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Development and validation of a gas chromatography/ion trap-mass spectrometry method for simultaneous quantification of cocaine and its metabolites benzoylecgonine and norcocaine: Application to the study of cocaine metabolism in human primary cultured renal cells

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ABSTRACT

Acute renal failure is a common finding in cocaine abusers. While cocaine metabolism may contribute to its nephrotoxic mechanisms, its pharmacokinetics in kidney cells is hitherto to be clarified. Primary cultures of human proximal tubular cells (HPTCs) provide a well-characterized *in vitro* model, phenotypically representative of HPTCs *in vivo*. Thus, the present work describes the first sensitive gas chromatography/ion trap-mass spectrometry (GC/IT-MS) method for measurement of cocaine and its metabolites benzoylecgonine (BE) and norcocaine (NCOC) using a primary culture of HPTCs as cellular matrix, following solid phase extraction (SPE) and derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). The application of this methodology also enables the identification of two other cocaine metabolites: ecgonine methyl ester (EME) and anhydroecgonine methyl ester (AEME). The validation of the method was performed through the evaluation of selectivity, linearity, precision and accuracy, limit of detection (LOD), and limit of quantification (LOQ). Its applicability was demonstrated through the quantification of cocaine, BE and NCOC in primary cultured HPTCs after incubation, at physiological conditions, with 1 mM cocaine for 72 h. The developed GC/IT-MS method was found to be linear ($r^2 > 0.99$). The intra-day precision varied between 3.6% and 13.5% and the values of accuracy between 92.7% and 111.9%. The LOD values for cocaine, BE and NCOC were 0.97 ± 0.09, 0.40 ± 0.04 and 20.89 ± 1.81 ng/mL, respectively, and 3.24 ± 0.30, 1.34 ± 0.14 and 69.62 ± 6.05 ng/mL as LOQ values.

1. Introduction

The most recent report of the United Nations Office on Drugs and Crime (UNODC) estimates that the highest prevalence of cocaine use remains in North America, affecting 2% of the adult population aged 15–64 years. In Europe, cocaine ranks second as the most used illicit drug after cannabis, though patterns of use vary greatly among countries, the annual prevalence of cocaine use in Europe ranging between 0.8% and 0.9% of the population aged 15–64 years, or around 4.5–5 million people who had used cocaine in the last year [1]. Cocaine is a naturally occurring alkaloid extracted from the *Erythroxylum coca* leaves, and is primarily abused for its neurological effects like euphoria, increased self-esteem and state of alert [2–4]. However, chronic use leads to side effects such as disorientation, paranoia and hallucinations. Toxic effects involving the central nervous system (CNS) [5–7], the cardiovascular [8,9] and respiratory systems [8,10], and direct or indirect toxicity at other target organs as the liver and the kidney [10–13] have also been reported.

As it enters the organism, cocaine is metabolized into two major metabolites, benzoylecgonine (BE) and ecgonine methyl ester (EME) that are excreted into the urine, and minor metabolites like norcocaine (NCOC), hydroxycocaine and hydroxybenzoylecgonine [3,14]. Two other metabolites, anhydroecgonine methyl ester (AEME) and cocaethylene (CE), can be produced when the drug is consumed in the free base form (as a result of thermal degradation of smoked "crack") [15], and with simultaneous use of alcohol [16], respectively (Fig. 1). The different cocaine metabolites present unique pharmacokinetic and toxicological profiles. It was demonstrated that cocaine metabolism in the liver into NCOC plays an important role in its hepatotoxic effects [17,18], while BE and EME are pharmacologically inactive and nontoxic metabolites. Therefore, the development of analytical techniques for identification and quantification of these analytes is highly relevant for the phar-

