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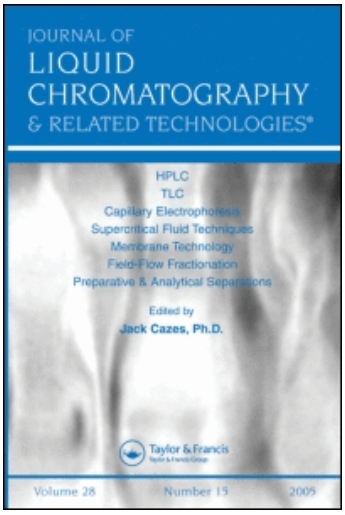

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VALIDATION OF A SOLILD PHASE EXTRACTION PROCEDURE FOR THE GC-MS IDENTIFICATION AND QUANTITATION OF COCAINE AND THREE METABOLITES IN BLOOD, URINE, AND MILK

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VALIDATION OF A SOLID PHASE EXTRACTION PROCEDURE FOR THE GC-MS IDENTIFICATION AND QUANTITATION OF COCAINE AND THREE METABOLITES IN BLOOD, URINE, AND MILK

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ABSTRACT

A simple and widely used solid-phase extraction procedure (United Chemical Technologies Method Handbook) was applied for the GC-MS identification and quantitation of cocaine (COC), benzoylecgonine (BE), cocaethylene (COCE), and *m*-hydroxybenzoylecgonine (HBE) in blood, urine, and milk. The method

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which utilizes BSTFA as a derivatizing agent yielded abundant diagnostic ions with high *m/z* values.

Linear quantitative response curves were generated for the analytes of interest over a concentration range of 5–1000 ng/mL. Linear regression analyses of the standard curve in the three specimen types exhibited correlation coefficients ranging from 0.997 to 1.000. The LOD values for COC, COCE, and derivatives of BE, and HBE in the three specimen types ranged from 2.5 to 5.0 ng/mL.

The LOQ values, however, ranged from 5.0 to 10.0 ng/mL. Intra-assay and inter-assay precision studies reflected a high level of reliability and reproducibility of the method. The applicability of the method for the detection and quantitation of COC, BE, COCE, and HBE was demonstrated successfully in human blood and urine samples, as well as blood samples obtained from cocaine-treated (subcutaneously) rats.

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INTRODUCTION

Cocaine (COC) is one of the most potent of the naturally-occurring central nervous system stimulants. It has been widely utilized in medicine as a local anesthetic (especially in opthalmological procedures) and increasingly by drug abusers for its stimulant properties. During the 1980s and 90s, cocaine abuse increased to epidemic proportions across the United States, and continues to be a major public concern.

Cocaine is rapidly metabolized in man by the hydrolysis of one or both of its ester linkages. At slightly basic pH, the drug is readily hydrolyzed to its primary metabolite benzoylecgonine (BE) (1–6). BE, in turn, is converted to ecgonine (E) by enzymatic hydrolysis, to *m*-hydroxybenzoylecgonine (HBE) by hydroxylation, and to benzoylnorecgonine (BNE) by N-methylation (1–6). In blood, cocaine is also hydrolyzed to ecgonine methyl ester (EME) by cholinesterase (1–6). Cocaethylene (COCE) is a neurologically active compound that provides the same degree of euphoria as COC, but for longer periods of time (7).

As reported by Hearn *et al.* (8) and others (9,10), COCE is a unique metabolite that occurs in blood as a result of concurrent use of cocaine and ethanol. Varying amounts of other metabolic products of cocaine, such as the active metabolite norcocaine (NCOC), and *meta*- hydroxycocaine (HCOC) have also been detected (11).

Because of the adverse health consequences and legal implications of cocaine abuse, it has become increasingly more important for analytical toxicologists to continue to improve upon the methods for the detection and quantitation of cocaine and its metabolites in biological specimens, to provide evidence of cocaine use

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and abuse. A number of methods for the detection and quantitation of cocaine in blood, urine, meconium, and hair, are documented in the literature.

These methods range from thin layer chromatography (TLC) to high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) (12–19). The Gas Chromatograph-Mass Spectrometer is readily available in most pharmaceutical and toxicology laboratories, and its main advantage over other instrumentation used in the laboratories, is that it provides appropriate sensitivity, specificity, and selectivity for the analytes of interest.

