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CASE REPORT

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Identification of Anhydroecgonine Ethyl Ester in the Urine of a Drug Overdose Victim

ABSTRACT: Toxicological evaluation of postmortem urine collected from a 41-year-old deceased white male detected anhydroecgonine ethyl ester (ethylecgonidine, AEEE), a transesterification product of smoked cocaine co-abused with ethanol. A solid phase extraction (SPE) method was used to extract cocaine, AEEE, and related metabolites from urine. SPE on a 1 mL urine sample from the decedent followed by GC-MS detected AEEE. Other metabolites identified by GC-MS included cocaine, cocaethylene, and anhydroecgonine methyl ester (AEME). To determine whether some or all of the AEEE was artifactually produced in the heated GC injector port, an alternative LC-MS method was developed. LC/MS following SPE found at least 50 ng/mL of AEEE in the extract. The mass fragmentation (MS/MS and MS^3) of AEEE detected in the urine was compared to spectra of authentic, synthesized compound. AEEE is a potential additional forensic marker for the co-abuse of smoked cocaine and ethanol.

KEYWORDS: forensic science, anhydroecgonine ethyl ester, ethylecgonidine, anhydroecgonine methyl ester, cocaethylene, pyrolysis, drugs of abuse

Free-base ("crack") cocaine smoking, popularized in America during the 1980's, continues to be a popular, toxic route of abuse and remains a problem of major public health and judicial law enforcement concern (1,2). Currently, there are an estimated 1.5 million chronic cocaine users over the age of 12 in the U.S.(2). The smoking of free-base cocaine forms primarily benzoic acid and anhydroecgonine methyl ester (AEME or methylecgonidine) (3,4). AEME has been reported to be an indicator of smoked cocaine use, and has been detected in forensic cases evaluating numerous matrices, including the urine, blood/plasma, saliva, perspiration, hair, brain, and liver of known crack cocaine users (5–9).

Several reports have documented the transesterification of cocaine in the presence of ethanol that converts a methyl ester to an ethyl ester (10,11). The ethyl ester analogue of cocaine is cocaethylene. This transesterification reaction is mediated by human carboxylesterase 1(hCE-1) (12,13), and is known to occur with other pharmaceutical agents, including meperidine, methylphenidate and acitretin (14–17) Cocaethylene, exhibiting similar pharmacologic properties to cocaine, has been detected in forensic cases (7,18,19). In an analogous fashion, AEME is postulated to transesterify in vivo to the ethyl ester, anhydroecgonine ethyl ester (AEEE), in the presence of ethanol. Fandino AS and co-workers (20) found that rat liver microsomes convert AEME to AEEE, and the transesterification is inhibited by NaF, a non-specific esterase inhibitor.

Ecgonine ethyl ester (EEE), a hydrolysis product of cocaethylene, has been detected in postmortem tissues/fluids (21) and toenail specimens (22). Although the presence of AEEE as a metabolite in cocaine and ethanol users was proposed earlier by Isenschmid DS (23), there are no documented peer-reviewed articles reporting the identification of AEEE in the urine (or other biological fluid) of known crack cocaine and ethanol users. Preliminary analysis of a drug overdose victim's urine by GC-MS at the Office of Chief Medical Examiner (OCME) of West Virginia identified relatively equally proportioned peaks corresponding to the appropriate parent mass and mass fragmentation patterns of AEME and AEEE. Our investigations, presented in this paper, verify these findings by GC-MS, quantify the relative amount of AEEE in the urine sample by liquid chromatography-mass spectrometry (LC-MS), and provide the mass fragmentation of this metabolite by LC-MS/MS, as compared to standard.

Case History

The decedent, a 41-year-old white male weighing 178 lb and 6′0″ tall, was found dead, collapsed outside his vehicle one morning. His wife stated that he had left home after an argument at approximately 2330 hours. A friend reported that the decedent had been out drinking the previous night. The decedent's wife stated that he had a history of alcohol and illicit drug use but that he had been "clean" for the past 6–7 weeks.