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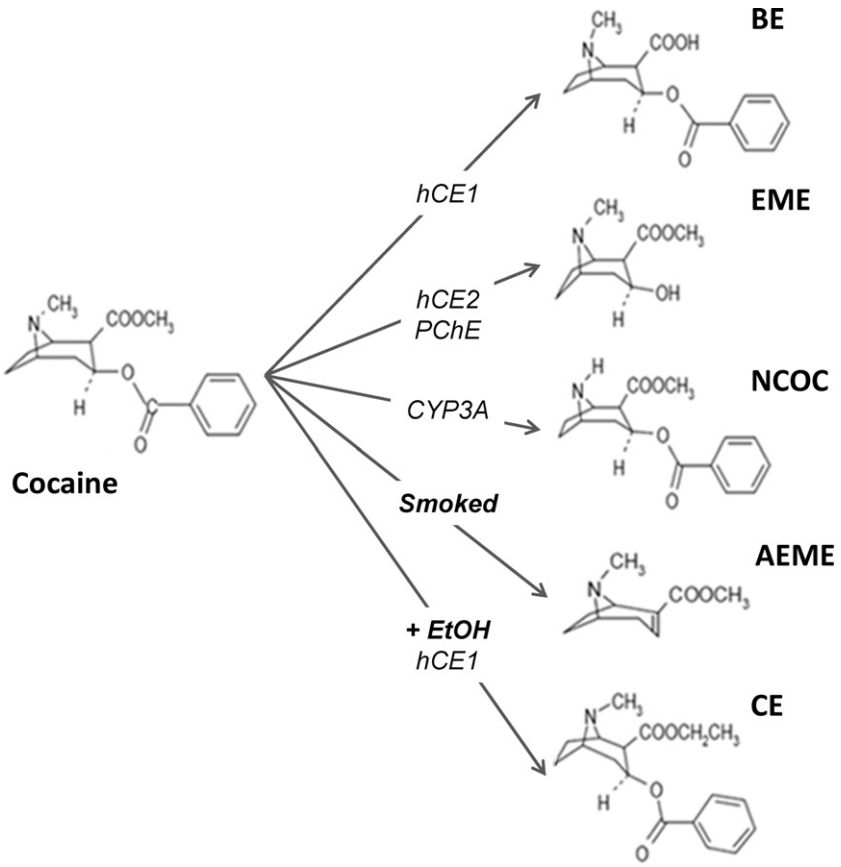

Fig. 1. Schematic representation of major metabolic pathways of cocaine in humans with the enzymes involved. The main *in vivo* metabolism of cocaine is its hydrolysis to benzoylecgonine (BE) mainly by liver carboxylesterase-1 (hCE-1). Cocaine may also be hydrolyzed to ecgonine methyl ester (EME) by liver carboxylesterase-2 (hCE-2) and plasma cholinesterase (PChE). Only a minor part is *N*-demethylated by *CYP*3A in humans to norcocaine (NCOC). During crack smoking, cocaine undergoes thermal breakdown to anhydroecgonine methyl ester (AEME). In the presence of ethanol (EtOH), hCE-1 catalyzes the ethyl transesterification of cocaine to cocaethylene (CE).

macokinetic and toxicological profiling of cocaine at the different target organs.

Acute renal failure is a common finding in cocaine abusers. In most reported cases, acute renal failure was found to be associated with rhabdomyolysis, ischemia due to vasoconstriction, or hyperthermia [10,19], with limited attention given to the possible direct toxic effects of the drug or its metabolites. In fact, the mechanism of cocaine toxicity to proximal epithelial cells is yet to be evaluated. Primary cultures of human proximal tubular cells (HPTCs) provide a well-characterized *in vitro* model, phenotypically representative of HPTCs *in vivo*, and therefore represent a suitable *in vitro* model to study the nephrotoxicity of xenobiotics [20].

Several GC/MS methods have been described in the literature for the determination of cocaine and its metabolites in biological fluids (blood, urine and sweat) [21–24]. However, to the best of our knowledge, the quantification of these compounds in a cellular matrix has not been investigated yet. Therefore, the aim of the present work was to develop and validate a suitable and sensitive gas chromatography/ion trap-mass spectrometry (GC/IT-MS) method for simultaneous quantification of cocaine, its main metabolite BE, and the known hepatotoxic metabolite NCOC, using a primary culture of HPTCs as cellular matrix, in order to assess the potential metabolism of cocaine in the human renal cortex.

