Simultaneous GC–MS Analysis of *meta*- and *para*-Hydroxybenzoylecgonine and Norbenzoylecgonine: A Secondary Method to Corroborate Cocaine Ingestion Using Nonhydrolytic Metabolites*

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Abstract

Positive benzoylecgonine (BZE) urinalysis results are sometimes challenged in legal and administrative proceedings on the grounds that the presence of BZE is due to the addition of cocaine to the urine sample with subsequent in vitro hydrolysis to BZE. Consequently, counsel for the respondent or defendant may move that an ecgonine methyl ester (EME) analysis be preformed because EME is presumed to be solely an in vivo cocaine metabolite. For these reasons, a sensitive and rapid gas chromatographic–mass spectrometric procedure was developed for the simultaneous analysis of *m*-hydroxybenzoylecgonine (*m*-OHBZE), *p*-hydroxybenzoylecgonine (*p*-OHBZE), and *N*-desmethyl benzoyl ecgonine (norBZE), all of which are cocaine metabolites believed to arise exclusively via in vivo metabolism. Analysis of human urine specimens previously reported positive for BZE using GC–MS at the Department of Defense cutoff of 100 ng/mL demonstrated that at least one of the three metabolites was present in 79 of the 82 specimens studied (96.3%). Thus, the simultaneous analysis of *m*-OHBZE, *p*-OHBZE, and norBZE could be used to substantiate that the presence of BZE in urine specimens is the result of cocaine ingestion. Additionally, the premise that EME is a "true" in vivo cocaine metabolite was investigated by assessing the stability of cocaine in unpreserved urine samples at several pHs ranging from 5.0 to 9.0.

Introduction

The human body converts cocaine to benzoylecgonine (BZE) via chemical hydrolysis under neutral and alkaline conditions and to ecgonine methyl ester (EME) by plasma and liver esterase mediated biotransformation (1–5) (Figure 1). Because the urinary elimination half-life of BZE (4.5 h) is greater than that of EME (3.1 h) (6), many urinalysis workplace drug-testing laboratories use quantitative gas chromatography–mass spectrometry (GC–MS) methods that target BZE to indicate the use of cocaine. However, challenges occasionally arise to the validity of BZE-positive urinalysis results on the grounds that BZE is not exclusively an in vivo metabolic product. Defense lawyers may move that the court order an EME analysis because this metabolite is presumed to be solely an in vivo metabolite resulting from hepatic enzymatic conversion. Thus, the presence of EME is alleged to be proof that a positive urinalysis test for cocaine is the result of ingestion of, and not contamination of, the urine sample with cocaine. The order for an EME analysis is problematic for two reasons. First, there is, to the

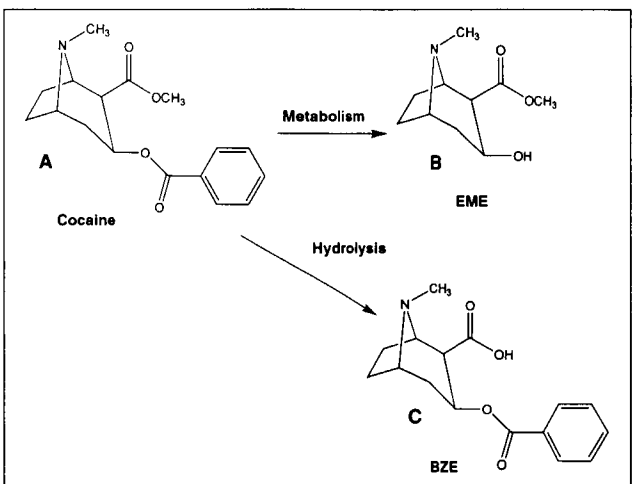

Figure 1. Metabolism of cocaine (A) to EME (B) and BZE (C).

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authors' knowledge, no published evidence that supports the notion that cocaine cannot be chemically hydrolyzed to EME. In contrast, 11.2% of cocaine (100,000 ng/mL) spiked into an aqueous buffered solution at pH 8.0 was chemically hydrolyzed to EME in as little as 8 h (7). Second, the detection of EME is not an easy task because EME has a shorter half-life and it is further spontaneously hydrolyzed to ecgonine (8). Thus, in workplace drug-testing specimens that contain low levels of BZE, it is unlikely that EME will be detected.

Although there have been several reports of other cocaine metabolites that arise from in vivo enzymatic processes, namely *n*-desmethylbenzoylecgonine (norBZE) and *para*-hydroxybenzoylecgonine (*p*-OHBZE), found in urine (9), meconium (10,11), and other human matrices (12), only one study (13) suggests a GC–MS analytical method using *meta*-hydroxybenzoylecgonine (*m*-OHBZE) as a potential marker for cocaine ingestion.