Preliminary Toxicological Evaluation

The body was brought to the state medical examiner's office and a complete autopsy was performed. Blood alcohol concentrations

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were determined by direct injection GC-FID analysis using *t*-butanol as an internal standard. Urine was screened for drugs of abuse and tricyclic antidepressants by enzyme mediated immunoassay (EMIT) using a Roche Cobas Mira with kits purchased from Dade Behring (Cupertino, CA). Urine was also screened for basic drugs using the Toxi-Lab A extraction system (Varian Inc.; Walnut Creek, CA) with Proadifen (SKF-525A) added as an internal standard. After a 20-min period in which the sample was inverted then centrifuged, the organic layer was evaporated to dryness and reconstituted with 75 μL of ethyl acetate. One microliter was injected onto an Agilent 6980/5973 GC-MS and analyzed in full scan mode. Peak identification was accomplished by comparison to a house mass spectral library, the AAFS drug library and the National Institute of Standards and Technology (NIST) 2002 library. Confirmation and quantitation was by GC-MS. Oxycodone was present in the subclavian blood at 4.40 mg/L and methadone at 0.25 mg/L. Cocaine was confirmed at <0.05 mg/L, benzoylecgonine at 0.18 mg/L and ethanol at 0.10%. Other compounds identified in the urine but not quantified include AEME, AEEE, cotinine, and two methadone metabolites, EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) and EMDP (2-ethyl-5-methyl-3,3-diphenylpyrroline). Cause of death was combined oxycodone, methadone, cocaine and alcohol intoxication. The manner of death was accidental.

Materials and Methods

Materials

Solvents, including acetonitrile, ethyl ether, methanol, methylene chloride, and 2-propanol, were purchased from Fisher Scientific (Fair Lawn, NJ). Optima grade solvents were used for mass spectrometry analysis and SPE elution, whereas laboratory or HPLC grade solvents were utilized for synthetic procedures. Additional chemicals purchased from Fisher Scientific included ammonium hydroxide (21%), glacial acetic acid, sulfuric acid, ammonium acetate and potassium phosphate. Absolute ethyl alcohol was obtained from Aaper Chemical Co. (Shelbyville, KY). Benzene, cocaine HCl, deuterated NMR solvents, fumaric acid, and hydrochloric acid (37%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Synthetic Procedures

Anhydroecgonine ethyl ester (AEEE, EEG, ethylecgonidine) was synthesized by modifications of reported procedures (24,25).

Anhydroecgonine (AE)—Briefly, 300 mg cocaine HCl (0.88 mmol) was dissolved in 15 mL concentrated HCl and refluxed at 110°C for 24 h. After cooling, the aqueous solution was extracted thrice with ethyl ether (30 mL) to remove benzoic acid. The resulting aqueous layer was evaporated in vacuo and the resultant residue was azeotroped with toluene (30 mL). An isomeric mixture of anhydroecgonine hydrochloride was obtained by precipitation of the residue from an acetone/water mixture. Product (149 mg, 0.73 mmole) had m.p. 235–240°C (lit. (26) m.p. 239–243°C); ESI-MS ($MH^+ = 168$; MS/MS = 150, 137, 122, 93, 82); and ^{1}H NMR spectroscopy (DMSO-d_6): δ 4.32 ppm, 4.3 ppm (H-1); 6.78, 6.88 (H-3); 3.00, 2.8 (H-4_{axial}); 2.39 (H-4_{eq}); 3.95, 4.07 (H-5); 1.79, 2.24 (H-6_{axial}); 2.30, 2.26 (H-6_{eq}); 2.34, 2.36 (H-7_{axial}); 2.0, 2.04 (H-7_{eq}); 2.73, 2.65 (N-CH_3).

Anhydroecgonine Ethyl Ester (AEEE)—Anhydroecgonine HCl (139 mg, 0.68 mmole) was dissolved in 10 mL absolute ethanol, 5 mL benzene and 0.75 mL H_2SO_4 and set up in a Dean-Stark apparatus. The mixture was refluxed for three hours and cooled, following which 10 mL of absolute ethanol were added, the Dean-Stark tube removed, and the mixture was refluxed for 2 h. Upon cooling, the organic layer was evaporated in vacuo, the residue was dissolved in 8 mL distilled H_2O and alkalinized with several drops of concentrated NH_4OH to about pH 10. The basic, aqueous layer was extracted several times with methylene chloride (30 mL), and then the combined organic layers were dried over sodium sulfate, vacuum filtered and evaporated in vacuo. AEEE free-base (54 mg, 0.28 mmole) was combined with fumaric acid (0.28 mmole) and dissolved in 1 mL of hot ethanol. The solution was cooled to room temperature, precipitated slowly by the dropwise addition of ethyl ether (5 mL), cooled overnight at 0°C, vacuum filtered and evaporated *in vacuo* to give AEEE fumarate (81 mg, 0.26 mmole): m.p. 138–141°C (lit. (25) m.p. 144–146°C); GC–MS (**195**, 166, 138, 122, 94, 82); ESI-MS ($MH^+ = 196$; MS/MS = 168, 150, 122, 108, 91; MS^3 of 150 = 132, 122, 109, 96, 93, 82); ^{1}H NMR spectroscopy (DMSO-d_6): δ 3.84 ppm (H-1); 6.78 (H-3); 2.0, 2.65 (H-4); 3.39 (H-5); 1.54, 2.10 (H-6); 1.78, 2.10 (H-7); 4.13 (O-$\mathbf{CH}_2CH_3$); 1.22 (O-$CH_2\mathbf{CH}_3$); 2.36 (N-CH_3). Proton chemical shift assignments were greatly assisted by a detailed NMR analysis (^{1}H, COSY, ^{13}C, HETCOR experiments) of the related congener, AEME (synthesis and proton and carbon shift assignments not reported), and previously reported ^{1}H NMR spectra of AEME (24,27).