This manuscript describes the validation and applicability of a simple and widely used solid-phase extraction procedure (20) for the extraction and GC-MS quantitation of COC, BE, HBE, and COCE in blood, urine, and milk, utilizing only one milliliter of sample. The applicability of the method to quantitate COC, BE, HBE, and COCE was demonstrated successfully in human whole blood specimens, human urine samples, as well as blood samples obtained from rats treated with cocaine subcutaneously.

To our knowledge, this manuscript is the first to describe method parameters and validation for testing COC, BE, HBE, and COCE in milk specimens.

EXPERIMENTAL

Materials

The COC, BE, HBE, and COCE standard materials and deuterated analogs were purchased from Radian Corporation (Austin, TX). Clean Screen extraction columns were purchased from World Wide Monitoring (Bristol, PA). *bis* (Trimethylsilyl) trifluoroacetamide, containing 1% trimethylchlorosilane, was purchased from Pierce Chemical Company (Rockford, IL). Monobasic and dibasic potassium phosphate, hydrochloric acid, methanol, dichloromethane, isopropanol, ammonium hydroxide, and ethyl acetate were purchased from Fisher Scientific (Suwanee, GA) and were of analytical grade or the highest purity available.

Autosampler vials (12 $\times$ 32 mm; clear crimp-top) with 100 μL limited volume inserts were purchased from Alltech Associates Inc. (Deerfield, IL). Water used in this study met Type II water criteria (21) and was filtered through Nanopure System (Barnstead, Dubuque, IA).

Methods

Extraction and Derivatization

Extraction and derivatization procedures were conducted essentially as described (20). Samples (1 mL) were spiked with 50 ng of deuterated (D_3) internal

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standards, corresponding to the analytes of interest. Then, 4 mL of deionized water were added, and blood samples were centrifuged at 2000 rpm for 10 min. Pellets were discarded and 2 mL of 100 mM potassium phosphate buffer was added to all specimen types and the pH was adjusted to 6–6.5.

The samples were then loaded on the extraction columns which had been pre-equilibrated by sequential treatment with 2 mL of methanol, 2 mL of deionized water, and 3 mL of 100 mM potassium phosphate buffer. After the samples passed through the bed of the columns, the columns were sequentially washed with 2 mL deionized water, 2 mL of 100 mM HCl, and 3 mL of methanol.

COC, BE, HBE, and COCE were eluted with 3 mL of freshly prepared elution mixture composed of dichloromethane, isopropyl alcohol, and ammonium hydroxide (78/20/2,v/v/v). Eluates were dried at room temperature under a continuous stream of nitrogen. To ensure complete removal of water, 0.5 mL dichloromethane was added to the residue and evaporated to dryness.

The analyte mixture was derivatized by the addition of 50 μL ethyl acetate and 50 μL BSTFA containing 1% TMCS. After 30 min incubation at 65°C, samples (2 μL) were injected onto the autosampler of an HP 5890 gas chromatograph (GC) equipped with a 5972 mass selective detector (MS) [Hewlett Packard (currently Agilent Technologies), Palo Alto, CA].

For the construction of standard curves, blank blood, urine, and milk samples were spiked with 50 ng internal standards and increasing concentrations of analytes (5, 50, 100, 500, and 1000 ng/mL).

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GC-MS Run Conditions

Samples (2 μL) were injected from the autosample vials into the GC-MS. The analytes were resolved with an HP 1MS capillary column crosslinked with 1% phenylmethylsilicone (15 m $\times$ 0.25 mm with 0.25•m film thickness) using inlet pressure programming. The electron multiplier was operated at 200 V above the tune value. The carrier gas used was ultra high purity helium (99.99%). Splitless injection was used, and the splitless valve remained closed for 1 min and the initial inlet pressure was 25 psi.

The column head pressure was held for 0.5 min, then decreased to 16 psi at a rate of 25 psi/min, and, finally, maintained at this pressure for the duration of the analysis time. This resulted in a flow rate of 1.1 mL/min during the run. Injector temperature was 280°C. The initial oven temperature was 150°C and was held for 1 min; the temperature was programmed to increase at 15°C/min to 215°C, and then increased to 300°C at 35°C/min, and held there for 2 min.

The transfer line temperature was held at 280°C. Selected ion monitoring (SIM) mode was used in all runs. The entire run time was less than 8.0 min.

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Selection of Ions

The quantitation and qualifier ions for this study were selected by examining the full scan mass spectra after injecting approximately 100 ng of each analyte and its deuterated analog or their TMS derivatives, as appropriate, into the HP 5970 MSD. The mass spectra of the analytes and their deuterated analogs were compared and ion pairs were selected.