Recently, El Sohly et al. (13) concluded that the analysis of urine specimens for EME and/or *m*-OHBZE may be used to prove that the presence of BZE is the result of cocaine ingestion. However, only 71% (17/24) of the specimens contained *m*-OHBZE at a level that exceeded the 5.0 ng/mL limit of quantitation (LOQ).

In this study, we present a GC–MS method for the simultaneous analysis of three cocaine metabolites believed to arise exclusively via biotransformation (Figure 2). This method can be used to confirm that the presence of BZE in urine specimens is due to the ingestion of cocaine. Additionally, data that demonstrate that cocaine can be chemically hydrolyzed under alkaline conditions to both BZE and EME but not to norBZE, *m*-OHBZE, or *p*-OHBZE are presented.

Materials and Methods

Chemicals and reagents

All solvents and reagents were analytical or high-performance liquid chromatography (HPLC) grade. Cocaine HCl was purchased from Merck (Rahway, NJ). BZE, BZE-d_3, EME, EME-d_3, norBZE, *m*-OHBZE, and *p*-OHBZE were purchased from Radian (Austin, TX). *m*-Hydroxybenzoylecgonine-d_3 (*m*-OHBZE-d_3), used as an internal standard (IS), was obtained from ElSohly Laboratories (Oxford, MS). The derivatizing reagents, tetramethylammonium hydroxide (TMAH), 24% in methanol, and trimethylphenylammonium hydroxide (TMPAH), 0.1M in methanol, were obtained from Fluka (Milwaukee, WI), and dimethyl sulfoxide (DMSO) was purchased from EM Science (Gibbstown, NJ). Pyridine was purchased from Baker (Phillipsburg, NJ), and iodopropane and acetic anhydride were purchased from Aldrich (Milwaukee, WI). Certified drug-free, negative urine, preserved with sodium azide (0.1%, w/v) and purchased from Utak (Valencia, CA), was used to prepare standards and controls.

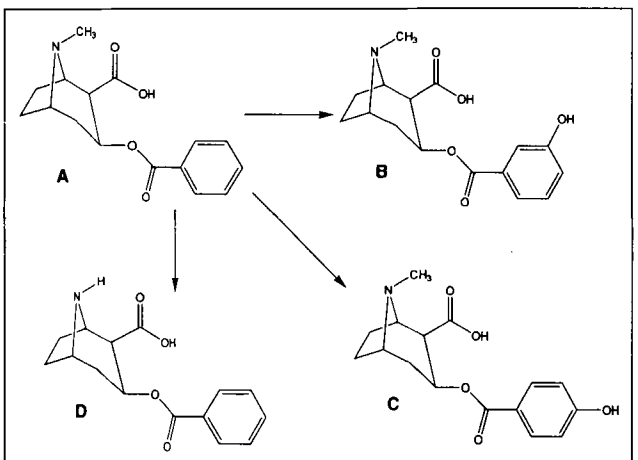

Figure 2. Metabolism of BZE (A) to *m*-OHBZE (B), *p*-OHBZE (C), and norBZE (D).

Forensic specimens

Eighty-nine urine specimens that had been collected under forensic conditions from active-duty United States Navy and Marine Corps personnel and subsequently tested positive for BZE using the Roche Online Kinetic Interaction of Microparticles in a Solution (KIMS) immunoassay at a cutoff concentration of 150 ng/mL were used for the study. Eighty-two of these specimens contained BZE at a concentration above the Department of Defense (DOD) GC–MS cutoff level of 100 ng/mL, and seven specimens contained BZE below the GC–MS cutoff but above the limit of detection (LOD).

Stability study preparation

Aliquots from urine determined to be negative for cocaine, BZE, and EME by GC–MS were pooled for the cocaine stability study. The pooled urine (300 mL) was spiked with 155,000 ng/mL of cocaine HCl. Full scan analysis of the cocaine HCl standard by GC–MS revealed no detectable amounts of norcocaine, BZE, EME, *m*-OHBZE, *p*-OHBZE, and norBZE. The resulting solution was divided into four 70-mL aliquots and the pH adjusted using acetic acid or potassium hydroxide to 5.0, 7.0, 8.0, and 9.0. Three-milliliter aliquots at each pH and time period (0, 3, 6, 12, 24, 48, and 72 h) were extracted and analyzed in triplicate by GC–MS as described in the Methods section.

