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Simultaneous Determination of Cocaine, Cocaethylene, and Their Possible Pentafluoropropylated Metabolites and Pyrolysis Products by Gas Chromatography/Mass Spectrometry

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16. Abstract

During the investigations of fatal transportation accidents, samples from victims are also analyzed for drugs, including cocaine (COC). COC is abused by smoking, nasal insufflantion, and intravenous injection, and it is also taken with ethanol. Therefore, it is important to determine concentrations of COC and its metabolites, ethanol analogs, and pyrolysis products for establishing the degree of toxicity, the possible ingestion of ethanol, and the possible route of administration. In this study, a sensitive and selective procedure is developed for the simultaneous analyses of COC, benzoylecgonine, norbenzoylecgonine, norcocaine, ecgonine, ecgonine methyl ester, *m*-hydroxybenzoylecgonine, anhydroecgonine methyl ester (AEME), anhydroecgonine (AECG), cocaethylene, norcocaethylene, and ecgonine ethyl ester in blood, urine, and muscle homogenate. In the analysis, available deuterated analogs of these analytes were used as internal standards. Proteins from blood and muscle homogenate were precipitated with cold acetonitrile. After the removal of acetonitrile by evaporation, the supernatants and urine were extracted by solid-phase chromatography. The eluted analytes were converted to hydrochloride salts and derivatized with pentafluoropropionic anhydride and 2,2,3,3,3-pentafluoro-1-propanol. The derivatized products were analyzed on a gas chromatograph (GC)/mass spectrometer system by selected ion monitoring. This method was successfully applied in analyzing 13 case specimens from aviation accident pilot fatalities and/or motor vehicle operators. AEME concentrations found in the 13 specimens were consistent with those produced solely by the GC inlet pyrolysis of COC controls, suggesting that COC was not abused in these cases by smoking. Although AEME remains a potential marker for establishing the abuse of COC by smoking, AECG was not a useful marker because of its low recovery and GC inlet production from COC metabolites. The developed procedure is unique because multiple analytes can be analyzed in urine, blood, and solid tissues by a single extraction with increased sensitivity through formation of hydrochloride salts and using a one-step derivatization.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AECG	anhydroecgonine
AEME	anhydroecgonine methyl ester
BEG	benzoylecgonine
BEG-D_3	benzoylecgonine-D_3
CAMI	Civil Aerospace Medical Institute
COE	cocaethylene
COE-D_3	cocaethylene-D_3
COC	cocaine
COC-D_3	cocaine-D_3
CYP	cytochrome P-450
ECG	ecgonine
EEE	ecgonine ethyl ester
EME	ecgonine methyl ester
EME-D_3	ecgonine methyl ester-D_3
FAA	Federal Aviation Administration
GC/MS	gas chromatograph/mass spectrometer
HBEG	*m*-hydroxybenzoylecgonine
LOD	limit of detection
LOQ	limit of quanititation
NBEG	norbenzoylecgonine
NCOE	norcocaethylene
NCOC	norcocaine
NCOC-D_3	norcocaine-D_3
OSBI	Oklahoma State Bureau of Investigation
PFP	pentafluoropropylated
PFPA	pentafluoropropionic anhydride
PFPOH	2,2,3,3,3-pentafluoro-1-propanol
SIM	selected ion monitoring
SPE	solid phase extraction.

Simultaneous Determination of Cocaine, Cocaethylene, and Their Possible Pentafluoropropylated Metabolites and Pyrolysis Products by Gas Chromatography/Mass Spectrometry

INTRODUCTION

The Federal Aviation Administration's (FAA's) Civil Aerospace Medical Institute (CAMI) is involved in the toxicological evaluation of samples collected from victims involved in fatal transportation accidents. During the investigations of such accidents, those biological samples are analyzed for the presence of combustion gases (carbon monoxide and hydrogen cyanide), alcohol and other volatiles, and drugs, including cocaine (COC). COC, a popular drug of abuse, is consumed by smoking, nasal insufflation, and intravenous injection (1). COC is also often taken in conjunction with ethanol, generating a transesterification product, cocaethylene (COE; 2). This COC analog has pharmacological activities similar to COC and may modestly prolong the COC-like effects (3). In recreational use, smoking may be the preferred route of ingestion of COC because its effects are immediate and intense. Furthermore, such effects are achieved without having to deal with needles and syringes, thereby minimizing the risk of contracting bloodborne diseases. Free-base COC (crack) is generally associated with smoking (1, 4, 5), while its salt form may be associated with other routes of administration.

Peak COC concentrations in blood usually occur 30–60 min after nasal insufflation and within minutes after smoking or intravenous injection ingestion (6). COC is rapidly inactivated by the hydrolysis of its ester groups, producing benzoylecgonine (BEG), ecgonine methyl ester (EME), and possibly ecgonine (ECG; Fig. 1). COC is further biotransformed by the cytochrome P-450 (CYP) enzyme system to norcocaine (NCOC), and BEG to norbenzoylecgonine (NBEG) and *m*-hydroxybenzoylecgonine (HBEG). Smoking produces the pyrolysis product anhydroecgonine methyl ester (AEME; 7, 8), which could be further hydrolyzed to anhydroecgonine (AECG). Furthermore, COE is metabolized into norcocaethylene (NCOE) and hydrolyzed into ecgonine ethyl ester (EEE), which could also be biosynthesized from EME in the presence of ethanol. COE could be converted into ECG and BEG, while NCOE and NCOC can generate NBEG. Additionally, NCOC can be converted into NCOE by transesterification (2).

Postmortem biochemical activity or improper specimen preservation and/or storage may also result in the conversion of COC into EME and BEG (Fig. 1). Even in water, at pH values greater than neutrality, COC is readily hydrolyzed to BEG. Cholinesterases in blood hydrolyze COC into EME, but this enzymatic reaction may be inhibited by freezing or by the addition of fluoride or cholinesterase inhibitors (9, 10, 11). A considerable amount of time may often pass before postmortem specimens are collected, chemically preserved, and refrigerated. Such a delay may possibly cause the conversion of all or some COC and COE into EME, BEG, and/or EEE. Therefore, concentrations of COC, COE, EME, BEG, other possible metabolites, and pyrolysis products in biological samples could be helpful in estimating the total amount of COC present in those samples at the time of their collection and/or at the time of an accident and/or death. Furthermore, the concentrations of COE, NCOE, and EEE may indicate concurrent use of COC and ethanol, and the concentration of AEME may assist in establishing whether COC was smoked or taken by other routes.

Numerous analytical methods for COC, COE, their metabolites, and other related products are reported in the literature (5, 7, 12, 13), but these methods involve multiple extraction and/or derivatization procedures and/or have not been shown to detect all the COC and possible related analytes in multiple specimen types. Although the pyrolytic product of COC, AEME, has been analyzed in blood, serum, and urine (5, 7, 12, 13), it has been demonstrated that COC can thermally degrade during gas chromatography, particularly at high injector port temperatures (8, 14).

In the present study, a selective and sensitive method, involving a single extraction and derivatization, was developed to simultaneously analyze COC, COE, their metabolites and pyrolysis products, and related compounds by gas chromatography/mass spectrometry in blood, urine, and solid tissues. Attempts were also made to establish a relationship between the concentration of COC and the production of AEME during gas chromatography and the suitability of AECG as an additional analytical marker for COC smoking. The developed method was successfully evaluated for analyzing blood, urine, and muscle specimens collected from 13 aviation accident pilot fatalities and/or motor vehicle operators.

