for cocaine, BZE, codeine and morphine when performing an external calibration (EC) or when spotting the dissolved targets mixed with blood (SAB). Regarding 6-MAM, both the ANOVA test and the Multiple Range Test verify that mean slopes for the external calibration and the standard addition technique shown statistically significant similarities. Therefore, the matrix effect can be assumed as unimportant when assessing 6-MAM in BDSs; however, there are matrix effect when measuring cocaine, BZE, codeine and morphine; thus, the standard addition technique must be used.

Table 2
Mean slopes and standard deviations for external calibration (EC) and the standard addition with blood (SAB).

	External calibration ($n=4$)		Standard addition technique ($n=4$)	
	Mean (ng^{-1} mL)	Sd (ng^{-1} mL)	Mean (ng^{-1} mL)	Sd (ng^{-1} mL)
Cocaine	4.4×10^{-2}	6.3×10^{-4}	2.9×10^{-2}	4.7×10^{-3}
BZE	3.1×10^{-2}	2.3×10^{-3}	1.3×10^{-2}	1.0×10^{-3}
Codeine	3.5×10^{-3}	4.1×10^{-4}	3.7×10^{-3}	5.8×10^{-4}
Morphine	3.3×10^{-4}	4.6×10^{-5}	4.9×10^{-3}	7.2×10^{-4}
6-MAM	3.0×10^{-3}	1.2×10^{-4}	4.8×10^{-3}	1.1×10^{-3}

3.4.2. Method validation

The developed DBS-ESI-MS/MS method was validated according to FDA guidelines [31]. Linearity was tested by obtaining nine different standard addition curves (on nine different days) by spiking 140 μL aliquots of drug-free whole blood (blank) with target standard solutions at increasing concentrations (20, 100, 200 and 400 μg L^{-1} for cocaine and BZE, and at 100, 200, 1000 and 2000 μg L^{-1} for codeine, morphine and 6-MAM, according with Section 2.6.). For all cases, deuterated analogs were fixed at 100 μg L^{-1} for cocaine-d_3 and BZE-d_3, and at 1000 μg L^{-1} for codeine-d_3, morphine-d_3 and 6-MAM-d_3. Each spiked whole blood (20 μL) was spotted twice.

Least-squares linear regression was used for fixing the slope, the intercept and the regression coefficient (r^2) for each target by using analyte counts to deuterated analogs counts ratio versus analyte concentrations. Regression coefficients between 0.995 and >0.999 were obtained for all cases, and slopes of standard addition graphs, expressed as mean ± standard deviation, were $2.7 \times 10^{-2} \pm 3.1 \times 10^{-3}$, $1.2 \times 10^{-2} \pm 1.1 \times 10^{-3}$, $2.6 \times 10^{-3} \pm 8.4 \times 10^{-4}$, $5.2 \times 10^{-3} \pm 7.5 \times 10^{-4}$, and $5.3 \times 10^{-3} \pm 1.5 \times 10^{-3}$ for cocaine, BZE, codeine, morphine and 6-MAM, respectively. Small variability for the slopes is observed, and good repeatability of the

calibration curves is proved. The limits of detection (LODs) and quantification (LOQs) were based on $3\sigma/10\sigma$ criterion (σ is the standard deviation of eleven measurements of a blank) [32]. Blanks were prepared from a drug-free whole blood sample that was subjected to the DBS-ESI-MS/MS process eleven times. After obtaining the standard deviation (σ), the three times (LOD) or ten times (LOQ) of the value was divided by the mean slope of the standard addition graphs. Calculated LODs were finally referred to the whole blood sample used for preparing the DBSs, and they were 1.0, 0.68, 2.8, 3.3, and 3.4 ng mL^{-1} for cocaine, BZE, codeine, morphine and 6-MAM, respectively. Similarly, LOQs were 3.5, 2.3, 9.4, 10, and 11 ng mL^{-1} (cocaine, BZE, codeine, morphine and 6-MAM, respectively).