Extraction procedure for BZE, EME, norBZE, *m*-OHBZE, and *p*-OHBZE

To 3-mL aliquots of each specimen or control was added 75 µL of a working internal standard, which resulted in an analytical concentration of 1000 ng/mL BZE-d_3 and 25 ng/mL of *m*-OHBZE-d_3. After the addition of 1.0 mL of 0.05M phosphate buffer (pH 6.0), the aliquots were loaded onto XTRX RP/W (Creative Technologies Systems) cross-linked styrene-divinyl benzene resin extraction cartridges. After the urine aliquots had passed through, the cartridges were washed with deionized water and the BZE and metabolites subsequently eluted with 3 mL of ethyl acetate/acetone (50:50, v/v). The resulting extracts were evaporated to dryness under a stream of nitrogen in a water bath at 55°C and then derivatized. Following the addition of 0.20 mL of base (TMAH/TMPAH/DMSO, 1:20:200) and vortex mixing, 0.02 mL of iodopropane was added and the tubes were again vortex mixed and heated at 55°C for 10 min. Two milliliters of 0.3M sulfuric acid and 3 mL of ethyl acetate were added followed by vortex mixing and centrifuging at 3000 rpm. The organic phase was removed by aspiration, and 0.4 mL of 1.0M NaOH, 1 mL of 1.5M carbonate buffer (pH 9.5), and 2.5

mL iso-octane were added. The tubes were vortex mixed and centrifuged, and the bottom aqueous layer was frozen in a dry ice/isopropanol bath. The upper layer was decanted and evaporated to dryness under nitrogen gas in a water bath at 55°C. The preceding procedure produced the *n*-propyl esters of the carboxylic acid moieties and the *n*-propyl ethers of the *meta* and *para* hydroxyl moieties. The specimens were then acetylated with acetic anhydride to form the *N*-acetyl (norBZE) and *O*-acetyl (EME) derivatives by adding 50 mL each of acetic anhydride and pyridine to each sample and heating at 70°C for 30 min. The specimens were then evaporated to dryness under nitrogen gas in a water bath at 55°C and reconstituted with 30 mL of ethyl acetate for GC–MS analysis.

GC–MS procedure for norBZE, *m*-OHBZE, and *p*-OHBZE

A Hewlett-Packard (HP) (Palo Alto, CA) 6890 GC coupled to a 5973 mass selective detector (MSD) operated in the electron-impact selected ion mode (SIM) was used for the analysis of BZE, norBZE, *m*-OHBZE, and *p*-OHBZE. Helium was the carrier gas flowing at 15.7 mL/min. The injector temperature was 280°C, and the transfer line was maintained at 280°C. An HP-5MS capillary column (15 m × 0.25-mm i.d., 0.25-µm film thickness) provided analytical separation. The oven temperature was maintained at 207°C for 7.0 min, ramped to 290°C at 20°C/min, and then held for 1 min. The analysis of BZE was conducted as described here previously except that the oven temperature remained under isothermal condition (207°C) for a total run time of 10.5 min.

GC–MS procedure for EME

An HP 6890 GC coupled to a 5973 MSD operated in the electron-impact SIM mode was used for the analysis of EME. Helium was the carrier gas flowing at 15.4 mL/min. The injector temperature was 280°C, and the transfer line was maintained at 280°C. An HP-5MS capillary column (15 m × 0.25-mm i.d., 0.25-µm film thickness) provided the analytical separation. The oven temperature program was maintained at 140°C for 3.0 min, ramped to 240°C at 20°C/min, and then held for 1 min.

Results

BZE analysis

Forensic identification of the cocaine metabolites was accomplished using retention time match and qualifying product ion ratios. Identification by retention time was considered acceptable if the specimens and controls exhibited retention times within ± 2% of the calibration standard. The analysis of BZE occurred independently from the analysis of norBZE, *m*-OHBZE, and *p*-OHBZE because of the high concentrations of BZE relative to the other metabolites. The total ion chromatogram (TIC) of a specimen containing BZE is shown in Figure 3A. The retention time of BZE and BZE-d_3 (4.1 min) was within ± 2% of the calibration standard in all runs.

When analyzed by GC–MS in electron-impact SIM, BZE produced the mass fragmentation spectra illustrated in Figure 3B. The ions chosen for monitoring were *m/z* 331, 272, and 210 for BZE and *m/z* 334 and 213 for BZE-d_3. The mass spectra for BZE observed in the actual specimens were the same as those observed in standards containing BZE (data not shown).

The quantitation ratio (BZE/BZE-d_3) was *m/z* 210/213, and the qualifiers for identification for BZE were the *m/z* 272/210 and *m/z* 331/210 ratios. The qualifier ion ratio for the internal standard, BZE-d_3, was *m/z* 334/213. BZE concentrations for specimens and controls were determined by single-point calibration against the 100-ng/mL BZE calibrator using the *m/z* 210/213 BZE/BZE-d_3 ion ratio. The spiked-urine BZE control concentrations were 0, 50, 200, and 400 ng/mL. Quantitative results were within ± 20% of the expected theoretical concentration for all runs, and the assay was linear (r^2 = 0.9994) from 50 to 400 ng/mL. Additionally, the limit of linearity (LOL) of the BZE assay (r^2 = 0.9993) was evaluated at concentrations ranging from 500 to 20,000 ng/mL for highly concentrated specimens resulting from the cocaine stability study.