EXPERIMENTAL

Materials

All solvents and reagents used during the analyses were of analytical grade and were of the highest available purity. These chemicals, analyte standards, and other reagents were obtained from commercial sources. Specifically, COC, BEG, NBEG, NCOC, ECG, EME, HBEG, AEME, AECG, COE, NCOE, and EEE were supplied by Cerilliant Corporation (Austin, TX) in certified 1.0 mg/mL solutions. Internal standards, cocaine-D_3 (COC-D_3), benzoylecgonine-D_3 (BEG-D_3), norcocaine-D_3 (NCOC-D_3), ecgonine methyl ester-D_3 (EME-D_3), and cocaethylene-D_3 (COE-D_3) were also supplied by the Cerilliant Corporation in certified 100 µg/mL solutions. Where a deuterated internal standard of an analyte was not available, the most structurally similar deuterated compound and/or the deuterated compound with the retention time nearest to the analyte in question was selected from the aforementioned internal standards for the quantitation of that particular analyte. For example, EME-D_3 was used as an internal standard for the analysis of ECG, AEME, AECG, and EEE. Pentafluoropropionic anhydride (PFPA) and 2,2,3,3,3-pentafluoro-1-propanol (PFPOH) were purchased from Pierce Chemical Company, Rockford, IL. The 10-mL Certify™ solid phase extraction (SPE) columns, with a 130-mg sorbent bed, were obtained from Varian Corporation, Harbor City, CA.

Case Samples

A muscle specimen and a second paired blood and urine specimen were obtained from two aviation accident pilot fatalities—these specimens were submitted to the FAA's Civil Aerospace Medical Institute by the National Transportation Safety Board for toxicological evaluation. Whole blood specimens from 10 motor vehicle operators suspected of being impaired by the abuse of COC were kindly provided by the Oklahoma State Bureau of Investigation (OSBI), Oklahoma City, OK. No historical information suggesting the abuse of COC by smoking was available for these 12 cases. The pilot fatality and motor vehicle operator specimens are referred herein as to "case samples" or "case specimens."

Calibrators

A solution of 6400 ng/mL AECG and 800 ng/mL AEME was prepared in bovine whole blood, preserved with 2 mg/mL potassium oxalate and 10 mg/mL sodium fluoride. Serial dilutions of this two-analyte mixture resulted in calibrators of 50, 100, 200, 400, 800, 1600, and 3200 ng/mL AECG and 6.25, 12.5, 25, 50, 100, 200, and 400 ng/mL AEME in the whole blood. To validate the precision of the calibrators, 80, 160, and 320 ng/mL controls of these two analytes were prepared together in human whole blood and preserved as above.

A second solution containing 6400 ng/mL ECG and 800 ng/mL each of COC, BEG, NBEG, NCOC, EME, HBEG, COE, NCOE, and EEE was prepared in bovine whole blood. This 10-analyte mixture was diluted in the same manner as the previously described AECG and AEME solution to obtain their respective calibrators. To validate the precision of the calibrators, 80, 160, and 320 ng/mL each of COC, BEG, NBEG, NCOC, EME, HBEG, COE, NCOE, and EEE controls were prepared together in human whole blood and preserved with potassium oxalate and sodium fluoride; these three controls also contained 800, 1600, and 3200 ng/mL ECG, respectively.

The generated AEME and AECG calibration curves were used to quantitate these analytes in the biological samples without any influence of their possible GC inlet pyrolytic production from COC and/or other possible derivatized/underivatized analytes. Similarly, the generated calibration curves of each of the 12 analytes were used for determining their concentrations in biological samples. Since AEME and AECG were not added to the 10-analyte mixture, any amount of these analytes found in the 10-analyte calibrators or controls resulted from the artifactual pyrolytic production in the GC inlet.

Based on the bovine blood calibrator and human blood control analytical data, it was concluded that both biological matrices were comparable to each other for the analyses. Therefore, bovine blood was used for the preparation of calibrators and controls in analyzing blood case samples. Bovine blood calibrators and controls were also used during the analysis of urine and muscle samples.

Gas Chromatographic AEME Production

For establishing a relationship between the amounts of AEME pyrolytically produced on the instrument during analysis, several COC controls in bovine blood were analyzed. The COC controls ranged from 25 to 6400 ng/mL. In addition, controls of other analytes were prepared and analyzed to determine the possible production of AEME.

Extraction

Three mL of whole blood or urine and 3.0 g of muscle homogenate, prepared in 1.0% aqueous sodium fluoride in a 1:2-proportion (w/w), were separately transferred into 150 mm × 16 mm screw-capped glass culture tubes. To each tube, 400 ng each of COC-D_3, BEG-D_3, NCOC-D_3, EME-D_3, and COE-D_3 was added as an internal standard. With the exception of urine, 10.0 mL of acetonitrile (−20°C) was added to each tube to precipitate protein from blood and homogenate samples. The tubes were capped and secured