Selections were based on the following criteria with decreasing order of importance: (**A**) The corresponding ion from the analog must not occur in the analyte, and the corresponding ion from the analyte must not occur in the analog. (**B**) The most abundant ions were selected. (**C**) The largest *m/z* ions were selected. Once the most abundant ions were selected for each analyte, their exact masses were determined periodically to ensure that the target ions remained the same.

RESULTS AND DISCUSSION

Solid-phase extraction has several advantages over liquid-liquid extraction. These advantages are: decreased solvent volumes, resulting in decreased solvent disposal costs; reduced operator time; high percent recovery of analytes; and low limits of detection and quantitation for the analytes (22). Additionally, solid-phase extraction yields clean extracts and minimizes the appearance of endogenous peaks, thereby increasing analyte selectivity (23).

The major ion peaks selected are shown in Table 1, and they are in agreement with previous reports (2,20). As shown in Figure 1, COC, BE, HBE, and COCE are easily separated under the above-described GC-MS conditions. The retention times for the four analytes ranged from 5.74 to 7.12 min (Table 1). The calibration curves obtained for each analyte were constructed using linear regression analysis (24). For COC, BE, and COCE in blood, urine, and milk, linearity was achieved

Table 1. Retention Times and Quantitative and Qualitative Ions Monitored for COC, COCE, and Derivatized BE and HBE

Analyte	Retention Time (min)	Quantitative Ion (m/z)	Qualitative Ion (m/z)	Qualitative Ion (m/z)
COC	5.74	182	198	303
TMS-BE	6.08	240	256	361
TMS-HBE	7.12	240	210	449
COCE	5.99	196	82	317

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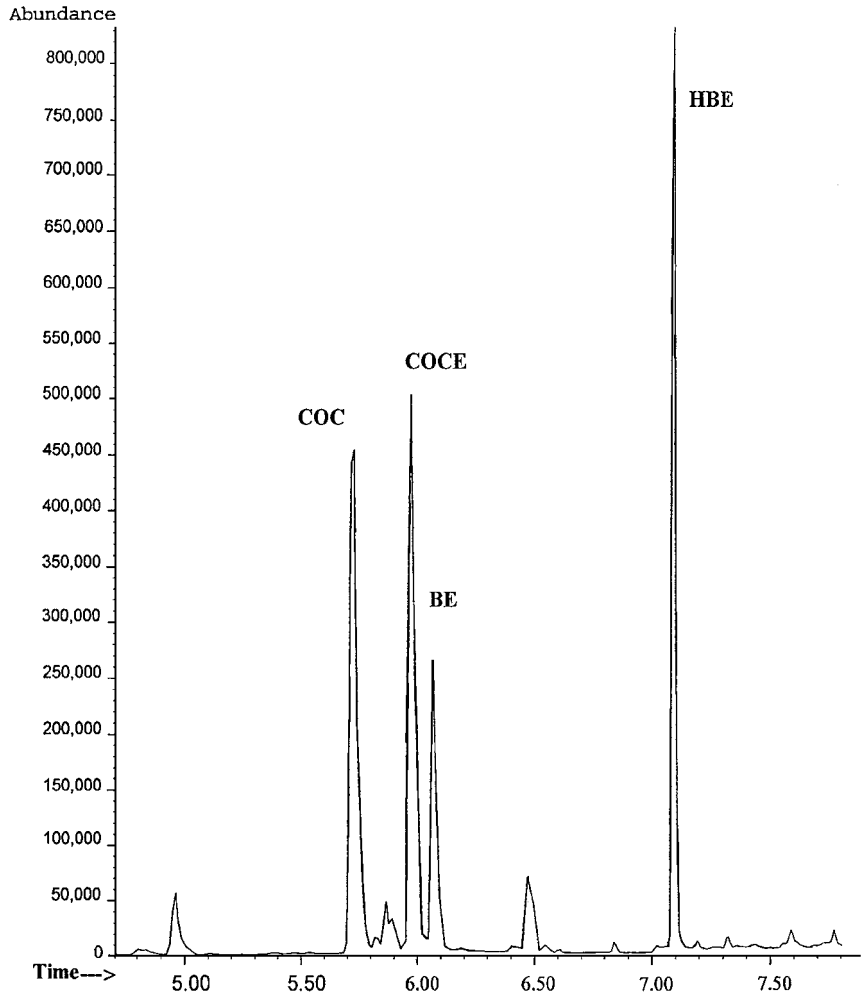

Figure 1. GC-MS Total Ion Chromatogram of 500 ng/mL COC, BE, HBE, and COCE.

over a concentration range of 5 to 1000 ng/mL, whereas linearity for HBE in all specimen types (blood, urine, and milk) ranged from 10 to 1000 ng/mL.

