

DETERMINATION OF COCAINE AND BENZOYLECGONINE IN HUMAN PLASMA BY LC-MS/MS

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Abstract

A high-throughput Liquid chromatography/ Mass spectrometry/ Mass spectrometry (LC/MS/MS) method for simultaneous quantification of cocaine (COC) and benzoylecgonine (BZE) in human plasma was elaborated. After the protein precipitation with methanol in the plasma samples, the analytes were separated on a Zorbax SB-C18 column under isocratic conditions using a 32:68 (v/v) mixture of methanol and 0.1% formic acid in water at 45°C, with a flow rate of 1 mL/min. The detection was in multiple reaction monitoring (MRM) mode (m/z 290 $\rightarrow$ m/z 168 for BZE and m/z 304 $\rightarrow$ m/z 182 for COC, respectively). Calibration curves were generated over the range of 20-2000 ng/mL for both BZE and COC ($r > 0.992$). In the laboratories that are not equipped with a LC/MS/MS system, the LC analysis of COC and BZE requires a previous isolation step. Therefore, the elaborated LC/MS/MS method was applied for the study of optimum experimental conditions for the isolation of COC and BZE from human plasma. Several solvent mixtures and solid-phase extraction (SPE) cartridges were tested at different pH values. The best results for both analytes were obtained by SPE on Oasis HLB cartridges (mean recovery of 71 % for COC and 92.4 % for BZE, $n = 3$). Both the elaborated LC/MS/MS method and the best extraction conditions can successfully be applied in clinical and forensic toxicology for the rapid quantification of COC and BZE in human plasma.

Rezumat

S-a elaborat o metodă rapidă LC/MS/MS pentru cuantificarea simultană a cocainei (COC) și benzoilecgoninei (BZE) din plasma umană. După precipitarea proteinelor cu metanol în probele de plasmă, analiții s-au separat pe o coloană Zorbax SB-C18 în condiții izocratice folosind un amestec 32:68 (v/v) de metanol și soluție apoasă de acid formic 0,1%, la 45°C cu un debit de 1 mL/min. Detecția s-a realizat în modul MRM (monitorizarea reacțiilor multiple) (m/z 290 $\rightarrow$ m/z 168 pentru BZE, respectiv m/z 304 $\rightarrow$ m/z 182 pentru COC). Curbele de calibrare s-au realizat pe domeniul de concentrații 20-2000 ng/mL atât pentru BZE cât și pentru COC ($r > 0.992$). În laboratoarele care nu sunt echipate cu un sistem LC/MS/MS, analiza LC a COC și BZE necesită anterior o etapă de izolare. Astfel, metoda LC/MS/MS elaborată s-a aplicat în studiul condițiilor experimentale optime necesare izolării COC și BZE din plasma umană. S-au testat mai multe amestecuri de solvenți și cartușe de extracție pe fază solidă (EFS) la diferite valori de pH. Cele mai bune rezultate s-au obținut prin EFS cu cartușe Oasis HLB (regăsiri medii de 71 % pentru COC și 92.4 % pentru BZE). Atât metoda LC/MS/MS elaborată cât și cele mai bune metode de extracție evidențiate se pot aplica cu succes în toxicologia clinică și medico-legală în vederea dozării rapide a COC și BZE din plasma umană.

Keywords: cocaine, benzoylecgonine, human plasma, LC-MS/MS

Introduction

Cocaine (COC) abuse has increased dramatically during the past three decades. Benzoylecgonine (BZE) is the main cocaine metabolite, formed either by a hepatic carboxylesterase or by spontaneous hydrolysis [6]. Several LC/MS/MS applications for COC have been published, but they have been either qualitative analysis in plasma [11] or quantitative analysis of COC and its metabolites in other matrices such as urine [4], oral fluid [1], hair [7, 8] and meconium [13]. Generally, the quantitative analysis of COC and BZE are performed by GC-MS, which requires derivatisation of BZE due to its polar nature [2, 3]. A new sensitive and selective LC/MS/MS method for the quantification of COC and BZE in human plasma was elaborated.