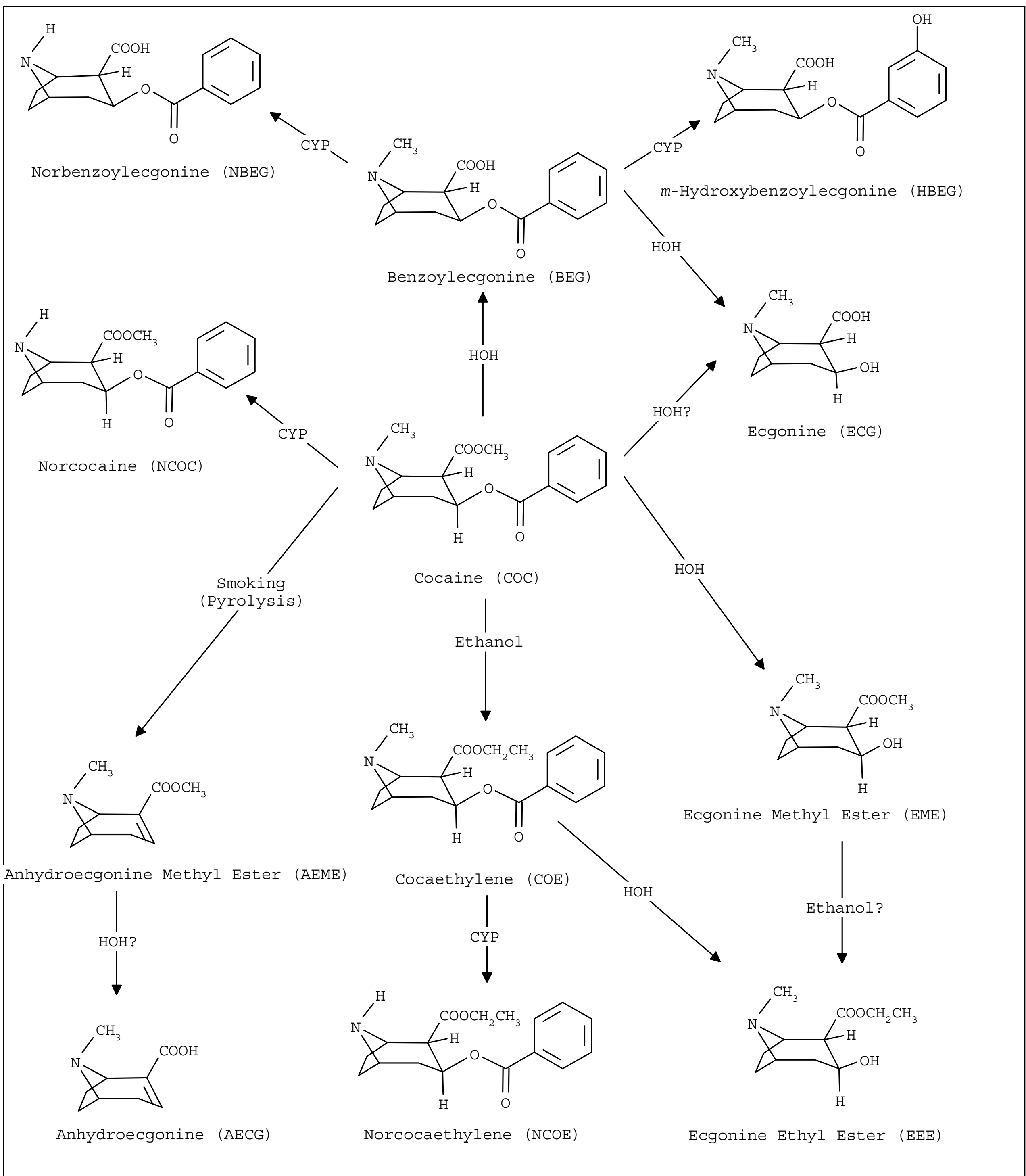

Figure 1: Possible biotransformation, pyrolysis, and ethanol products of COC (CYP = cytochrome P-450; HOH = hydrolysis).

in a test tube rack. The entire rack was subsequently shaken manually for 2 min. The tubes were centrifuged at 770 × *g* for 5 min, and the obtained supernatants were transferred into 16 mm × 125 mm glass culture tubes. The volumes of the supernatants were reduced to approximately 2 mL by placing the tubes in an evaporator with a water bath set at 40°C, while purging with nitrogen. Two mL of phosphate buffer (0.1 M, pH 6.00) was added to each supernatant and to the urine sample. The solutions from each tube were then decanted into the SPE columns, which had previously been solvated with 2.0 mL of methanol, then with 2.0 mL of the buffer. Once the supernatants and urine had passed through the columns, they were washed with 6.0 mL of deionized water, then with 3.0 mL of 0.1 N HCl. The columns were dried with 25 lb of pressure for 5 min. Subsequently, 9.0 mL of methanol was passed through the columns and the 25-lb pressure was applied to the columns for 5 sec. The analytes were eluted from the columns by using 4.0 mL of a mixture of ammonium hydroxide: isopropyl alcohol: methylene chloride (1:10: 40) into 10-ml screw-capped, glass conical tubes.

Derivatization

To improve the analytical sensitivity, it was first necessary to convert all basic analytes into their hydrochloride salts prior to the PFPA/PFPOH derivatization. This was achieved by bubbling through the eluates hydrochloric acid vapors from a hydrochloric acid vapor generator (Fig. 2). An unused Pasteur pipette was attached to the generator for each eluate. The pipette tip was placed into the eluate, and the bulb of the generator was gently squeezed once. It is crucial to emphasize that only vapors from a generator with conditioned hydrochloric acid should be used during this step. Hydrochloric acid, which has not been previously conditioned, results in a large production of inorganic salts suspended in the eluates, leading to little or no recovery of the analytes of interest and possible damage to the GC column. The acid in the generator is conditioned by filling the generator approximately one-third with concentrated hydrochloric acid and bubbling air through the solution with the bulb 5 to 10 times daily for at least 10 days. Purging the acid solution for several hours with nitrogen or diluting the concentrated acid solution could not produce the same analytical result as the conditioning process produced. The conditioned acid solution is stable for at least two months. Therefore, by having two generators, one may be used for one month after conditioning while the other is being conditioned. Switching monthly to the generator with newly conditioned hydrochloric acid while the other is being freshly prepared and being conditioned with hydrochloric acid will ensure that a generator with the conditioned acid is always available for the hydrochloric salt formation prior to the PFPA/PFPOH derivatization.

After bubbling acid vapors through the eluates, they were taken to dryness by using a water bath evaporator set at 40°C while purging with nitrogen. To the residues, 50.0 µL each of PFPA and PFPOH was added. The tubes were capped and incubated in a heating block at 70°C for 20 min. The reaction mixtures were cooled, then taken to dryness by using an evaporator set at 40°C while purging with nitrogen. Subsequently, the residues were reconstituted in 50.0 µL of ethyl acetate and analyzed.

Instrumentation

Using the electron impact mode, a gas chromatograph/ mass spectrometer (GC/MS; Model 6890/5973; Agilent Technologies, Wilmington, DE) was employed for the analyses of the reconstituted residues by selected ion monitoring (SIM; Table I). This system was configured with electronic pressure control. A bonded fused silica

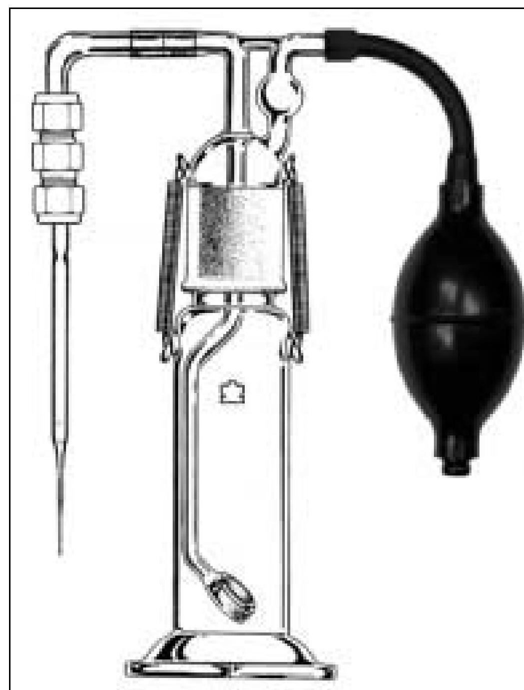

Figure 2: Hydrochloric acid vapor generator. This unit was assembled in the CAMI laboratory from a gas washing bottle (No. 657750, Kimble Glass, Inc., Vineland, NJ), a vacuum/pressure bulb (No. 14-085, Fisher Scientific, Springfield, NJ), a small piece of polypropylene tubing, a bent piece of glass tubing, a Pasteur pipette polypropylene fitting (No. P0635-R, Organomation Associates, Inc., Berlin, MA), and disposable Pasteur pipettes. The hydrochloric acid vapors from this type of generator were used for converting various basic analytes into their hydrochloride salts prior to the PFP derivatization. Details are given in the Derivatization subsection of the Experimental section.