The data, upon the analysis of the corresponding regression lines (24), exhibited correlation coefficients ranging from 0.999 to 1.0 for blood, 0.998 to 0.999 for urine, and 0.997 to 0.999 for milk (Table 2). The formulas of the lines for each analyte in the three specimen types are also shown in Table 2. The limits of detection (LOD) and limits of quantitation (LOQ) for each analyte in each specimen type were determined experimentally (N = 5).

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Table 2. Analysis of the Regression Line for Analytes in Blood, Urine, and Milk

Analyte	Category	Blood	Urine	Milk
COC	RR*	$1.70 \times AR^{¶} + 4.86 \times 10^{-2}$	$0.954 \times AR + 1.39 \times 10^{-2}$	$1.12 \times AR + 8.09 \times 10^{-3}$
	r^{2**}	0.999	0.999	0.999
	Linearity (ng/mL)	5–1000	5–1000	5–1000
TMS-BE	RR	$0.85 \times AR + 8.69 \times 10^{-2}$	$1.08 \times AR + 5.01 \times 10^{-2}$	$1.04 \times AR + 6.23 \times 10^{-2}$
	r^2	0.999	0.998	0.999
	Linearity (ng/mL)	5–1000	5–1000	5–1000
TMS-HBE	RR	$1.70 \times AR + 4.9 \times 10^{-2}$	$2.62 \times AR + 9 \times 10^{-2}$	$2.41 \times AR + 9.8 \times 10^{-1}$
	r^2	1.000	0.999	0.997
	Linearity (ng/mL)	10–1000	10–1000	10–1000
COCE	RR	$0.896 \times AR + 3.1 \times 10^{-2}$	$1.08 \times AR + 1.97 \times 10^{-2}$	$8.0 \times AR + 1.39 \times 10^{-2}$
	r^2	1.000	0.999	0.999
	Linearity (ng/mL)	5–1000	5–1000	5–1000

*RR = Response Ratio; Area of Analyte/Area of Internal Standard.
¶AR = Amount Ratio; Concentration of Analyte/Concentration of Internal Standard.
**r^2 = correlation coefficient.

For LOQ, blank specimens were spiked with a series of decreasing concentrations of analytes and a constant concentration (50 ng/mL) of their corresponding internal standards. LOD was defined as the concentration corresponding to a signal to noise ratio of 3. LOQ was defined as the lowest quantitated concentration that was within 20% of the target concentration. As shown in Table 3, the LOD values obtained for COC, BE, HBE, and COCE extracted from blood were 2.5, 2.5, 4.0, and 2.5 ng/mL, respectively. These values were comparable to those obtained for the same analytes extracted from urine or milk.

The LOQ values obtained for COC, BE, HBE, and COCE extracted from blood were 5.0, 5.0, 8.0, and 5.0 ng/mL, respectively. Similarly, LOQ values obtained for COC, BE, HBE, and COCE extracted from urine or milk ranged from 5.0 to 10 ng/mL (Table 3).

Table 4 illustrates the extraction efficiency of various analytes at three concentrations (10, 500, and 1000 ng/mL) in blood, urine, and milk. These three concentration points represent the low, middle, and high portions of the standard

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Table 3. Limits of Detection and Quantitation, and Percent Recovery of Analytes in Blood, Urine, and Milk

	Blood**		Urine**		Milk**	
Analyte	LOD*	LOQ¶	LOD	LOQ	LOD	LOQ
COC	2.5	5.0	2.5	5.0	2.5	5.0
TMS-BE	2.5	5.0	2.5	5.0	2.5	5.0
TMS-HBE	4.0	8.0	5.0	10.0	5.0	10.0
COCE	2.5	5.0	2.5	5.0	2.5	5.0

**n = 5.
*LOD = Limit of Detection, defined as the concentration of analyte at which mean signal to noise ratio is 3:1.
¶LOQ = Limit of Quantitation, defined as the lowest standard that was within 20% of the target concentration concentration of the analyte.