In the laboratories that are not equipped with a LC/MS/MS system, the liquid chromatographic analysis of COC and BZE requires a previous isolation step [5, 12]. Therefore, the elaborated LC/MS/MS method was applied for the study of optimum experimental conditions for the isolation of COC and BZE from human plasma by liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

Materials and methods

Reagents and materials

COC hydrochloride and BZE were standard references purchased from Lipomed AG (Arlesheim, Switzerland). All chemicals were of analytical-reagent grade. HPLC-grade methanol and 98% formic acid were purchased from Merck (Darmstadt, Germany), concentrated ammonia from Fluka (Buchs, Switzerland), ethyl acetate, hexane, dichloromethane, isopropanol, disodium phosphate and monopotassium phosphate from Sigma-Aldrich (Steinheim, Germany) and Tris hydrochloride from Riedel de Haën (Germany). Bidistilled, deionised water *pro injectiones* was purchased from Infusion Solution Laboratory of University of Medicine and Pharmacy Cluj-Napoca, Romania. The human blank plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania. For SPE the following cartridge types were used: Oasis[®] MCX, 30 mg and Oasis[®] HLB, 30 mg (Waters Corporation, Massachusetts, Ireland), Isolute[®] HCX, 130 mg (International Sorbent Technology, Mid Glamorgan, UK), Chroma-bond C18ec (Macherey-Nagel, Düren, Germany) and SepPak C18, 300 mg (Lida Manufacturing Corporation, Kenosha, WI, USA).

Apparatus

The following apparatus were used in this study: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus Balance (Mettler-Toledo, Switzerland) and Discovery Balance (Ohaus, Pine Brook, NJ, SUA); Vortex Genie 2 (Scientific Industries, New York, USA); ultrasonic bath Elma Transsonic 700/H (Singen, Germany); SPE automated extractor Chromabond® Vacuum manifolds (Macherey-Nagel, Düren, Germany); evaporator with nitrogen stream (Cole-Parmer, USA).

The HPLC system was a 1100 series model (Agilent Technologies, Darmstadt, Germany) consisting of a G1312A binary pump, an in-line G1379A degasser, a G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector (1100 VL; Agilent Technologies).

Chromatographic separation was performed at 45°C on a Zorbax SB-C18 (100 mm x 3.0 mm, 3.5 µm I.D) column (Agilent Technologies) protected by an in-line filter under isocratic conditions. The mobile phase was a 32:68 (v/v) mixture of methanol and 0.1% formic acid in water. The flow rate of the mobile phase was 1 mL/min.

Chromatograms were processed using Quant Analysis software.

The detection of BZE and COC was in the multiple-reaction monitoring (MRM) mode using an ion trap mass spectrometer equipped with an atmospheric pressure chemical ionization ion source, in positive mode. The ion transitions monitored were m/z 290→ m/z 168 for BZE and m/z 304→ m/z 182 for COC, respectively.

Standard solutions

1 mg/mL stock solutions of COC and BZE were prepared by dissolving the appropriate quantities of reference substances in 5 mL methanol. The marked plasma samples with the concentrations of 300 and 1000 ng/mL were obtained by diluting specific volumes of COC and BZE stock solutions, respectively, with blank plasma.

Sample preparation

All analysis were performed in triplicate.

For LLE, spiked plasma samples (0.5 mL) were mixed with 2.5 mL buffer solution (0.1 M phosphate buffer pH 6 or 0.1 M Tris buffer pH 8.4) and 5 mL organic solvent (Table I) at vortex-mixer for 5 minutes. After centrifugation, the organic phase was separated and evaporated to dryness at 50°C under a nitrogen stream. The dry extract was reconstituted in 500 µL of water:methanol 80:20 (v/v) and a 4 µL aliquot was injected into the chromatograph.

For SPE, spiked plasma samples (0.5 mL) were mixed with 2.5 mL 0.1 M phosphate buffer (pH 6) at vortex-mixer for 30 seconds, than

centrifugated. The tested cartridges were successively conditioned with methanol, bidistillated water and 0.1 M phosphate buffer (pH 6). After sample supernatant addition, the cartridges were washed with 0.001 M phosphate buffer (pH 6). The volumes of the solutions used for conditioning and washing were 3 mL for Oasis MCX, Oasis HLB and Isolute HXC, and 5 mL for Chroma-bond C18ec and SepPak C18, respectively. The elution was performed in two consecutive phases, with: a) methanol : conc. ammonia (19:1, v/v) (2 mL) and b) dichlormethane : isopropanol (9:1, v/v) (2 mL). Each eluate was evaporated to dryness at 45°C under a nitrogen stream. The dry extract was reconstituted in 500 µL water:methanol 80:20 (v/v) and a 4 µL aliquot was injected into the chromatograph.