Analyte	Molecular Formula	Molecular Weight	Retention Index	Retention Time (Min)	Target Ion (m/z)	Qualifying Ion I (m/z)	Qualifying Ion II (m/z)	% Change in Analytical Response Due to HCl Salt	% Recovery	LOD ng/mL	LOQ ng/mL
AECG-PFP	$C_{12}H_{14}F_5NO_2$	299.24	1329	3.38	270	299	284	-31	0.2	†640	†640
AEME	$C_{10}H_{15}NO_2$	181.23	1369	3.85	152	181	166	+233	40	13	13
ECG-D3-2PFP	$C_{15}H_{15}D_3F_{10}NO_4$	466.26	1418	4.25	303	466	317	–	0.03	–	–
ECG-2PFP	$C_{15}H_{15}F_{10}NO_4$	463.28	1418	4.26	300	463	314	–	1.5	640	800
EME-D3-PFP	$C_{13}H_{13}D_3F_5NO_4$	348.25	1445	4.67	185	317	348	–	–	–	–
EME-PFP	$C_{13}H_{16}F_5NO_4$	345.27	1446	4.68	182	314	345	+278	1.5	2	2
EEE-PFP	$C_{14}H_{18}F_5NO_4$	359.29	1505	5.32	196	314	359	+254	5	2	2
BEG-D3-PFP	$C_{19}H_{17}D_3F_5NO_4$	424.34	2080	8.21	303	424	319	–	–	–	–
BEG-PFP	$C_{19}H_{20}F_5NO_4$	421.37	2081	8.22	300	421	316	+13	26	2	2
NBEG-2PFP	$C_{21}H_{17}F_{10}NO_5$	553.35	2105	8.45	214	312	431	0	–	25	25
HBEG-2PFP	$C_{22}H_{20}F_{10}NO_6$	584.38	2160	8.62	300	434	583	+57	25	50	50
COC-D3	$C_{17}H_{18}D_3NO_4$	306.33	2174	8.76	185	85	306	–	–	–	–
COC	$C_{17}H_{21}NO_4$	303.36	2174	8.77	182	82	303	+95	99	2	2
COE-D3	$C_{18}H_{20}D_3NO_4$	320.36	2232	9.09	199	275	320	–	–	–	–
COE	$C_{18}H_{23}NO_4$	317.38	2235	9.10	196	212	272	0	77	2	2
NCOC-D3-PFP	$C_{19}H_{15}D_3F_5NO_5$	438.32	2240	9.12	316	438	333	–	–	–	–
NCOC-PFP	$C_{19}H_{18}F_5NO_5$	435.35	2242	9.12	313	435	330	+57	25	2	2
NCOE-PFP	$C_{20}H_{20}F_5NO_5$	449.38	2290	9.44	327	344	449	+48	22	2	2

*Analytes are listed in the order of their elution from the GC column. Cut-offs for AEME in the presence of COC and its metabolites were greater than the LOD due to its inlet pyrolytic production. The percent analytical response change due to HCl salt, percent recovery, LOD, and LOQ are based on extracted analytes from analyte fortified bovine blood samples. Details are given in the Experimental and Results sections.

†AECG-PFP LOD and LOQ values in the absence of other analytes. AECG-PFP can also be artifactually produced from BEG-PFP, ECG-2PFP, and HBEG-2PFP (Fig. 6).

GC capillary column (12 m × 0.2 mm i.d., with a 0.33-μm film thickness of 100% methyl siloxane liquid phase) was used for the analyses. The carrier gas was helium and was set at 1 mL/min constant flow. The inlet temperature was set at 210°C. The initial oven temperature was 70°C. The temperature was then programmed to 135°C at 30°C/min, then to 140°C at 2°C/min, followed by 230°C at 30°C/min, then to 234°C at 2°C/min, followed by 250°C at 50°C/min. There were no oven temperature hold times.

RESULTS

Analytical Sensitivity

As exhibited in Fig. 3, COC, COE, AEME, and the pentafluoropropylated (PFP) derivatives of other analytes chromatographed symmetrically with good response and analytically acceptable separation. Each of the derivatives produced significant molecular weight ions and characteristic mass fragmentation patterns, which allowed selection of unique target and qualifying ions during the SIM analyses (Table I; Fig. 4a-c). Although COE and NCOC-PFP overlapped chromatographically (Fig. 3), unique ions were selected for the identification of both analytes. Analyses of COC, BEG, EME, AEME, COE, NCOE, and EEE analyses produced calibration curves of good linearity in the range of 6.25 to 800 ng/mL. The calibration curves for the analyses of NBEG, NCOC, ECG, HBEG, and AECG were linear in the range of 25 – 600 ng/mL, 6–100 ng/mL, 800–3200 ng/mL, 50–400 ng/mL, and 800–6400 ng/mL, respectively. The calibration curves of all the analytes demonstrated coefficients of linearity ≥ 0.995. Limit of detection (LOD) and limit of quantitation (LOQ) values for these analytes are given in Table I. Some of the CAMI/OSBI case specimens contained analytes in concentrations greater than their respective assay's highest calibrators. Those samples were diluted by a factor of 10 or 100 with 1.0% aqueous sodium fluoride. Three mL of the various diluted specimens were used to bring their concentrations within the linear range of their respective calibration curves prior to re-extraction. Where necessary, 1.0 mL aliquots of some of the case specimens were used for the analysis.

Pyrolytic Products

During the GC/MS analysis with the GC inlet temperature of 210°C, COC thermally degraded and predictably generated AEME (Table II; Fig. 5). The AEME production was COC concentration-dependent, as the net amount of AEME increased as the COC concentration increased in blood controls—13 to 18 ng/mL AEME was produced from 25 to 400 ng/mL of COC. However, the percentage of the pyrolytic production of AEME decreased as COC concentration increased in blood controls. Analytes—such as BEG-PFP, NBEG-2PFP, NCOC-PFP, ECG-2PFP, EME-PFP, HBEG-2PFP, COE, NCOE-PFP, and EEE-PFP—did not increase the production of AEME over that observed with COC alone. Based on the chemical structures of AECG and AECG-PFP, it is chemically inconceivable that these compounds could pyrolytically produce AEME (Figs. 1 & 6).

The COC-AEME concentration relationship established under the current experimental conditions was used to predict artifactual production of AEME at a given blood COC concentration (Table II; Fig. 5). The predicted AEME value was subtracted from the AEME value in a specimen determined from the AEME calibration curve constructed from the AEME and AECG mixture. Therefore, the derived subtracted value would represent a true concentration of AEME in a biological sample.

GC inlet temperatures greater than 210°C produced an excessive amount of AEME, while the inlet temperatures of less than 210°C caused a decrease in the responses of all the analytes, negatively affecting the LODs and LOQs of the assay. Therefore, an injector temperature of 210°C was found to be most suitable for the analysis. Lower GC inlet temperatures sacrificed the sensitivity of the method for detecting analytes with no significant reduction in production of AEME. Higher GC inlet temperatures produced even more AEME but did not significantly increase sensitivity of the method enough to justify the higher inlet temperatures. The heated inlet artifactually produced large amounts of AECG-PFP from the pyrolysis of PFP derivatized BEG, ECG, and HBEG (Fig. 5). Authentic AECG-PFP can only be produced *in vitro* from the PFP derivatization of AECG since AECG is a possible hydrolytic product of AEME (Fig. 1).

Case Sample Analyses

As is given in Table III, COC was detected in seven samples and NCOC was found in one sample. The COC/COE hydrolysis product, BEG, was present in considerable amounts in all the samples, including the muscle sample. Excluding the muscle and the blood samples of the CAMI cases, all samples were found to contain ECG in large amounts. Out of the 13 samples, seven samples were found to contain AEME ranging from 6–35 ng/mL. COE and NCOE were not detected in any of the case samples, but EEE was detected in small concentrations only in urine and muscle samples. Since AECG was artifactually produced in large amounts, concentrations of this analyte were not included in the table.

