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Research Article

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Development and validation of a HPLC/MS/MS method for the quantification of Cocaine and its principal metabolite

B. Belafkih[1,2], S. Belouafa[1]*, M. Charrouf[1], A. Bennamara[1], A. Skalli[2] and A. Abourriche[1]

[1]*Laboratoire Biomolécules et Synthèse Organique, Faculté des Sciences Ben M'Sik, Université Hassan II – Casablanca. Avenue Cdt D. El harti, BP 7955, Casablanca, Morocco*
[2]*DGSN/DPJ/DPTS/Laboratoire de Police Scientifique, Casablanca, Morocco*

ABSTRACT

A sensitive and fast HPLC/MS/MS method for measurement of cocaine in seized powders and of cocaine and benzoylecgonine in human urine was developed and validated. Good selectivity, linearity, accuracy and precision were obtained for the developed method. The validated method is very simple and more rapid than other similar methods.

Key words: Cocaine, benzoylecgonine, seizures, human urine, quantification, HPLC-MS/MS, validation.

INTRODUCTION

Cocaine (Figure 1) was first isolated, in 1861 by Albert Niemann, from the leaves of Eritroxylum coca via extraction with organic solvents followed by purification. 20 years later, Karl Koller discovered the anesthetic properties. Its writings and other papers promoted the use of the refined cocaine for a variety of ailments including depression, gastric disorders, asthma, and morphine or alcohol addiction. Its use as local anesthetic or as an aphrodisiac was also suggested [1].

In 1914, cocaine entered the list of U.S. controlled substances [2]. Cocaine increases alertness, wakefulness, elevates the mood, induces a high degree of euphoria, decreases fatigue, improves thinking, and increases concentration and energy. In large doses, users often display symptoms of psychosis with confused and disorganized behavior, irritability, fear and paranoia [3]. Cocaine is a highly addictive substance developing a strong tolerance and psychological dependence and moderate physical dependence. Illicit cocaine is usually distributed as a white crystalline powder in hydrochloride salt form or as an off white chunky material which is cocaine free base and commonly named as crack. Cocaine powder is often diluted with sugars and local anesthetics like lidocaine [2,4-7]. The hydrochloride salt form of cocaine can be snorted or dissolved and injected. Crack cocaine comes in a rock crystal that can be heated and its vapors smoked; the term "crack" refers to the crackling sound heard when it is heated [8,9].

Due to the clandestine development of new drugs and the ever increasing number of samples to be analyzed by forensic science laboratories, the scientist is required to employ powerful hyphenated and fast techniques like capillary electrophoresis, liquid chromatography with mass spectrometry (HPLC-MS), gas chromatography with Fourier transform infrared spectroscopy (GC-FTIR) and Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR/ATR), ... [6].

Also, numerous analytical methods for cocaine have been reported in plasma [10,11], whole blood [12,13], brain [14], nails [15,16], saliva [17,18], urine [19], hair [20-23]. They include gas chromatography–mass spectrometry (GC/MS), gas chromatography nitrogen phosphorous detection (GC/NPD) and HPLC/MS/MS techniques.

An isolation step of analytes often required prior to instrument analysis, performed generally by liquid–liquid extraction (LLE) [24] or solid phase extraction (SPE) [25,26], but it is a time consuming step that also increases the cost of the assay and can affect the recovery.

In this study, the advances of a high performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) technique with particular emphasis on the use of electrospray ionization source have been exploited for the quantization of cocaine and its main metabolite (benzoylecgonine) in seized materials and human urine.

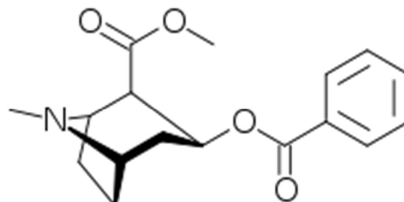

Figure 1: Structure of Cocaine

EXPERIMENTAL SECTION

Reagents and samples

Simples of cocaine white powder were seized by the police in Morocco and were received by the forensic science laboratory for analysis and human urine was procured from Department of Forensic Medicine and was stored at -20°C.