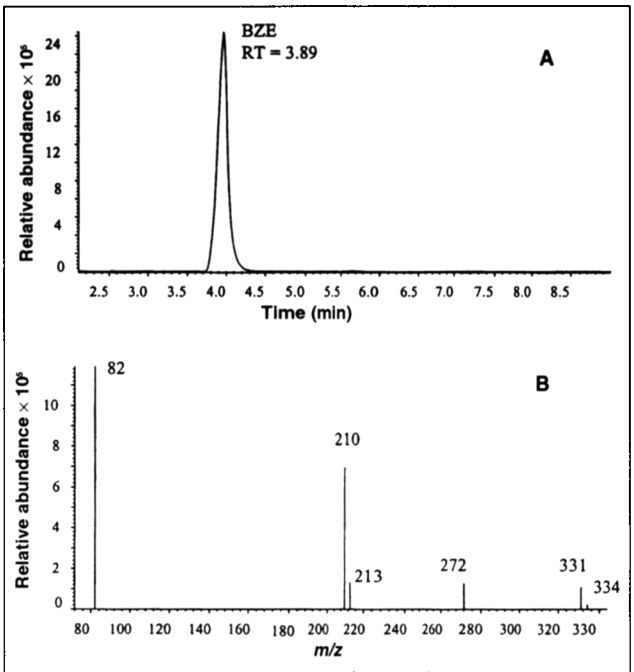

Figure 3. Total ion chromatogram (TIC) of an actual specimen (# 86) containing BZE (A) and the mass spectrum of BZE found in the specimen as analyzed by GC–MS (B).

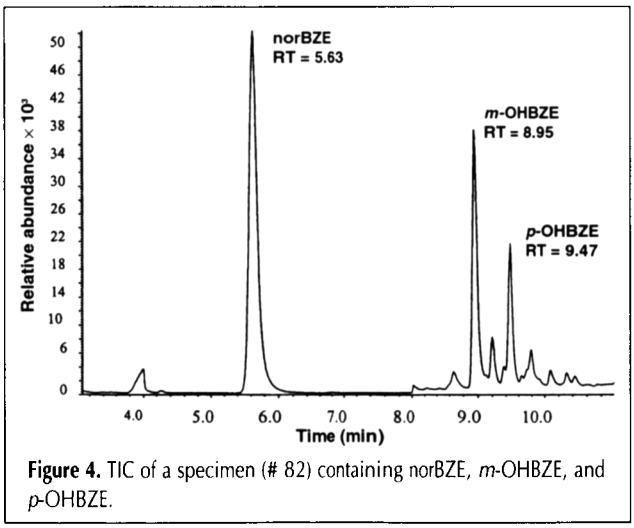

Figure 4. TIC of a specimen (# 82) containing norBZE, *m*-OHBZE, and *p*-OHBZE.

NorBZE, *m*-OHBZE, and *p*-OHBZE analysis

The total ion chromatogram of a specimen containing norBZE, *m*-OHBZE, and *p*-OHBZE is shown in Figure 4. NorBZE, *m*-OHBZE and *m*-OHBZE-d_3, and *p*-OHBZE were detected at retention times of 5.6, 9.0, and 9.5 min, respectively. The retention times for all three metabolites were within ± 2% of the calibration standard.

When analyzed by GC–MS in the electron impact SIM, the metabolites resulted in the mass fragmentation spectra illustrated in Figures 5A–5C. Unlike BZE and EME, the *m/z* 210 ion (the most prevalent ion for both *m*- and *p*-OHBZE) was not used for quantitation or as a qualifier ion for *m*-OHBZE and *p*-OHBZE. This was because interferences were observed around the retention time for these metabolites in the specimens. The ions chosen for monitoring norBZE were the prominent ions at *m/z* 238, 330, and 359. The ions for *m*-OHBZE and *p*-OHBZE were *m/z* 389, 330, and 226. The mass spectra for the respective metabolites observed in the actual specimen were the same as those observed in standards containing norBZE, *m*-OHBZE, and *p*-OHBZE (data not shown).

Identification of norBZE, *m*-OHBZE, and *p*-OHBZE was accomplished in a manner similar to that described previously for BZE. The qualifying product ion ratios for norBZE were *m/z* 330/238 and *m/z* 359/238, and the qualifiers for identification for *m*-OHBZE and *p*-OHBZE were *m/z* 226/389 and *m/z* 330/389 ratios. All qualifying identity ratios for specimens and controls with analyte concentrations above the assays LOQ were within ± 20% of the calibration standard.