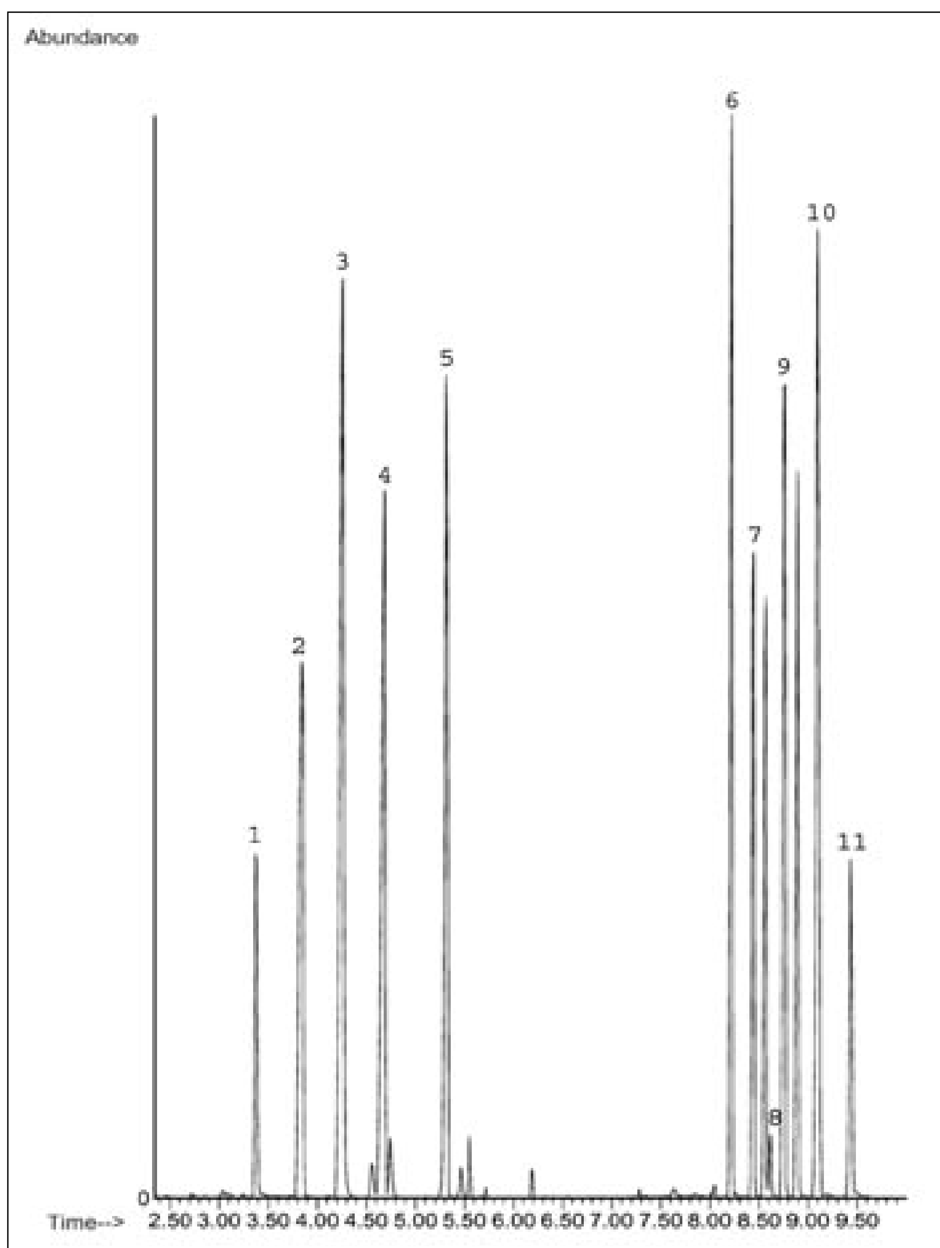

Figure 3: A total ion chromatogram of a mixture of COC, COE, AEME, and their PFP-derivatized metabolite and related products:
1 = AECG-PFP; 2 = AEME; 3 = ECG-2PFP; 4 = EME-PFP; 5 = EEE-PFP;
6 = BEG-PFP; 7 = NBEG-2PFP; 8 = HBEG-2PFP; 9 = COC, 10 = COE;
and NCOC-PFP, 11 = NCOE-PFP. This chromatogram depicts resolution, sensitivity, and peak shape of 50 ng of each of these unextracted analytes on the GC column. Other unlabeled peaks are artifacts from some or all of the 12 analytes. Although COE and NCOC-PFP overlapped chromatographically, their distinctive mass spectra allowed clear-cut identification of both analytes.

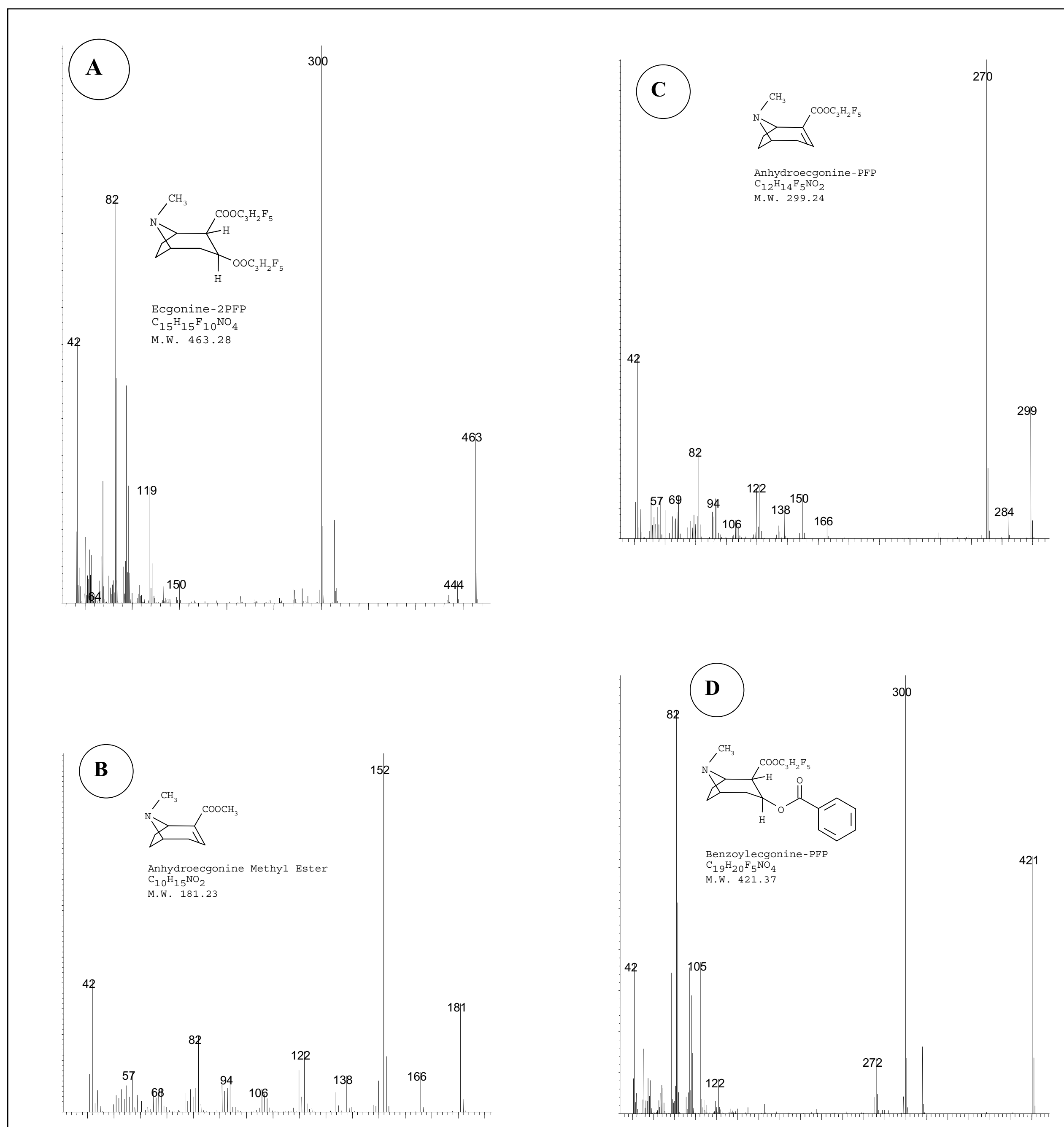

Figure 4a: Mass spectra of (A) ECG-PFP, (B) AEME, (C) AECG-PFP, and (D) BEG-PFP.