Cocaine standard was purchase from LGC Standards S.A.R.L. France.

Ammonium formate, formic acid (98%), methanol (99,9%) and HPLC grade acetonitrile, were obtained from Sigma Aldrich (Germany).

Ultrapure Water of HPLC was prepared using dispositive labconco (Seriel N°:130982243F)

Standard solutions

Cocaine solution was prepared by dissolving the cocaine standard (1 mg/mL) with methanol.

Two working solutions 10 µg/mL of cocaine and 10 ng/mL of benzoylecgonine were prepared by appropriate dilutions in methanol. These solutions were used to prepare calibration standards with the concentrations of high range assay (0.1, 1, 10, 100, and 500 ng/mL), median range assay (0.1, 1, 10, 100, and 250 ng/mL) and low range assay (1, 10, 50, 80, and 100 ng/mL) for cocaine and (1, 10, 50, 75, and 100 ng/ml) cocaine and benzoylecgonine in free human urine.

Simple preparation

1 mg of each cocaine white powder samples was dissolved in 1 mL of methanol. After centrifugation and filtration, the obtained solutions were diluted to 100 ng/mL with methanol and transferred in the auto sampler vials. 20 µL were injected into the HPLC system.

1mL urine sample was added to 1mL of acetonitrile. After centrifugation and filtration, the obtained solution was diluted (1:100) with acetonitrile and transferred in the autosampler vials. 20 µL were injected into the HPLC system.

Apparatus

An HPLC-MS/MS QTRAP 3200 system equipped with a Perkin Elmer Series 200 chromatographic pump and a Perkin Elmer Series 200 auto sampler (PE Sciex, Concord, Canada) was used for direct ESI-MS/MS determinations, operating in positive ion and multiple reactions monitoring (MRM) acquisition mode.
The chromatograms were acquired using the analyst software (version 1.4.1).

Procedure

A liquid chromatographic system consisted of a solvent delivery system (pumps identified as A and B) and an autosampler was used. Chromatographic separation of the analytes was achieved on PHENOMENEX column (50x2, 2mm; 4µm) equipped with a pre-column filter.

For preparation of mobile phase, 63g of ammonium format was dissolved in 1L water HPLC grade.

Mobile phase A: 2 mL of 1M ammonium format solution and 2 mL formic acid in 996 mL of water HPLC grade.

Mobile phase B: 2 mL of 1M ammonium format solution and 2 mL formic acid in 996 mL of acetonitrile HPLC grade.

Eluents were sonicated before use. The flow rate was set at 0.5 mL/mn.

The used gradient program time was presented in Table 1. The total run time was 8 mn and the column temperature was maintained at 40°C.

The autosampler injection needle was washed with methanol/water (1:1, v/v) after each injection.

The mass spectrometer was run in positive ion ESI mode using multiple-reaction monitoring (MRM) to monitor the mass transitions. The ion spray voltage and the source temperature were set at 5500 V and 600°C, respectively. Nitrogen gas was used as the curtain gas and set at 10. The ion source gas 1 and 2 were set at 50. A summary of the ion transitions, declustering potentials, collision energies, and collision cell exit potentials for all range assays were presented in Table 2.

The concentration of cocaine and benzoylecgonine were determined automatically by the instrument data system using peak areas and external standard method.

Table 1: Gradient program time

Total time (mn)	Flow rate (mL/mn)	Mobile phase A (%)	Mobile phase B (%)
0	0.5	80	20
3	0.5	10	90
5	0.5	10	90
5.5	0.5	80	20
8	0.5	80	20

Table 2: Optimal positive ion ESI mass spectrometric conditions for multiple-reaction monitoring (MRM)

Drugs	Ion transitions	Declustering potentials	Collision energies	Collision cell exit potentials
Cocaine	304.1→182	40	35	3
Benzoylecgonine	290.1→168.2	40	25	5.74

Method validation

The method was validated to comply with specified requirements using the most recommended guidelines for analytical validation in Europe [27,28], including the most widely applied analytical-performance characteristics such as linearity, limit of detection (LOD) and quantitation (LOQ), precision and accuracy.