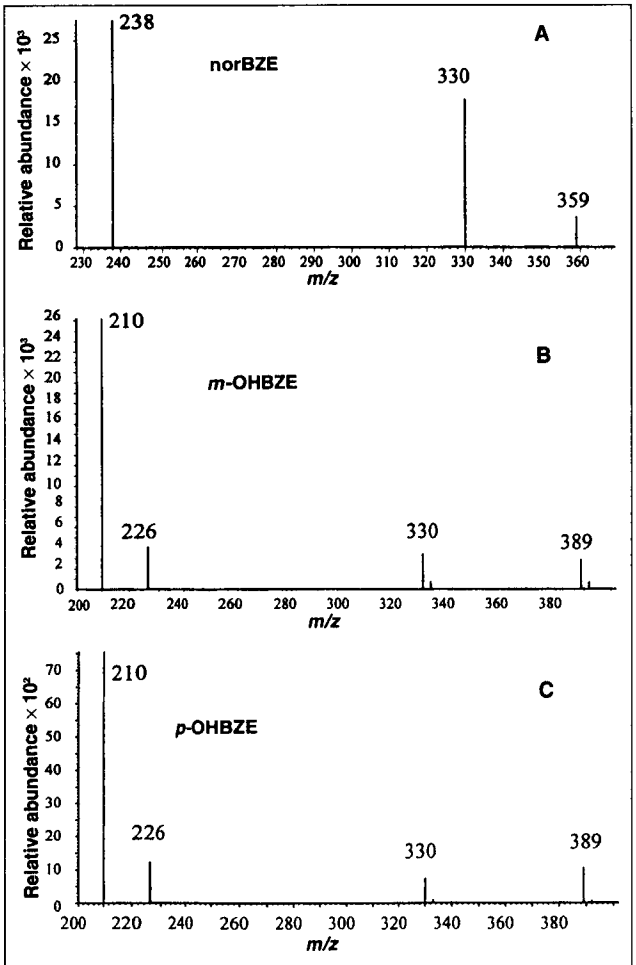

Figure 5. Mass spectrum of norBZE (A), *m*-OHBZE (B), and *p*-OHBZE (C) found in a specimen (#82) as analyzed by GC–MS.

Quantitation of the cocaine metabolites in both specimens and controls was accomplished by single-point calibration against the 25-ng/mL calibrator using the *m/z* 238/392 (norBZE/*m*-OHBZE-d_3) ion ratio and the *m/z* 389/392 (*m*- and *p*-OHBZE/*m*-OHBZE-d_3) ion ratio. Each analytical run included a set of urine controls prepared at 0, 5, 10, 15, 25, and 50 ng/mL for each of the three cocaine metabolites. All of the controls quantitated within ± 20% of the expected theoretical concentration. The linearity range for norBZE, *m*-OHBZE, and *p*-OHBZE was from 5 to 50 ng/mL and exhibited correlation coefficients of 0.9991, 0.9999, and 0.9819, respectively.

The LOD and LOQ of the method were determined by analyzing three replicates of urine standards spiked with the cocaine metabolites at decreasing concentrations from 25 to 1 ng/mL. The LOD was defined as the level where one or more of the analyte qualifying ion ratios fell outside of the ± 20% limit established by the calibrator. The LOQ was defined at the level where one or more of the analyte qualifying ion ratios fell outside of the ± 20% limit established by the calibrator or the experimentally determined concentration fell outside ± 20% of the theoretical concentration. The resulting LOD and LOQ for all cocaine metabolites were both 5 ng/mL.

A total of 89 human urine specimens that screened positive for BZE by immunoassay (KIMS) at 150 ng/mL were analyzed for BZE by GC–MS. Each sample was first analyzed for BZE and then later analyzed for norBZE, *m*-OHBZE, and *p*-OHBZE. The population of specimens covered a wide range of BZE concentrations. Seven of the 89 specimens were below the DOD BZE cutoff concentration of 100 ng/mL, demonstrating a mean of 74 ng/mL with values ranging from 32 to 97 ng/mL. The mean BZE concentration for the remaining 82 specimens was 3220 ng/mL with values ranging from 112 to 100,118 (Table I).

The prevalence of each of the three proposed in vivo cocaine metabolites detected at concentrations above the LOQ (5 ng/mL) ranged from a low of 67% (60/89) for norBZE to 83% (74/89) for *m*-OHBZE in the 89 specimens. *p*-OHBZE was detected in 79% (70/89) of the 89 specimens (Table I). However, if the prevalence of either one or both of the hydroxylated metabolites was considered, 92% (82/89) of the specimens were positive. Furthermore, if any of the three metabolites in any combination was considered, then 94% (84/89) of the specimens were positive. When samples that were initially below the DOD GC–MS cutoff (100 ng/mL) for BZE were removed from the data set, the percentage of samples containing at least one of the three metabolites was 96% (79/82). Further removal of specimens that quantitated below the HHS GC–MS cutoff (150 ng/mL) for BZE enhanced the correlation to 97% (65/67).