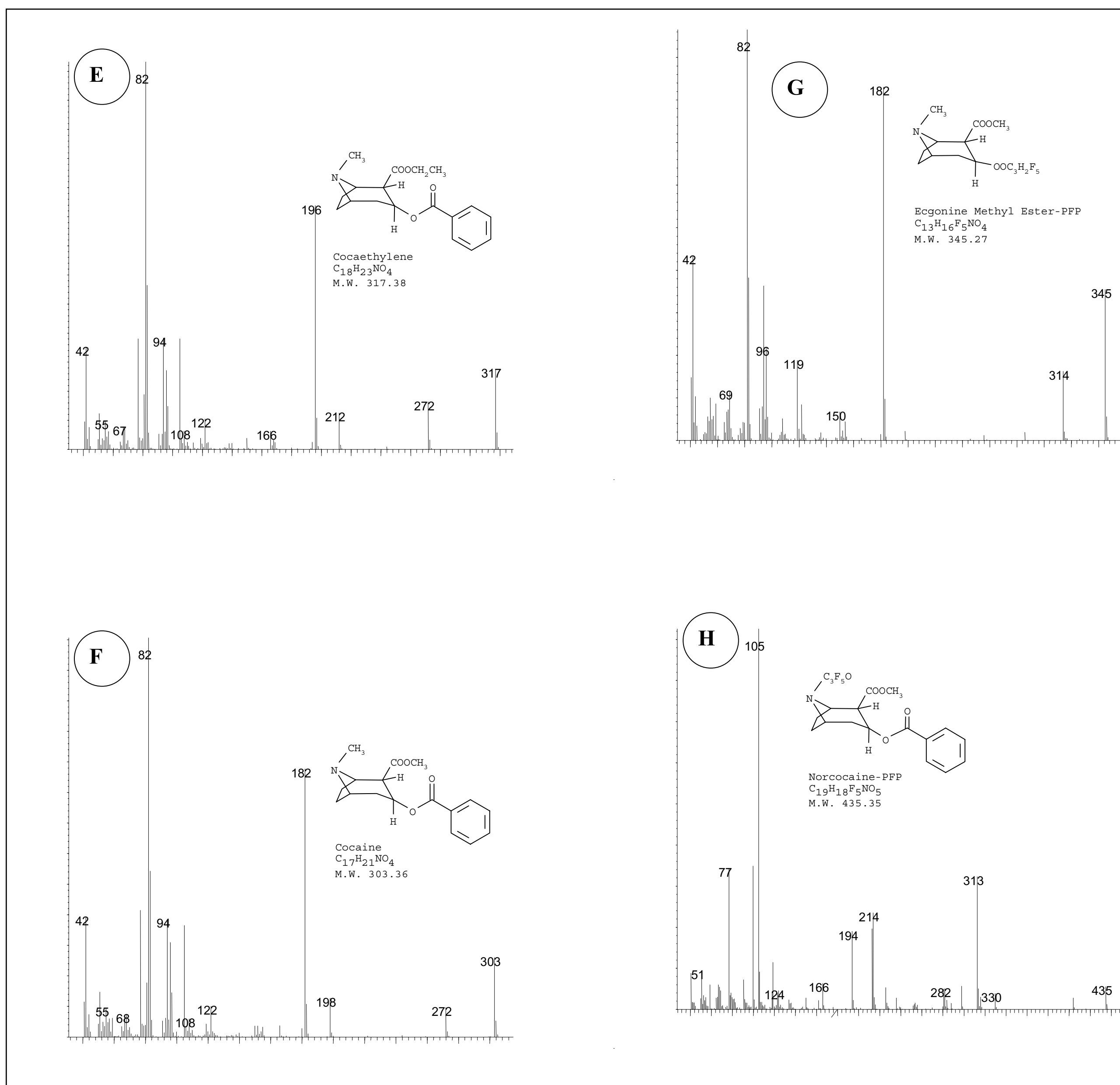

Figure 4b: Mass spectra of (E) COE, (F) COC, (G) EME-PFP, and (H) NCOC-PFP.