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curve. With the exception of HBE, extraction efficiencies (recoveries) of all analytes from the three biological matrices (blood, urine, and milk) were comparable (Table 4). At a low concentration (10 ng/mL), percent recoveries for COC, BE, and COCE ranged from 90.9 to 94.1%, whereas recoveries for HBE in the three matrices ranged from 80.4 to 86.6% (Table 4). At 500 ng/mL, the highest percent recovery achieved was for COC (98.2%), and the lowest recovery was for HBE (81.2%). At a concentration of 1000 ng/mL, recoveries of COC, BE, and COCE ranged from 92.7 to 97.8%, whereas recoveries for HBE ranged from 82.6 to 87.8% (Table 4).

Generally, recoveries of HBE from blood, urine, and milk at the three concentration levels (10, 500, and 1000 ng/mL) were less than those observed with the other analytes (Table 4). This suggests that HBE either did not extract as efficiently as the other analytes, or underwent stereochemical configuration with

Table 4. Percent Recoveries of Analytes in at Three Concentrations (ng/mL) in Blood, Urine, and Milk

	Blood*			Urine*			Milk*		
Analyte	10	500	1000	10	500	1000	10	500	1000
COC	92.8	98.2	96.6	91.9	96.1	97.2	90.9	95.8	96.4
TMS-BE	94.1	97.6	97.8	93.5	95.6	96.1	93.3	NA¶	93.5
TMS-HBE	82.8	83.5	87.8	80.4	81.2	82.6	86.6	87.9	84.8
COCE	92.1	94.0	94.8	93.3	97.1	94.6	91.9	93.2	92.7

*n = 5.
¶NA = Not Analyzed.

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a different spatial configuration. Whether HBE was converted from the *meta*-hydroxy configuration to *para*-hydroxy configuration, or not, is currently under intensive investigation in our laboratory. Preliminary data (not shown) suggest that conversion of the *meta*-hydroxy configuration of HBE to *para*-hydroxy configuration (*para*-HBE) did not occur during the extraction or the GC-MS run conditions for there was no evidence for the occurrence of the m/z 82, usually abundant in the *para*-HBE mass spectrum (2).

Intra- and inter-assay precision of the analytical procedure in blood, urine, and milk, as represented by percent correlation of variance (% C.V.), is illustrated in Table 5 (A,B,C). Precision was determined experimentally (n = 5) by spiking negative blood, urine, and milk samples with COC, BE, HBE, and COCE at concentrations of 10, 500, and 1000 ng/mL. For all the analytes, at concentrations of 500 and 1000 ng/mL, intra-assay precision (% C.V.) values were less than 10 (Table 5 A,B,C). At a concentration near the limit of quantitation (10 ng/mL), % C.V. values reflected a greater variation of precision for COC, BE, HBE, and COCE which ranged between 2.3 to 14.1 (Table 5 A,B,C).

Inter-assay precision of the method was determined experimentally in a manner similar to that of intra-assay precision. Spiked blood, urine, and milk samples were analyzed on a daily basis for two weeks. With the exception of HBE, inter-assay precision ranged from 2.6 to 12.

Table 5. Intra- and Inter-Assay Precision for Cocaine (COC), Cocaethylene (COCE), Derivatized Benzoylecgonine (TMS-BE), and Derivatized *m*-Hydroxybenzoylecgonine (TMS-HBE) in (A) Blood, (B) Urine, and (C) Milk

Target Conc. (ng/mL)	Analyte	Intra-Assay (mean conc)*	Precision (% C.V.)	Inter-Assay (mean conc.)*	Precision (% C.V.)
A. Blood					
10	COC	9.3	2.3	9.1	5.5
	TMS-BE	9.4	2.8	9.2	4.3
	TMS-HBE	8.8	14.1	7.9	18.3
	COCE	9.6	7.4	9.3	8.7
500	COC	491	2.2	472	3.1
	TMS-BE	488	2.1	465	2.6
	TMS-HBE	416	9.8	381	11.6
	COCE	470	4.3	411	6.7
1000	COC	966	3.4	921	7.4
	TMS-BE	978	5.2	908	8.8
	TMS-HBE	878	9.9	816	12.9
	COCE	948	4.6	919	7.1

(continued)