The *linearity* of analytical method was determined by studying standard calibration curves. The range of analytical method was decided from the interval between upper and lower level of calibration curves. Thus, three concentrations of reference material were analyzed 3 times. The results of linearity study are shown in Tables 3 and 4.

LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. It corresponds to 3 times the standard deviation of five replicates of samples.

LOQ is the lowest amount of analyte in a simple which can be quantitatively determined with suitable precision and accuracy (less than 20% [29]). It corresponds to 10 times the standard deviation of five replicates of samples.

Table 3: Linear regression analysis of standard solutions for cocaine and benzoylecgonine in urine

Analyte	Range	Equation	Range (ng/mL)	r
Cocaine Only	1	$Y=5.43.10^3$ X- $1.05.10^5$	0,1-500	0.983
	2	$Y=7.82.10^3$ X $+7.27.10^4$	0,1-250	0.9905
	3	$Y=2.71.10^4$ X $+4.59.10^4$	1-100	0.9966
Cocaine in urine		$Y=4.94.10^4$ X $-7.78.10^3$	1-100	0.9989
Benzoylecgonine in urine		$Y=1.39.10^4$ X $+2.34.10^3$	1-100	0.9915

Table 4: Linear regression analysis of standard solutions for a 1-100 ng/mL concentration range, performed in 3 different days for cocaine

Analyte	day	Equation	Range (ng/mL)	r
Cocaine	1	$Y=1.54.10^4$ X+ 6.5510^3	1-100	0.9919
	2	$Y=1.53.10^4$ X	1-100	0.9921
	3	$Y=2.71.10^4$ X+ 4.5910^4	1-100	0.9966

Table 5: Results of Intra-day and Inter-day precision assay for cocaine, cocaine and benzoylecgonine in human urine

Analyte	Concentration (ng/mL)	Intra-day precision (%)	Inter-day precision (%)
Cocaine	1	12.3	2.24
	10	4.7	1.23
	50	2.7	1.6
	80	5.1	0.8
	100	4	1.12
Cocaine in urine	1	10.76	2.86
	10	0.88	2.52
	50	2.03	3.45
	75	0.46	2.30
	100	0.64	0.62
Benzoylecgonine in urine	1	25	6.56
	10	2.41	1.39
	50	2.60	1.32
	75	1.44	1.24
	100	0.64	0.90

The intra-day precision (expressed as coefficient of variation) and accuracy (expressed as relative difference between obtained and theoretical concentrations (%)) were determined by analyzing, on the same day, six replicates of three different samples from each standards (20, 40 and 80 ng/mL) for cocaine and (20, 40 and 70 ng/mL) for cocaine and benzoylecgonine.

The inter-day precision was evaluated by repeating the intra-day precision study in 3 different days. Data of precision and accuracy was presented in Tables 5 and 6.

Table 6: Intra-day and Inter-day Accuracy data for cocaine, cocaine and benzoylecgonine in human urine

Analyte	Concentration (ng/mL)	Intra-day Accuracy (%)	Inter-day Accuracy (%)
Cocaine	10	84.7	95
	50	101.9	103
	80	85.8	101.5
Cocaine in urine	20	111	106
	40	98.4	102
	70	99.8	93.5
Benzoylecgonine in urine	20	107.7	119
	40	90.35	93.5
	70	79	77

RESULTS AND DISCUSSION

Selectivity

To verify the selectivity of the method are made with injections of low concentration standard solution until a well-resolved peak (Figures 2,3). No interfering peaks were observed at the retention times of both analytes, confirming the good selectivity of the method.

Linearity

The calibration curves (Tables 3,4) were linear over all the studied concentration ranges with coefficients of determination (r) greater than 0.99 for the calibration curves of cocaine only and cocaine and benzoylecgonine in urine.

LOD and LOQ

The LOQ and LOD of cocaine were estimated following EMEA criteria [20].

LOD defined as the signal-to-noise ratio ($\frac{S}{N}.3$) is equal to 4.5 ng/ml for cocaine and 3.6 ng/mL for benzoylecgonine.
LOQ defined as the signal-to-noise ratio ($\frac{S}{N}.10$) is equal to 15 ng/ml for cocaine and 12 ng/mL for benzoylecgonine.