2. Materials and methods

2.1. Reagents and standards

All chemicals and reagents were of analytical grade. Cocaine hydrochloride, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), benzophenone and hydrochloric acid were obtained from Sigma–Aldrich (St. Louis, MO). Methanol and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). Benzoylecgonine tetrahydrate was obtained from LGC Standards (Molsheim, France). Norcocaine (NCOC) was kindly supplied by Dr. John Munson (Center for Environmental and Human Toxicology, University of Florida). Dulbecco's modified Eagle's medium with nutrient mixture F-12 (DMEM/F-12) and GlutaMAX-I™, heat-inactivated fetal bovine serum (FBS), trypsin 0.05%–EDTA, antibiotic mixture of penicillin/streptomycin (10.000 U/mL/10.000 μg/mL), fungizone (250 μg/mL) and human transferrin (4 mg/mL) were obtained from GIBCO Invitrogen (Barcelona, Spain). Phosphate buffer solution (PBS) was obtained from Lonza Laboratories (Verviers, Belgium).

2.2. Human renal proximal tubular cells

The validation of the method was performed using HPTCs as matrix. Primary cultures of HPTCs were prepared from human kidney tissue samples as previously described [25]. HPTCs were cultured in 75 cm^2 culture flasks, using DMEM/F-12 medium supplemented with 10% FBS, 50 U/mL/50 μg/mL penicillin/streptomycin, 2.5 μg/mL fungizone, and 5 μg/mL human transferrin, in a 5% CO_2 atmosphere at 37 °C. Aliquots of 1 mL HPTCs suspensions (7.5×10^5 cells/mL) were obtained by trypsinization of the confluent adherent culture, followed by sonication and centrifugation ($400 \times g$, 5 min, 4 °C). The supernatant was then used as the matrix where the standards of cocaine, BE and NCOC were prepared.

2.3. Calibrators and quality control samples

Cocaine, BE and NCOC standards were prepared by dissolving the analytes directly into the supernatant of HPTCs, to yield 5 working calibration solutions (1, 5, 10, 50, and 100 μg/mL). As an internal standard (IS) for quantitative analysis, a stock solution of benzophenone (1 mg/mL) was prepared in acetonitrile.

2.4. Sample preparation for GC/IT-MS analysis

2.4.1. Solid phase extraction (SPE)

The extraction of standards, blanks (supernatant without analytes) and cellular samples were performed in OASIS SPE columns obtained from WATERS (Milford, MA). Samples (total volume, 1 mL) were passed through the columns, and then washed with 2 mL of 0.1 N HCl, followed by 2 mL methanol. Finally, the compounds of interest were eluted into glass tubes using 2 mL of a 5% NH_4OH methanolic solution. The obtained solutions were evaporated to dryness under nitrogen flow.

2.4.2. Derivatization procedure and choice of suitable derivatization temperature

Three different temperatures of silylation, a low (37 °C), a medium (55 °C) and a high (80 °C) temperature were analyzed in order to choose the appropriate one. For each temperature, 3 replicates of supernatant spiked with 250 μg/mL of cocaine were analyzed. Dried residues were submitted to derivatization with 90 μL of MSTFA and 10 μL of the IS at a final concentration of 100 μg/mL, and incubated at the different temperature conditions for 10 min. After cooling to room temperature, the samples (1 μL) were injected into the GC/IT-MS system.