Intra-day precision studies were performed by preparing seven DBSs from a drug-free whole blood spiked with different target concentrations: 10 ng mL^{-1} of cocaine and BZE, and 100 ng mL^{-1} of codeine, morphine and 6-MAM (low value), 100 ng mL^{-1} of cocaine and BZE, and 500 ng mL^{-1} of codeine, morphine and 6-MAM (medium value), and with 200 ng mL^{-1} of cocaine and BZE, and 1000 ng mL^{-1} of codeine, morphine and 6-MAM (high value). Cocaine-d_3 and BZE-d_3 at 500 ng mL^{-1}, and codeine-d_3, morphine-d_3 and 6-MAM-d_3 at 100 ng mL^{-1} were used as internal standards. Table 3 lists the relative standard deviation (RSD) values obtained for experiments analyzed in the same run. It can be seen that RSDs vary from 2% to 10%. The analytical recovery (intra-day accuracy), as well as the added and measured concentrations, are also listed in Table 3. Analytical recovery values close to 100% was obtained, which proves good intra-day accuracy of the procedure.

Similarly, inter-day precision and inter-day accuracy studies were performed. In this case, five different standard addition curves from DBSs prepared from a drug-free whole blood sample spiked with the low, medium and high target concentrations used for intra-day experiments were prepared in five different days. RSDs and inter-day analytical recovery values are listed in Table 3. Results show good inter-day precision (RSD values within the 4–12 range) and accuracy (analytical recoveries between 96% and 104%).

The proposed method was finally applied to six different drug-free whole blood samples (blanks) for testing the selectivity of the method. The MRMs for the different selected precursor ion→product ion transitions were negligible in all cases, which proves the specificity of the developed procedure.

3.5. Applications

Thirteen whole blood samples from polydrug abusers were analyzed by the proposed DBS-ESI-MS/MS method. Each sample was subjected to the DBS sample pre-treatment method in triplicate by dispersing 20 μL of the whole blood sample mixed with the internal standards as described in Section 2.4. Each extract obtained was measured once by ESI-MS/MS. Samples were also analyzed by a conventional method based on SPE procedure and GC–MS quantification (Sections 2.5 and 2.7). Results obtained after both procedures (Table 4) are quite similar for those samples positive for cocaine and/or heroin abuse, and only three samples offered detectable values for codeine, morphine and 6-MAM. In addition, the high sensitivity of ESI-MS/MS measurements allowed the determination of targets in some samples positive to cocaine/heroin abuse for which GC–MS offered values lower than the LOQs. The high sensitivity of the proposed DBS-ESI-MS/MS method required the 5-fold dilution of one whole blood sample for an accurate assessment of BZE (concentration of 766 ng mL^{-1}).

4. Conclusions

The application of the DBS technique has been found adequate for the screening and confirmation of cocaine and heroin abuse. The methodology is inexpensive, simple and reliable, and can be used instead of multistage sample pre-treatments such as SPE procedures. Solvent volume consumption is also minimized, and small blood sample volume (140 μL or lower) can be used. The technique also offers absence of matrix effects for 6-MAM (a metabolite directly

Table 3
Intra-day precision and inter-day precision (RSD/%), intra-day and inter-day analytical recovery (AR/%) of the method.