Precision and accuracy

The CV (%) values calculated for intra- and inter-day precision studies of cocaine and benzoylecgonine (Table 5) did not exceed 15%. Thus, the developed method is considered precise for these analytes [30].

The % recovery varies from 77% to 119%, which is less than 120% (Table 6). The calculated accuracy error is less than 5. So, it is considered insignificant [31]. These results show the high quality of quantitative results achievable with this method.

Analytical application

Following the previously described general procedure, the method was successfully applied to the determination of cocaine in seized powder samples and in human urine with benzoylecgonine.

Table 7 shows contents of cocaine in 8 seizures and Figure 4 presents a chromatograms of an extract obtained from urine sampled by Forensic Medicine.

The % RDS, less than 5 [31], indicates that the method was accurate, precise and selective. Then, this method is suitable and can be successfully applied by the police.

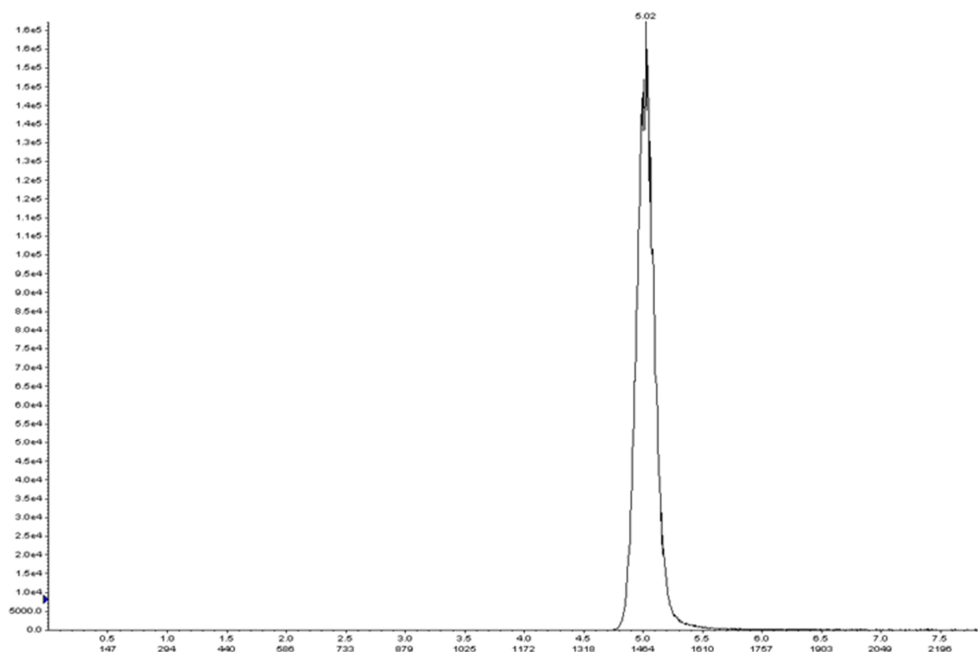

Figure 2: Representative chromatographic of cocaine

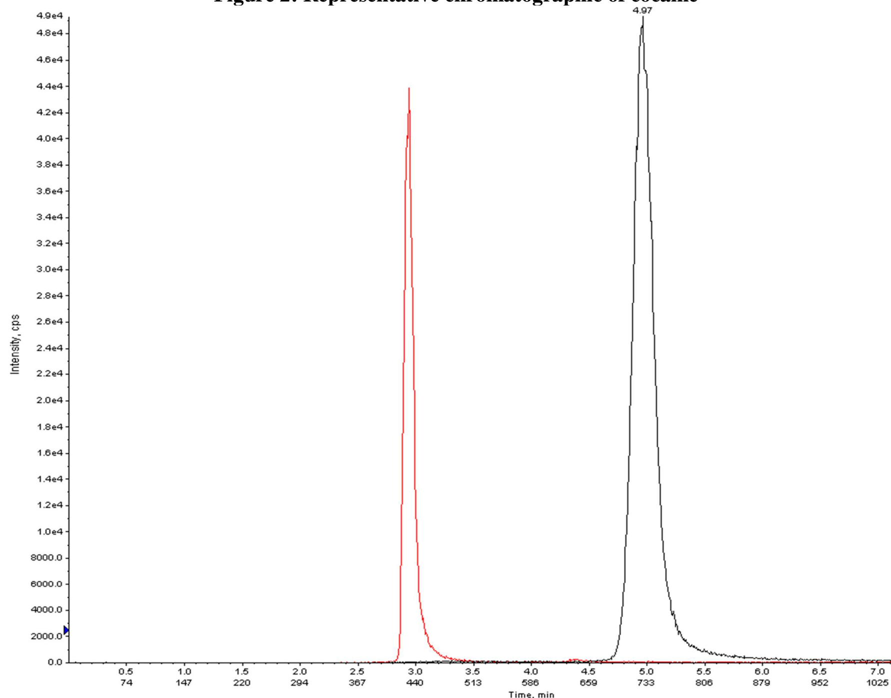