Results and discussion

We propose a very simple and rapid pretreatment of plasma samples including only protein precipitation by methanol and direct injection into the chromatographic system of the supernatant obtained after centrifugation.

The chromatographic conditions, especially the composition of the mobile phase, were optimized in several trials in order to achieve a good MS signal, a short retention time for COC and BZE with an adequate separation and consequently a high-throughput analysis. The best results were obtained with the mixture of methanol and 0.1% formic acid in water 32:68 (v/v) under isocratic conditions at 45°C, with a flow rate of 1 mL/min. In the selected chromatographic conditions the retention times of COC and BZE were 1.35 min and 1.15 min, respectively, and the analytical run-time was 1.7 min. Representative chromatograms of drug-free plasma and plasma spiked with COC and BZE at low limit of quantification (LLOQ) are shown in Fig. 1 and Fig. 2, respectively. No interfering peaks from the endogenous plasma components were observed at the retention times of COC and BZE. The calibration curves were linear over the concentration range of 20-2000 ng/mL in human plasma, with a correlation coefficient greater than 0.992. The LLOQ was 20 ng/mL.

The values for the recovery obtained after LLE and SPE extractions of COC and BZE from human plasma are presented in Tables I and II, respectively. COC and BZE are the hydrophylic basic compounds with $pK_a > 8$, but BZE is more polar ($\log P_{o/w}$ of -1.3) than COC ($\log P_{o/w}$ of 2.3) [10] and cannot be efficiently isolated by LLE [9].