2.5. Chromatographic and detection system conditions

Quantitative GC/IT-MS analysis were performed on a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 ion trap mass detector (USA) and a Saturn GC/IT-MS workstation software version 6.8. Chromatographic separation was achieved using a capillary column VF-5ms (30 m × 0.25 mm × 0.25 μm) from VARIAN and a high purity helium C-60 (Gasin, Portugal) as carrier gas at a constant flow of 0.7 mL/min, in splitless injection mode. One μL of each derivatized sample was injected. An initial oven temperature of 100 °C was held for 1 min, followed by a ramp of 15 °C/min to 300 °C, holding for 10 min. The injection port temperature was maintained at 250 °C. Total chromatographic separation was achieved in 15 min. The ion trap detector was set as follows: the transfer line, manifold, and trap temperatures were 280, 50, and 180 °C, respectively. All mass spectra were acquired in the electron impact mode. Ionization was maintained off during the first 4 min, to avoid solvent overloading. The mass range was 35–600 *m*/*z*, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25,000 μs, with an ionization storage level of 35 *m*/*z*. The analysis was performed in full scan mode. The obtained full scan chromatogram was reprocessed using the following selected qualifier ions: *m*/*z* 105 and *m*/*z* 182 for IS; *m*/*z* 182 and *m*/*z* 303 for cocaine; *m*/*z* 82 and *m*/*z* 240 for BE; and *m*/*z* 140 and *m*/*z* 240 for NCOC.

2.6. Method validation

2.6.1. Selectivity

In order to detect possible interferences from the medium or endogenous components, blank (no analyte or IS added) and standard samples were extracted by SPE, derivatized, and analyzed by GC/IT-MS. The presence or absence of co-eluting peaks at the retention times of the target analytes determined the chromatographic selectivity. The IS solution was also analyzed alone, under equal conditions, with the same purpose.

2.6.2. Linearity

The method linearity was determined by evaluation of the regression curve (ratio of analyte peak area and IS peak area versus analyte concentration) and expressed by the squared correlation coefficient (r^2). All standards of cocaine, BE and NCOC were extracted by SPE, before derivatization in the presence of the IS. The linear range of the method was analyzed by performing calibration curves in 3 days, using 6 different concentration levels of the analytes (0, 1, 5, 10, 50, and 100 μg/mL) and the minimal accepted r^2 for cocaine and its metabolites was 0.99.

2.6.3. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined from calibration curves data, following European Medicines Agency (EMEA) criteria [26]. The LOQ was defined as the lowest concentration of the calibration curve and it was estimated after 3 injections based on a signal-to-noise ratio of 10. A signal-to-noise ratio of 3 was considered acceptable for estimating the LOD [26].

2.6.4. Precision and accuracy

The intra-day precision was determined by preparing and analyzing by GC/IT-MS, on the same day, 3 replicates of 3 different concentrations (1, 10, and 100 μg/mL) of the 3 analytes. The inter-day precision was evaluated by repeating the intra-day precision study in 3 different days, for cocaine and BE, and in 2 different days for NCOC. Precision was assessed by calculating the mean, standard deviation, and coefficient of variation (CV) of those values. The accuracy of the method was determined through the calculation of the percent deviation between the calculated value and the nominal value [27].

2.7. Proof of applicability

2.7.1. Evaluation of cocaine metabolism in HPTCs

To provide proof of applicability for this analytical method, it was used herein to evaluate the metabolism of cocaine in the HPTCs model. At about 80–90% of confluence, HPTCs were detached with trypsin and subcultured in 6-well plates at a density of 5.0×10^4 cells/cm^2. After reaching the confluence, the monolayer was washed with PBS buffer and then incubated with 1 mL of 1 mM cocaine at 37 °C for 72 h. This study was performed in 3 independent experiments. At the end of incubation time, the medium was recovered, the cells were detached, and the total suspension – medium and cells – was sonicated and centrifuged (400 × *g*, 5 min, 4 °C). The obtained supernatant (1 mL) was treated as described in Section 2.4 for subsequent GC/IT-MS analysis.

2.7.2. Cell viability

The cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. HPTCs were exposed to 1 mM cocaine for 72 h in a humidified incubator at 37 °C with an atmosphere of 5% CO_2. The MTT assay was performed as previously described [25], in 3 independent experiments.