EME analysis

The total ion chromatogram of a specimen containing EME is shown in Figure 6A. The retention time of EME and EME-d_3 (7.9 min), was within ± 2% of the calibration standard in all runs. The MS was operated in the electron-impact SIM mode with the following ions being monitored: *m/z* 210, 182, and 82 for EME and 213 and 185 for EME-d_3. The quantitation ratio (EME/

EME-d_3) was *m/z* 210/213 and the qualifying ratios for identification of EME were *m/z* 82/210 and *m/z* 182/210. The qualifying ratio for the internal standard, EME-d_3 was 185/213. EME concentrations for specimens and controls were determined by single-point calibration against the 100-ng/mL EME calibrator using the *m/z* 210/213 EME/EME-d_3 ion ratio. The spiked urine EME control concentrations were 0, 50, 100, 200, and 400 ng/mL. Quantitative results were within ± 20% of the expected theoretical concentration for all runs and the assay was linear (r^2 = 0.9998) from 50 to 400 ng/mL. The LOL of the EME assay (r^2 = 0.9996) was evaluated at concentrations ranging from 500 to 20,000 ng/mL for highly concentrated specimens resulting from the cocaine stability study.

Cocaine stability study

Figure 7 shows the results of cocaine-spiked urine (155,000 ng/mL) in which the pH was adjusted from pH 5.0 to 9.0 and stored at 25°C over a 72-h period. The samples were sub-

Table I. Specimen Profile

Specimen #	BZE (ng/mL)	norBZE (ng/mL)	*m*-OHBZE (ng/mL)	*p*-OHBZE (ng/mL)	Specimen #	BZE (ng/mL)	norBZE (ng/mL)	*m*-OHBZE (ng/mL)	*p*-OHBZE (ng/mL)
1	34	2.2	9	7.4	45	317	9.7	28.2	29.4
2	45	64.8	9.1	7.5	46	318	3.9	19.9	4.2
3	66	0.8	3.2	ND*	47	346	1.1	7.9	ND
4	82	0.5	ND	0.3	48	348	17.6	7.9	62.9
5	95	26.9	1.8	11.2	49	369	22.3	ND	ND
6	97	88.2	126.3	183.1	50	371	52.7	15.1	ND
7	97	2.5	38.6	5.7	51	375	8.6	13.2	28.8
8	112	2.1	30.9	6.5	52	409	4.7	10.9	45
9	114	2	4.9	21.8	53	437	4.1	16.1	105.5
10	115	30.5	196	10.7	54	532	4.9	16	ND
11	118	3.9	7.3	5.5	55	542	7.4	90	38.1
12	120	2.6	2.8	8.5	56	560	8.1	9.8	ND
13	121	2.8	2.3	12.6	57	560	89.4	106	109.9
14	123	10.5	41	ND	58	614	7.9	5.8	3.3
15	123	4.7	34.9	2.7	59	679	45.2	39.9	37.4
16	133	4.4	ND	ND	60	740	1277	5.8	17
17	135	26.6	ND	96.6	61	943	10.2	38.7	4.2
18	136	15.3	31.4	53.8	62	967	1307.4	187.7	452.6
19	138	26.6	ND	96.6	63	1032	47.7	19.5	24.1
20	141	3.1	5.3	10.1	64	1270	30	24.9	19.8
21	147	5.2	9	11.7	65	1404	87.1	49.3	128
22	148	4.8	8.9	15.3	66	1428	53.6	18	14.5
23	152	40.8	81.5	47.1	67	1428	7.2	6.1	25.9
24	160	0.8	1.2	ND	68	1492	21.6	73.6	25.3
25	162	5.3	22.8	45.4	69	1525	33.6	39.1	73.5
26	172	24.8	22.1	30	70	1640	25.5	47.9	13.7
27	190	9.5	ND	ND	71	1688	10.4	12.5	25
28	194	3	1.7	17.7	72	1740	8	25.5	9.9
29	208	7.2	8.8	10.4	73	1740	104.5	41.2	48.5
30	218	16.7	17.8	57.9	74	1773	316.6	201.6	395.1
31	221	7.6	11.5	29.6	75	2271	56.3	22.9	91.6
32	226	3.6	ND	ND	76	3099	169.7	46.2	162.6
33	232	0.9	19.7	5.4	77	3797	117.7	171.7	114.6
34	243	16.5	16.7	21.1	78	4597	197	50	622.8
35	255	2.6	5.9	ND	79	6203	2.5	3.1	11.8
36	268	ND	18.4	ND	80	6203	1.6	8.5	18
37	278	7.1	157.2	117.8	81	7209	609.1	68	687.5
38	279	6.7	67.4	42	82	8149	803.4	150.2	29.9
39	294	7.9	14.4	89.9	83	9001	487	144.9	229.2
40	300	6.3	26.5	ND	84	9459	227.2	263.8	283.7
41	310	3.8	68.8	64.2	85	10233	331.8	82.8	92
42	313	6.2	14.2	10.9	86	11919	8.4	39.8	482.8
43	314	6.4	15	16	87	15048	1776.4	104.5	1639.1
44	316	0.3	9.5	9	88	32127	2265.4	2038.5	859.3
					89	100118	3.3	2932.9	1579.2