Added concentration (ng mL^{-1})	RSD[a] (%)	RSD[b] (%)	Measured concentration (ng mL^{-1})			
			Intra-day assay[a]	Inter-day assay[b]	AR[a] (%)	AR[b] (%)
Cocaine						
10	5	8	10 ± 1	10 ± 1	104 ± 5	101 ± 8
100	3	4	102 ± 4	104 ± 5	102 ± 4	104 ± 5
200	2	4	209 ± 5	206 ± 8	104 ± 2	103 ± 4
BZE						
10	7	8	10 ± 1	10 ± 1	104 ± 7	101 ± 8
100	6	6	104 ± 6	102 ± 6	104 ± 6	102 ± 6
200	5	6	98 ± 11	198 ± 11	99 ± 5	99 ± 5
Codeine						
100	9	10	106 ± 9	105 ± 10	106 ± 9	105 ± 10
500	3	4	486 ± 16	496 ± 21	97 ± 3	99 ± 4
1000	3	4	961 ± 31	1000 ± 38	96 ± 3	100 ± 4
Morphine						
100	7	9	93 ± 6	96 ± 9	93 ± 6	96 ± 9
500	6	7	479 ± 16	498 ± 34	96 ± 5	100 ± 7
1000	5	5	991 ± 52	966 ± 46	99 ± 5	97 ± 5
6-MAM						
100	10	12	103 ± 10	101 ± 12	103 ± 10	101 ± 12
500	8	8	518 ± 41	487 ± 38	104 ± 6	97 ± 6
1000	5	5	1045 ± 53	1011 ± 48	104 ± 5	101 ± 5

[a] Intra-day assay (n=9).
[b] Inter-day assay (n=5).

Table 4
Concentrations of cocaine, BZE, codeine, morphine and 6-MAM in thirteen whole blood samples after DBS-ESI-MS/MS and SPE–GC–MS.

Case	Drug concentration (ng mL^{-1})									
	Cocaine		BZE		Codeine		Morphine		6-MAM	
	DBS-ESI-MS/MS	SPE–GC–MS	DBS-ESI-MS/MS	SPE–GC–MS	DBS-ESI-MS/MS	SPE–GC–MS	DBS-ESI-MS/MS	SPE–GC–MS	DBS-ESI-MS/MS	SPE–GC–MS
1	< 3.5	< 30	6.4 ± 0.4	< 30	37 ± 2	< 30	< 10	< 130	< 11	< 30
2	< 3.5	< 30	5.1 ± 0.3	< 30	< 9.4	< 30	< 10	< 130	< 11	< 30
3	4.6 ± 0.3	< 30	6.0 ± 0.1	< 30	33 ± 2	35 ± 2	< 10	< 130	< 11	< 30
4	5.6 ± 0.2	< 30	32 ± 3	< 30	43 ± 3	40 ± 2	27 ± 3	< 130	11 ± 1	< 30
5	< 3.5	< 30	238 ± 15	225 ± 20	< 9.4	< 30	< 10	< 130	< 11	< 30
6	< 3.5	< 30	9.7 ± 0.4	< 30	< 9.4	< 30	< 10	< 130	< 11	< 30
7	< 3.5	< 30	13.4 ± 0.6	< 30	< 9.4	< 30	90 ± 5	< 130	12 ± 1	< 30
8	16 ± 1	< 30	776 ± 23	779 ± 45	< 9.4	< 30	< 10	< 130	< 11	< 30
9	< 3.5	< 30	9.1 ± 0.3	< 30	< 9.4	< 30	130 ± 8	134 ± 4	12 ± 1	< 30
10	< 3.5	< 30	124 ± 10	131 ± 12	< 9.4	< 30	< 10	< 130	< 11	< 30
11	< 3.5	< 30	12 ± 0.4	< 30	< 9.4	< 30	< 10	< 130	< 11	< 30
12	< 3.5	< 30	27 ± 2	< 30	< 9.4	< 30	< 10	< 130	< 11	< 30
13	4.0 ± 0.2	< 30	113 ± 10	107 ± 7	< 9.4	< 30	< 10	< 130	< 11	< 30

related to heroin abuse). Therefore, heroin abuse screening/confirmation can be performed by using external calibrations, which shortens the whole procedure even more. However, cocaine abuse through cocaine and metabolites assessment in DBSs must be performed by using the standard addition technique. Finally, the fast direct ESI-MS/MS measurement, the high degree of specificity, and the high sensitivity allow for short and highly sensitive analysis, resulting in quick laboratory response.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.010.

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