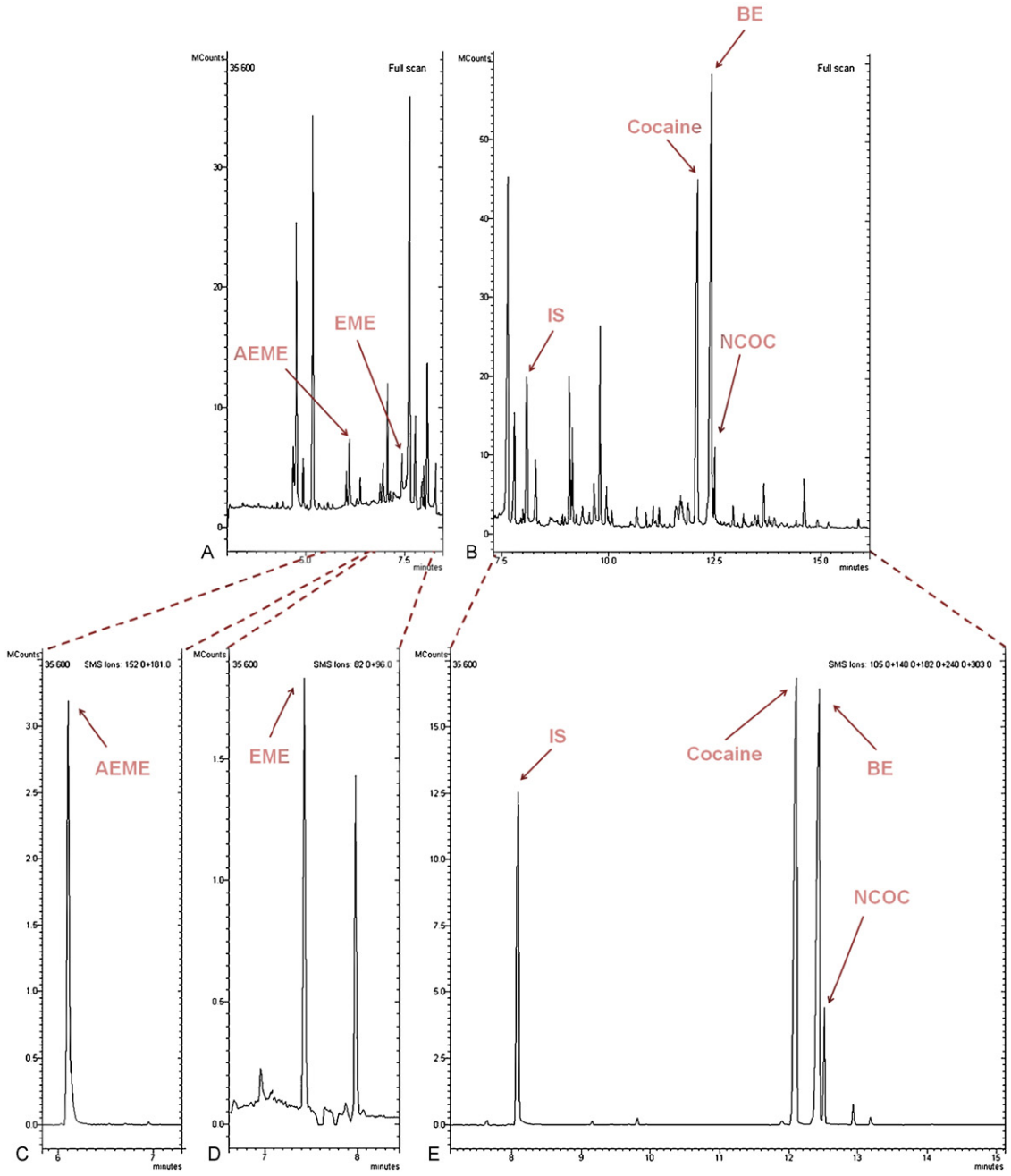

Fig. 2. Representative chromatographic run, from (A) an extract of HPTCs incubated with 1 mM cocaine for 72 h (full scan) and (B) a calibration sample of HPTCs spiked with 50 μg/mL of cocaine, BE and NCOC (full scan). Reconstructed chromatograms with selected ions for (C) AEME (*m*/*z* 152 and *m*/*z* 181), (D) EME (*m*/*z* 82 and *m*/*z* 96), and (E) IS (benzophenone), cocaine, BE and NCOC (*m*/*z* 105, *m*/*z* 140, *m*/*z* 182, *m*/*z* 240, *m*/*z* 303) in HPTCs by GC/IT-MS.

3. Results and discussion

3.1. Method validation

3.1.1. SPE extraction, chromatographic separation and selectivity

The applied SPE procedure allowed the pre-concentration of the target analytes. In spite of the elution of a large number of peaks along the chromatogram (Fig. 2B), most likely from constituent compounds of the incubation medium, no interference peaks were detected at the analytes or IS retention times, confirming the selectivity of the method. The total time of analysis was 15 min. Moreover, the analytes and IS peaks presented a good resolution (Fig. 2E).

3.1.2. Choice of suitable derivatization temperature

In GC analysis, it is known that BE and NCOC need a derivatization step to improve its volatility due to the presence of a polar functional group in its molecular structure [28,29]. As the temperature of incubation for the chosen silylating agent, MSTFA, varies from author to author [30–34], the effectiveness of this derivatization procedure was evaluated using 3 different temperatures: 37, 55, and 80 °C. The main objective was to verify if cocaine chemical stability would be affected by the derivatization temperature. The observed variations were not significant, neither for cocaine, nor for the amount of BE formed along the preparation of the samples, which corresponds to about 2% for all tested temperatures. Furthermore, all 3 conditions generated well resolved chromato-