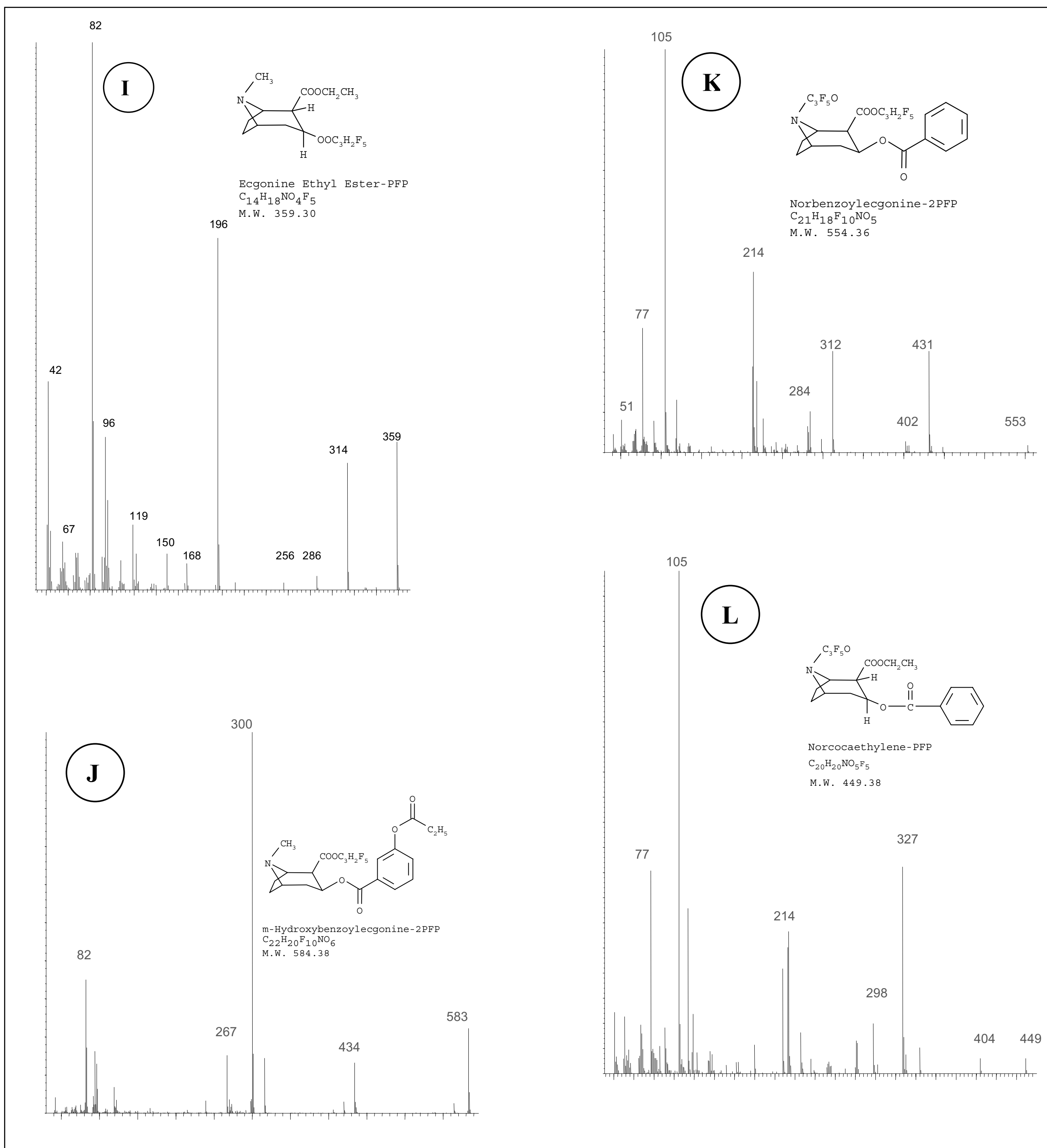

Figure 4c: Mass spectra of (I) EEE-PFP, (J) HBEG-2PFP, (K) NBEG-2PFP, and (L) NCOE-PFP.

DISCUSSION

This study describes a sensitive and specific method for simultaneously analyzing COC, COE, their metabolites and pyrolysis products, and other related compounds. In this method, PFPA and PFPOH were used for the derivatization of the analytes. PFPA reacts with the hydroxyl and primary/secondary amino groups to form pentafluoropropionyl ester and amide derivatives, respectively, while PFPOH reacts with the carboxyl groups to produce pentafluoropropionyl ester derivatives. Therefore, the combined use of PFPA and PFPOH becomes an efficient reaction mixture for simultaneously derivatizing hydroxyl, amino, and carboxyl functional groups. Such derivatization was effective in selecting unique analyte ions for the SIM analysis of various analytes, particularly of those having more than one of these functional groups.

Findings of this study suggest that one must be cognizant of the potential misinterpretations of the presence of AEME when high concentrations of COC are encountered in biological samples. However, the relationship established between the AEME production and the COC concentration could be effectively utilized for calculating the amount of true AEME present in a biological sample. Additionally, AECG was not found to be a viable analyte for establishing whether COC had been smoked, because the inlet pyrolytic production of AECG-PFP from the PFP derivatives of BEG, ECG, and HBEG would completely mask the AECG originally present in a biological specimen. The artifactual GC inlet production of AECG-PFP is most likely mediated via a pyrolytic cleavage of one of the ester groups of the PFP derivatives of BEG, ECG, and HBEG, leading to the formation of the C=C containing AECG-PFP.

Neither the specimens from the pilot fatalities nor the blood specimens from the OSBI were found to have concentrations of AEME greater than those pyrolytically produced from the COC-spiked bovine blood samples (Table II). Although the AEME concentration in urine was higher than that in any other CAMI/OSBI case specimens, the urine AEME concentration of 35 ng/mL in the presence of 846 ng/mL of COC was consistent with the amount of AEME produced from the bovine blood sample fortified with 800 ng/mL COC (Table II; Fig. 5). These observations suggested that the individuals probably consumed COC by a route other than smoking. In addition, the absence of COE, NCOE, and EEE in the blood case specimens indicated that ethanol was not consumed during the ingestion of COC, since these compounds are synthesized *in vivo* in the presence of COC and ethanol (10). However, the presence of EEE—a possible hydrolytic product of COE and a biosynthetic product of EME—in the absence of COE and NCOE in the urine and muscle samples may not necessarily indicate that ethanol was consumed with COC in these two cases, but ethanol might have been consumed much later after the metabolism of COC and/or produced postmortem. This conclusion is based on the facts that EME was present in both samples, particularly in the urine wherein the EME concentration was very high (5203 ng/mL), and ethanol was found in the muscle and in the companion blood sample of the case with urine. Therefore, the production of EEE could have been attributed to the transesterification reaction of EME and ethanol.

COC, COE, and PFP derivatives of EME and BEG have been analyzed previously in meconium, whole blood, and plasma (15), but the applicability of this method for the analysis of other COC and COE metabolites was not

Table II: GC Inlet Pyrolytic Production of AEME at 210°C From Various Concentrations of COC in Bovine Blood*

Cocaine (ng/mL)	Pyrolytically Produced AEME (ng/mL)	Pyrolytically Produced AEME (%, COC Concentration)
25	13	52.0
50	13	26.0
100	13	13.0
200	15	7.5
400	18	4.5
800	30	3.8
1600	40	2.5
3200	75	2.3
6400	132	2.1

*Details are given in the Experimental and Results sections.

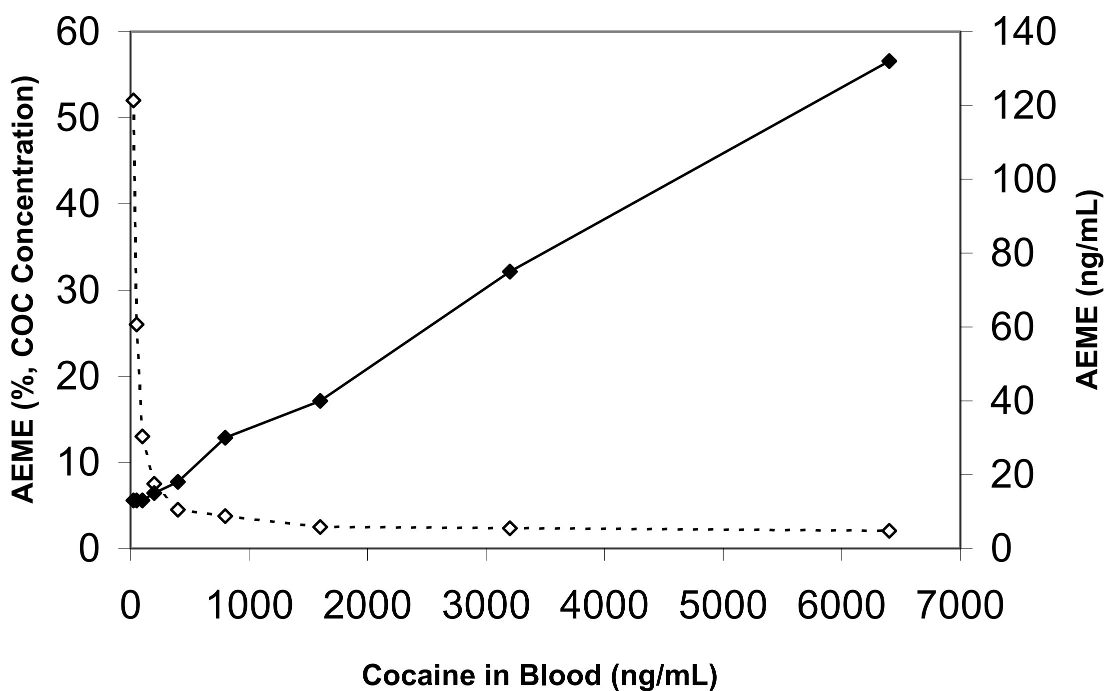

Figure 5: A graphic representation of the relationship between the amount of AEME produced pyrolytically from various concentrations of COC in bovine blood at the 210°C GC inlet temperatue.