* ND, not detected.

sequently analyzed at various time intervals for the presence of EME and BZE. There was no significant loss of cocaine observed in the pH 5.0 and 7.0 urine throughout the 72 h of incubation (i.e., only 0.6–0.7% of the cocaine was hydrolyzed to

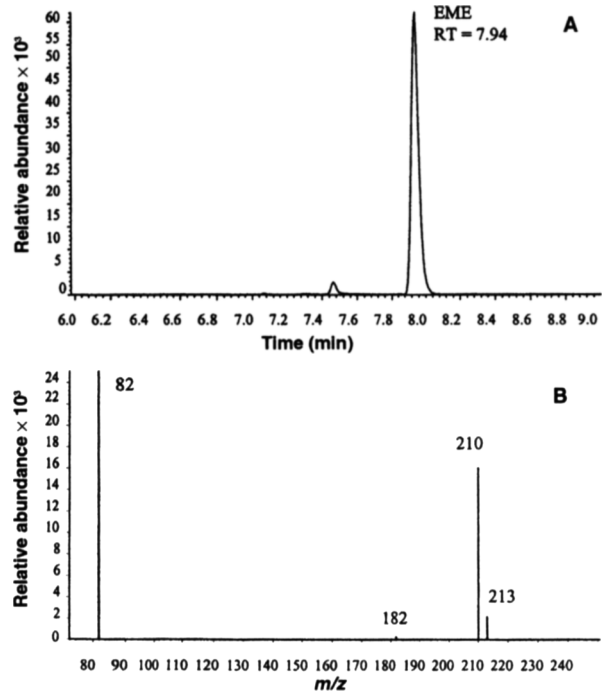

Figure 6. TIC of a specimen treated with cocaine (155,000 ng/mL) maintained at pH 9.0 and 25°C for 72 h showing EME (A) and mass spectrum of EME found in the specimen as analyzed by GC–MS (B).

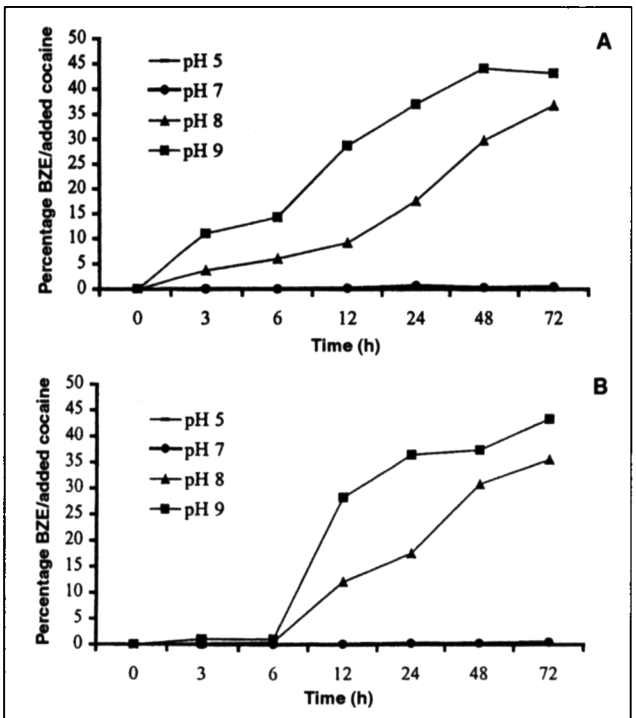

Figure 7. The effect of pH (5.0–9.0), length of storage (0–72 h), and temperature (25°C) on the stability of cocaine (155,000 ng/mL). Production of BZE (A) and EME (B) expressed as the percentage of cocaine initially added.

EME and BZE under acidic and neutral conditions). In contrast, the data clearly demonstrated that at pH 8.0 and 9.0, both EME and BZE increased dramatically with respect to time. Expressed as a percentage of the initial amount of cocaine added, the detected amounts of BZE were 34% and 43% after 72 h for pH 8.0 and pH 9.0, respectively (Figure 7A). The comparable data for EME were 36% and 43% (Figure 7B). Importantly, norBZE, *m*-OHBZE, and *p*-OHBZE were not detected under any of the described conditions.