Table 1
Linear regression analysis of standard solutions in HPTCs matrix for a 0–100 μg/mL concentration range, performed in 3 different days for cocaine and BE, and 2 different days for NCOC.

Analyte	Day	Equation	Range (μg/mL)	r^2
Cocaine	1	$y = 0.0570x - 0.0209$	0–100	0.9999
	2	$y = 0.0652x - 0.0431$	0–100	0.9996
	3	$y = 0.0636x - 0.0441$	0–100	0.9997
BE	1	$y = 0.0363x + 0.0537$	0–100	0.9992
	2	$y = 0.0403x + 0.0353$	0–100	0.9998
	3	$y = 0.0395x + 0.0244$	0–100	0.9999
NCOC	1	$y = 0.0035x - 0.0006$	0–100	0.9999
	2	$y = 0.0046x - 0.0063$	0–100	0.9989

Table 2
Precision and accuracy for the determination of cocaine, BE and NCOC in HPTCs matrix.

Analyte	Concentration (μg/mL)	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)
Cocaine	1	13.5	12.0	107.1
	10	6.0	9.5	94.6
	100	3.6	6.5	100.6
BE	1	6.6	14.9	104.1
	10	10.6	3.2	100.6
	100	8.0	12.2	101.8
NCOC	1	10.2	33.1	111.9
	10	12.6	11.0	92.7
	100	11.6	18.8	104.5

graphic peaks (data not shown). Therefore, in the present work, the lowest temperature (37 °C) was chosen for the derivatization procedure.

3.1.3. Linearity, LOD, and LOQ

The method was linear at the concentration range of 0–100 μg/mL, with correlation coefficients (r^2) greater than 0.99 for the calibration curves of cocaine and both metabolites. Table 1 summarizes the results from the linearity studies. The LOQ and LOD of cocaine, BE and NCOC were estimated following EMEA criteria [26]. The LOD values were 0.97 ± 0.09, 0.40 ± 0.04 and 20.89 ± 1.81 ng/mL for cocaine, BE and NCOC, respectively, while the LOQ values were estimated to be 3.24 ± 0.30, 1.34 ± 0.14 and 69.62 ± 6.05 ng/mL. These LOD values are lower than the ones previously described for chromatographic analysis of these analytes in biological matrices like plasma, blood, saliva and urine, with either SPE or solid phase microextraction (SPME) pre-concentration treatment [35–39].