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Table 5. Continued

Target Conc. (ng/mL)	Analyte	Intra-Assay (mean conc)*	Precision (% C.V.)	Inter-Assay (mean conc.)*	Precision (% C.V.)
B. Urine					
10	COC	9.2	4.3	9.1	8.9
	TMS-BE	NA¶	—	NA¶	—
	TMS-HBE	8.0	12.3	7.6	14.9
	COCE	9.3	6.7	8.9	12.0
500	COC	481	4.1	448	7.9
	TMS-BE	478	4.9	449	8.3
	TMS-HBE	406	8.8	373	13.1
	COCE	NA¶	—	NA¶	—
1000	COC	972	4.2	921	9.9
	TMS-BE	961	4.7	908	9.8
	TMS-HBE	826	9.3	781	12.5
	COCE	946	5.2	889	6.4
C. Milk					
10	COC	8.7	5.1	9.0	8.9
	TMS-BE	9.3	4.7	9.1	7.6
	TMS-HBE	9.0	12.4	7.9	15.7
	COCE	9.5	6.1	8.6	9.8
500	COC	479	5.3	427	8.4
	TMS-BE	481	6.1	428	9.3
	TMS-HBE	440	9.0	383	10.8
	COCE	466	4.5	461	9.7
1000	COC	964	5.1	NA¶	—
	TMS-BE	935	5.2	931	7.8
	TMS-HBE	848	9.7	801	11.2
	COCE	927	6.0	902	10.0

*n = 5.
¶NA = Not Analyzed.

The coefficients of variation (% CV) for HBE in all specimen types were higher than those obtained for COC, BE, and COCE (Table 5). This may be due to the lower abundance of the quantitation ion of TMS-HBE and the relatively lower recovery. The higher % C.V. values observed with the 10.0 ng/mL samples are because this concentration is near the limit of quantitation where greater variation should be expected.

The applicability of this validated method was tested on human and rat blood samples, as well as on human urine samples obtained from alcohol and/or

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Table 6. Analysis of Rat Blood for COC, BE, HBE, and COCE

Specimen I.D.	Analyte	Concentration (ng/mL)
1	COCE	<LOQ*
	BE	0.00
	HBE	0.00
	COC	34.8
2	COCE	11.4
	BE	113.6
	HBE	0.00
	COC	28
3	COCE	<LOQ*
	BE	0.00
	HBE	0.00
	COC	31
4	COCE	<LOQ*
	BE	0.00
	HBE	0.00
	COC	20.0

*<LOQ = the concentration was less than the limit of quantitation.

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cocaine-abusing individuals. Unfortunately, we were unable to obtain human milk samples from cocaine-abusing nursing mothers. As shown in Table 6, four control rat blood samples obtained from rats not treated with cocaine did not reveal any presence of cocaine analytes.

On the other hand, analysis of samples obtained from rats treated with cocaine (10 μg/kg) subcutaneously revealed the presence of COC and BE. Because the rats were not administered alcohol concomitantly with cocaine, cocaethylene, a metabolite produced as a result of a concomitant administration of cocaine and alcohol (7), was not detected. HBE was detected in rat blood samples spiked with the compound. The reason for the absence of HBE in samples obtained from cocaine-treated rats may have been due to the fact that blood samples were drawn one week (longer than the half-lives of most cocaine metabolites including HBE) after cocaine treatment. Another possibility is the possible inability of rats to form HBE.

Comprehensive literature searches conducted at the time of submitting this manuscript failed to produce any literature on the presence of HBE in rats.

As shown in Table 7, all five human urine samples tested contained all four cocaine analytes in various concentrations. The corresponding blood samples, however, contained BE and traces of HBE. This was not surprising for COC's half-life is significantly shorter than BE.

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Table 7. Analysis of Human Blood and Urine Samples for COC, BE, HBE, and COCE

Specimen I.D.	Analyte	Blood Concentration (ng/mL)	Urine Concentration (ng/mL)
	COCE	18.4	11.1
1	BE	921	801
	OH-BE	218	0.4*
	COC	1930	1272
	COCE	49	210
2	BE	714	998
	OH-BE	78	13
	COC	557	719
	COCE	171	21
3	BE	391	433
	OH-BE	30	2.9*
	COC	57	44
	COCE	7	13
4	BE	914	2013
	OH-BE	111	17
	COC	257	57
	COCE	34	66
5	BE	502	871
	OH-BE	49	152
	COC	53	128

* = less than the limit of quantitation.

Oyler et al. (2) have shown that both the *p*- and *m*-HBE are excreted in adult urine. The presence of HBE in adult urine and blood (trace amounts) indicates that our data further supports Oyler et al. (2) findings that *p*- and *m*-HBE are not unique fetal metabolites of COC as initially reported by other investigators (25).

In conclusion, a practical and reliable analytical method for the detection and quantitation of COC, BE, HBE, and COCE is validated. The applicability of the method to extract COC, BE, HBE, and COCE from blood, urine, and milk specimens was demonstrated successfully. The method can be utilized for the detection of these cocaine analytes in a research setting, as well as in forensic drug testing laboratories.

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