Discussion

Previous publications regarding the metabolism of cocaine support the hypothesis that the benzoyl ester of cocaine is hydrolyzed to EME by pseudocholinesterase, whereas the methyl ester is hydrolyzed to BZE nonenzymatically (1–5). The implication that BZE is not formed enzymatically has resulted in several defense strategies in forensic urine drug-testing cases. One such defense alleges that the presence of BZE does not exclude the possibility that the urine was spiked with cocaine during the collection process and that the added cocaine was subsequently hydrolyzed to BZE. As a result, the defense may argue that BZE-positive test results are inconclusive with regards to ingestion because the laboratory did not also assay for EME to demonstrate that cocaine was indeed ingested. The argument may be further supported because pseudocholinesterase is not present in urine (1–5,14) and as such could not be expected to contribute to the hydrolysis of cocaine in this scenario. This defense strategy may be successfully used even though there is evidence that BZE is also formed enzymatically (15) and there is no published evidence that cocaine does not hydrolyze to EME in vitro.

The premise that EME is a "true" in vivo cocaine metabolite was investigated by assessing the stability of cocaine in unpreserved urine samples at several pHs ranging from 5.0 to 9.0. The target concentration of cocaine (155,000 ng/mL) used in the cocaine stability study was based on the premise that a small but measurable amount of cocaine (approximately 5 mg) may be spiked into a urine specimen or bottle ultimately containing a volume of urine (30–85 mL) typically received by urinalysis workplace drug-testing laboratories. The data presented in this study clearly demonstrate that cocaine undergoes spontaneous chemical hydrolysis to form both EME and BZE under alkaline conditions. Specifically, at pH 9.0, 43% of the cocaine in urine specimens maintained at 25°C underwent in vitro chemical hydrolysis to form both BZE and EME after 72 h. The observation that cocaine can be chemically hydrolyzed to EME is consistent with the findings of Mitchell (7). Specifically, in a cocaine stability study conducted at the Jacksonville Navy Drug Screening Laboratory, it was determined that aqueous samples buffered at pH 8.0 and spiked with 100,000 ng/mL of cocaine produced concentrations of EME as high as 10,000 ng/mL in only 8 h (7).

In consideration of these data, evidence of cocaine use could still be subject to challenge as it could be alleged that both BZE and EME are present because of in vitro addition of cocaine, rather than in vivo production of these compounds. For

this reason, a methodological approach is presented to determine if the presence of BZE resulted from the use of cocaine or from the spiking of cocaine into a urine specimen. The method targets cocaine metabolites believed to be produced only via metabolic (biotransformation) mechanisms. Importantly, none of these in vivo metabolites were detected in cocaine spiked specimens at pH 5.0–9.0 for up to 72 h. In regulated drug testing, criteria for normal urine has been established as pH 4.8–8.0. Occasionally, specimens with a higher pH have been observed. The simultaneous analysis of all three metabolites (*m*-OHBZE, *p*-OHBZE, and norBZE) dramatically increases the chances of establishing the ingestion of cocaine compared with analyzing for only one of the metabolites. This is an improvement over the results of ElSohly et al. (13), which demonstrated that, although *m*-OHBZE was indeed more prevalent than EME, only 71% of the specimens contained *m*-OHBZE at a level that exceeded the LOQ (5.0 ng/mL).

It should be noted, however, that Casale and Waggoner (16) reported that norcocaine is present in trace amounts in cocaine samples that were analyzed by GC and is produced mostly as a manufacturing by-product. The mean amount detected was 0.12% with a standard deviation of 0.24 and a range of 0.01–2.4. This means that norBZE could arise from the hydrolysis of nor cocaine. If norBZE is the sole metabolite detected, the ratio of norBZE to BZE should be examined because ratios of 0.025 or less might be questioned regarding their significance as to the origin of norBZE. One specimen from the data in Table I (specimen #27) showed only norBZE. Its norBZE/BZE ratio was 0.05. Of the 60 samples that contained norBZE above the LOQ, 39 had a norBZE/BZE ratio of 0.025 or higher.

Conclusions

This study demonstrates that a GC–MS method for the simultaneous identification of the three in vivo metabolites of cocaine (norBZE, *m*-OHBZE, and *p*-OHBZE) can provide conclusive evidence of cocaine ingestion and that at least one of these metabolites was nearly always present in a specimen that contained BZE above either the DOD or HHS GC–MS cutoffs. In addition, because it was also demonstrated that EME could be formed in vitro under conditions which may be encountered in regulated drug-testing samples, the use of EME as an indicator of cocaine ingestion based on the assumption that it is not a hydrolytic product is not valid. This procedure is recommended in instances where the reported BZE-positive urinalysis results are being challenged on the premise that the subject's urine was spiked with cocaine or contaminated with cocaine during the collection or drug-testing processes.

Acknowledgments

The authors acknowledge with thanks Marilyn Manglicmot, Patrick Neff, and Eleuterio Brillo of the San Diego Navy Drug Screening Laboratory for their valuable assistance in the analysis of the cocaine specimens. The authors are grateful to John Mitchell, Ph.D. for his insightful discussions and providing data from his cocaine stability studies. The authors also wish to thank Mahmoud ElSohly, Ph.D. for providing the internal standard OHBZE-d_3.

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Manuscript received March 30, 2000;
revision received May 23, 2000.