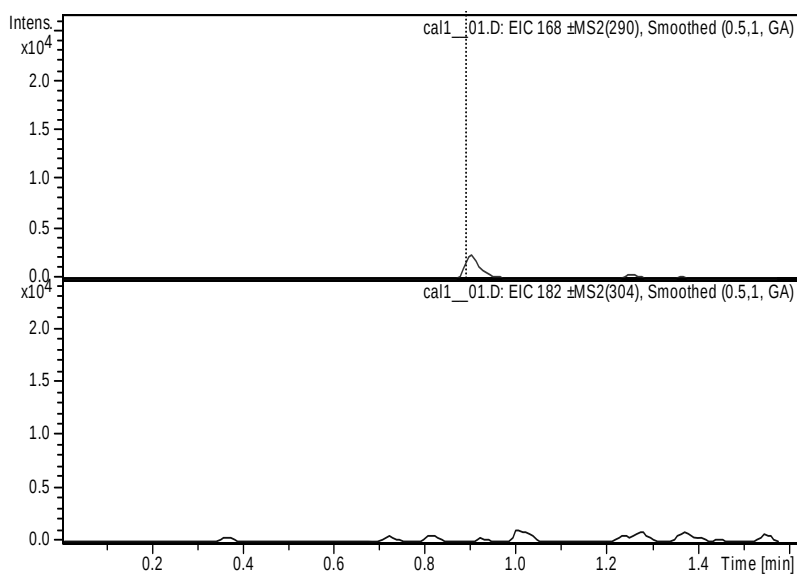


Figure 1
Chromatogram of plasma blank

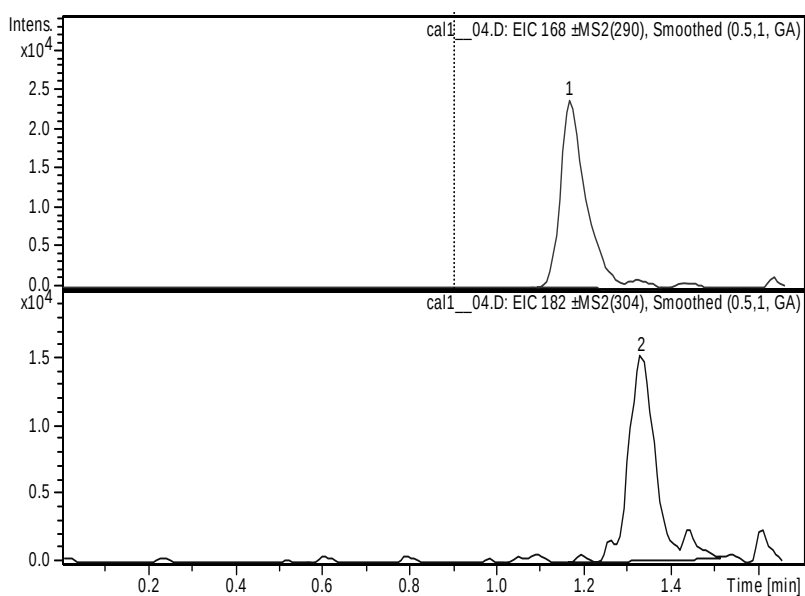


Figure 2
Chromatograms of plasma spiked with COC (1) and BZE (2)
at LLOQ level (20 ng/mL)

Besides the higher polarity of BZE, its LC/MS signal may be also suppressed by endogenous interferents from alkaline extracts, so the apparent calculated recovery can be consequently lowered [14, 15]. The

recoveries obtained with the tested solvents were poor (< 7.3%), except the extraction in dichloromethane at pH 6, that was better but insufficient (~16%) (Table I).

The SPE extraction from plasma was performed at pH 6. Both analytes were eluted with the first eluent (Table II). The concentrations of COC and BZE were insignificant in the second eluent. The best results for both analytes were obtained with Oasis HLB cartridges (68.57% recovery for COC and 91.85% for BZE at 300 ng/mL, and 73.47% for COC and 92.96% for BZE at 1000 ng/mL, respectively, $n = 3$).

Table I

Recovery of cocaine and benzoylecgonine from human plasma by LLE using different solvent mixtures at different plasma pH ($n=3$)

Solvent mixture	pH	Nominal C ng/mL	COC		BZE	
			Recovery %	CV %	Recovery %	CV %
CH ₂ Cl ₂	6	300	66.65	9.70	17.18	1.19
		1000	63.57	3.15	15.01	1.76
Ethyl acetate	6	300	54.27	1.47	1.94	1.68
		1000	69.50	1.88	1.21	0.43
CH ₂ Cl ₂ :IP*:hexane (5:1:1, v/v)	6	300	57.04	3.81	2.74	2.41
		1000	64.54	10.03	3.14	0.56
CH ₂ Cl ₂ :IP:ethyl acetate (5:1:1, v/v)	8.4	300	57.84	3.64	5.00	0.97
		1000	65.47	2.98	4.86	0.31
CH ₂ Cl ₂ :IP:hexane (5:1:1, v/v)	8.4	300	41.59	6.31	5.24	0.21
		1000	57.43	18.40	7.27	2.19

*IP = isopropanol

Table II

Recovery of cocaine and benzoylecgonine from human plasma by SPE using different cartridges at pH 6 ($n=3$) in the first eluent, methanol:ammonia conc. (19:1, v/v)

SPE cartridge	Nominal C ng/mL	COC		BZE	
		Recovery %	CV %	Recovery %	CV %
Chroma-bond C18 ec	300	56.03	3.21	80.38	6.61
	1000	71.71	6.30	95.42	3.85
SepPak C18	300	61.78	1.11	82.38	2.82
	1000	68.41	6.00	85.63	6.04
Izolute	300	60.38	14.37	84.68	2.47
	1000	78.28	2.87	89.34	6.68
Oasis MCX	300	60.08	2.70	91.66	8.93
	1000	67.74	8.04	90.25	11.64
Oasis HLB	300	68.57	9.96	91.85	4.78
	1000	73.47	9.99	92.96	12.45

Conclusions

The elaborated LC/MS/MS method can be useful for the rapid quantification of COC and its metabolite in human plasma in different toxicological studies.

The best results for the isolation of both analytes from human plasma were obtained by solid-phase extraction with Oasis HLB cartridges. These optimum experimental conditions for the isolation of COC and BZE from plasma samples can be used in other classical HPLC determinations of these analytes.

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