Figure 3: Representative chromatographic of an urine sample spiked with cocaine and benzoylecgonine

Table 7: Assay of cocaine in presence of adulterants

Seizures	Cocaine (%)	Adulterants
1	84	-
2	93	-
3	70	tetramisole
4	75	phenacetin
5	87	-
6	47	phenacetin
7	73	levamisole
8	50	phenacetin

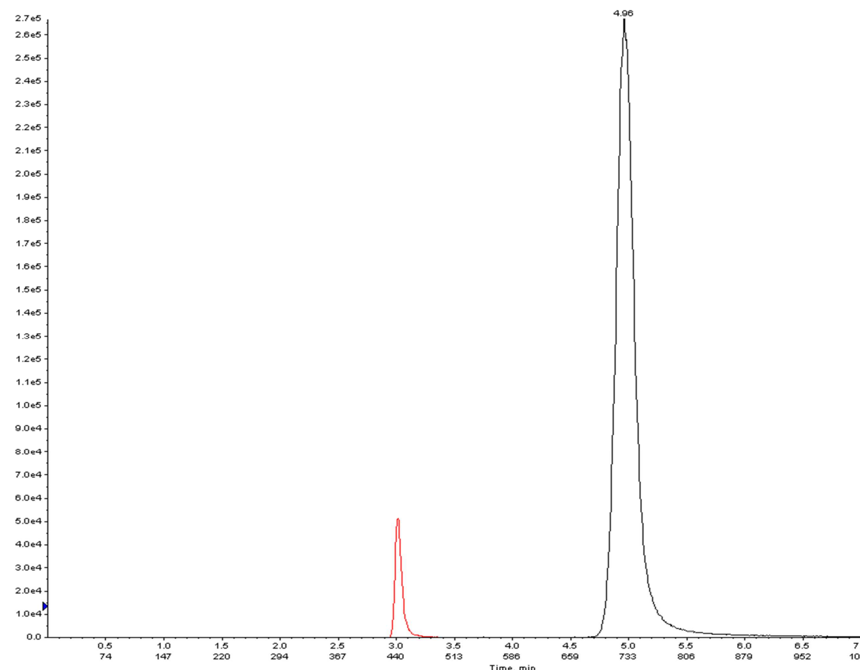

Figure 4: Representative chromatographic of an extract obtained from urine sampled by Forensic Medicine in order to further investigate the cause of death

Concentration of cocaine is 84 ng/ml and concentration of benzoylecgonine is 38ng/ml

CONCLUSION

The experimental results demonstrate that the proposed HPLC-MS/MS method is simple and less time consuming, accurate, precise and selective. It offers preferential advantages over most of the established procedures. The adulterants usually present with cocaine in drugs did not interfere with determination of cocaine in seizures and cocaine and benzoylecgonine in urine. Therefore, the introduced method can be recommended for routine quality control of seized cocaine and cocaine with benzoylecgonine in human urine in order to further investigate the cause of death and in monitoring program of cocaine abuse.

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