Table III: Analysis of CAMI and OSBI Case Specimens*

Specimens		Analytes (ng/mL)								
Source	Type	COC	BEG	NBEG	NCOC	ECG	EME	HBEG	AEME	EEE
OSBI 1	Blood	24	1205	<1	†ND	5497	18	25	11	ND
OSBI 2	Blood	25	1318	23	ND	15826	10	86	ND	ND
OSBI 3	Blood	ND	69	ND	ND	1119	ND	5	6	ND
OSBI 4	Blood	ND	1130	ND	ND	8497	<1	5	ND	ND
OSBI 5	Blood	10	1949	49	ND	21231	43	46	ND	ND
OSBI 6	Blood	2	1270	ND	ND	10148	2	23	13	ND
OSBI 7	Blood	2	696	ND	ND	3344	9	ND	12	ND
OSBI 8	Blood	ND	349	ND	ND	4311	ND	ND	ND	ND
OSBI 9	Blood	ND	505	ND	ND	3727	ND	ND	10	ND
OSBI 10	Blood	ND	218	ND	ND	1215	ND	ND	ND	ND
CAMI-1A	Blood	62	159	ND	ND	ND	78	ND	12	ND
CAMI-1B	Urine	846	7760	511	20	15126	5203	19	35	44
CAMI-2	Muscle	ND	48	ND	ND	ND	8	ND	ND	7

*All samples were negative for COE and NCOE. AEME concentrations are uncorrected with respect to the COC concentrations. AECG values are not included in this table because large amounts of AECG-PFP can also be artifactually produced from BEG-PFP, ECG-2PFP, and HBEG-2PFP (Fig. 6).

†ND = Not detected.

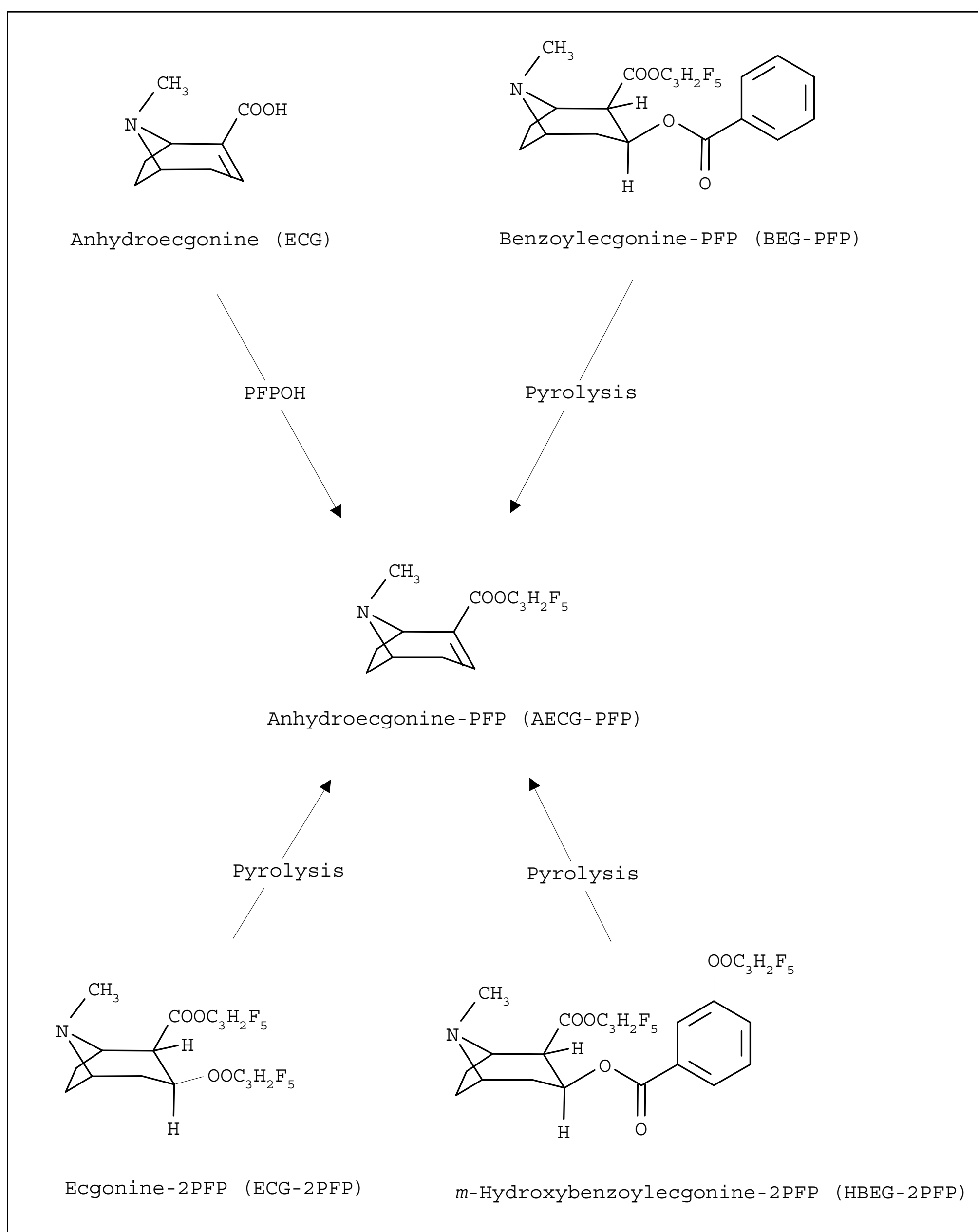

Figure 6: PFPOH derivatization of AECG into AECG-PFP and predicted artifactual production of AECG-PFP from the GC inlet pyrolysis of three PFP metabolite derivatives.

demonstrated in the study. In another method, 11 COC metabolites in urine were analyzed by performing a single extraction and by converting them into their PFPA and hexafluoropropyl derivatives (13). However, the effectiveness of this method in other types of biological samples was not demonstrated. An analytical procedure for the pyrolysis product AEME was reported using *N,O-bis-*(trimethylsilyl)trifluoroacetamide as a derivatizing reagent (7), but derivatives of this reagent are considered less stable than the PFP derivatives. Additionally, the methods reported in the literature lack sensitivity for analyzing the 11 analytes (not including AECG) in whole blood and other tissue types, as reported in the present study. In several methods (7, 8, 12, 14), the GC inlet temperature was ≥ 250°C, and concerns have been raised for the artifactual pyrolytic production of AEME during gas chromatography/mass spectrometry (4, 8, 14). Therefore, the AEME concentration reported in the literature may not represent the true concentrations of the analyte in the samples (7, 12). Although at 140°C no pyrolytic production of AEME was observed from COC (8), the sensitivity of the method at this temperature might not be analytically acceptable for detecting other analytes. The 210°C-inlet temperature adopted in the present study was suitable for minimizing the production of AEME without compromising the sensitivity of the method for other analytes.

Overall, the present study describes a selective and sensitive method for the simultaneous analysis of COC, COE, and their metabolite and pyrolysis products, involving solid-phase column chromatographic isolation, hydrochloride salt formation, PFP derivatization, and GC/MS analyzation. Converting the analytes into their hydrochloride salts by bubbling hydrochloric acid vapors through the column eluates, prior to the derivatization, significantly increased the sensitivity for detecting COC, BEG, NCOC, EME, HBEG, AEME, NCOE, and EEE. Additionally, the method could be used to analyze NBEG, AECG, COE, and, in particular, ECG in biological samples. ECG found in large amounts in case samples further suggests that this metabolite is a quantitatively prominent analyte that can be used as a marker for COC abuse. The developed method allows the analysis of specimen types—such as whole blood and muscle—wherein the concentration of these analytes are relatively less in comparison to such analytes present in urine.

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