3.1.4. Precision and accuracy

Precision and accuracy results are presented in Table 2. As the CV (%) values calculated for intra- and inter-day precision studies of cocaine and BE did not exceed 15%, the developed method is considered precise for these two analytes [27]. The exception was NCOC, which presented inter-day precision values over 15%, most likely due to the instability of the salt compound. The accuracy values calculated for all 3 analytes were always within 15% of the nominal value, which means that the method is considered accurate [27].

3.2. Proof of applicability

3.2.1. Evaluation of cocaine metabolism in HPTCs

For proof of applicability, primary cultured HPTCs were exposed to 1 mM cocaine for 72 h at 37 °C in order to study the metabolism of cocaine in those cells. The results for three independent experiments are shown in the Table 3. Previous human pharmacokinetic studies showed that, as it enters the organism, a major part of cocaine dose is converted into BE, EME and ecgonine, and only a minor part originates the *N*-demethylated metabolite NCOC. Moreover, less than 20% of the drug is eliminated in the urine, while the three major metabolites correspond to 80–90% of the urinary metabolites [3,14,40,41]. In agreement, the results obtained in our *in vitro* human kidney cell model show that BE is produced in large amounts, while NCOC represents less than 2% of the initial cocaine concentration. In full scan mode, we were able to detect another two cocaine metabolites, namely AEME (Fig. 2C) and EME (Fig. 2D). Both compounds were identified by mass spectra database search using the National Institute of Standards and Technology (NIST) MS 05 spectral database, and the MS fragmentation of EME was also compared with the one of the pure compound. While EME is a major metabolite of cocaine in the organism, AEME is formed only when the drug is smoked. It has been demonstrated that cocaine thermally degrades during gas chromatography, mainly at high GC injection port temperatures [42]. Therefore, the GC inlet temperature used in our method (250 °C) was most likely responsible for the artifactual pyrolytic production of AEME during GC/IT-MS.

Table 3
Cocaine, BE, and NCOC concentrations in HPTCs exposed to 1 mM cocaine for 72 h.

	Cocaine (μM)	BE (μM)	NCOC (μM)
Concentration	435.5	564.8	17.6
	442.7	487.2	15.4
	459.2	584.2	14.0
Mean	445.8	545.4	15.7
SD	12.2	51.4	1.8

3.2.2. Cell viability

Cell viability, evaluated by the MTT assay, was 79.6 ± 6.4% of control after incubation with 1 mM cocaine for 72 h at 37 °C.

4. Conclusions

The current study presents a valid, highly sensitive and robust GC/IT-MS method for the simultaneous quantification of cocaine and its metabolites BE and NCOC in primary cultured human renal cells. The method proved to be precise and highly accurate, sensi-

tive for a wide linearity range, and presented LOD and LOQ values lower than those published in the literature for other biological matrices. The SPE procedure allowed the pre-concentration of the analytes of interest, and the derivatization process with MSTFA resulted in the elution of well resolved peaks. Furthermore, the total chromatographic run time was 15 min, which is a relatively short time of analysis.

In addition, the present method was further applied to a pharmacokinetic study of cocaine metabolism in HPTCs, allowing the identification and precise quantification of the drug and its metabolites formed in these cells. Therefore, as cocaine metabolites are expected to exhibit different toxic effects in the kidney, the developed and validated GC/IT-MS method presently described can help to elucidate cocaine pharmacokinetic profile and toxicity in this target organ or other organs to which it is likely to apply the method to.

To our best knowledge, this is the first sensitive method described for simultaneous measurement of cocaine and its metabolites by GC/IT-MS in a cellular matrix.

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