Instrumentation

The ^{1}H and COSY NMR data were recorded on a Varian Inova (Palo Alto, CA) 600 MHz spectrometer. GC-MS analysis of the metabolites was performed with an Agilent (Palo Alto, CA) 5973 Electron-Impact-gas chromatograph/mass spectrometer. Samples were analyzed on a Supelco Equity 1 (St. Louis, MO) capillary column with an oven temperature of 90°C held for 1 min and then programmed at 20°C/min to 280°C, with a total run time of 12.50 min. Helium was the carrier gas, and the injection volume was 2 μL per injection.

LC-MS: LC analysis of compounds was performed with a Waters 2695 (Milford, MA) separation module, using a Zorbax SB-Phenyl reversed phase analytical column (4.6 × 150 mm ID, 5 micron) and a Waters 996 Photodiode Array Detector with UV scanning from 200 to 400 nm. The mobile phase consisted of acetonitrile:ammonium acetate buffer (10 mM, pH 6.80) in a 70:30 ratio, and was run at 0.5 mL/min. This HPLC system was coupled to a Waters Micromass ZMD mass spectrometer programmed to utilize electrospray ionization in a positive ion mode. Source conditions: capillary 3.35 kV; sample cone 20 V; extraction cone 8 V; source block temperature of 100°C and desolvation gas temperature of 300°C; and desolvation gas flow and cone gas flow (both N_2) were 250 L/h and 95 L/h, respectively

Multistage mass spectrometry LC-MS: A Shimadzu (Columbia, MD) LC-$10AD_{vp}$ LC system (equipped with a SPD-10A UV/VIS detector, SIL-$10AD_{vp}$ auto injector and DGU-14A degasser) coupled to a Thermoquest LCQ DECA (San Jose, CA) ion trap mass spectrometer was used for MS, MS/MS and MS^3 analyses. The electrospray source (ESI) parameters were the following: positive ion detection; sheath N_2 gas flow rate 80 (arbitrary units); spray voltage 2.5 kV; capillary temperature 240°C; capillary voltage 7.00 V; and tube lens offset 15.0 V. The LC column and mobile phase were identical to the column and solvent system described above.

Extraction Procedure

Urine collected from the overdose victim was kept at −80°C in a vacuum-sealed container. Sample was completely thawed before

use by placing in a laminar airflow hood (Labconco, Kansas City, MO) at room temperature for several hours.

The solid phase extraction (SPE) method was modified from a literature procedure (28). Solid phase extraction was performed with Varian Bond Elut Certify (130 mg/3 mL) cartridges containing a mixed mode sorbent with non-polar and cation exchange mechanisms. Extraction cartridges were subjected to reduced pressure (to facilitate solvent flow) on a PrepTorr (Fisher Scientific) vacuum manifold connected to a water aspirator. Extraction was initiated by preconditioning the cartridge with 2 mL of acetonitrile at a flow rate under vacuum (8 kPa) of 0.75 mL/minute, followed by 3 mL of potassium phosphate buffer (0.1 M, pH = 6.0). Next, a well-mixed, pre-prepared solution (5 mL) consisting of 1 mL thawed urine from the decedent diluted in 4 mL phosphate buffer, was loaded onto the extraction cartridge. The cartridge was rinsed with 2 mL acetic acid solution (0.1 M) followed by 3 mL of acetonitrile, and the sorbent was dried under vacuum for one minute. The analytes of interest were eluted with 6 mL of elution solvent consisting of a freshly prepared mixture of methylene chloride:2-propanol:22% ammonium hydroxide (78:20:2). Elution solvent washed through the column was initially collected in 1.5 mL centrifuge tubes, then transferred to a round bottom flask (10 mL), evaporated in vacuo at room temperature using a rotavapor apparatus, and further dried in a vacuum dessicator. The small residue was carefully reconstituted in appropriate solvent and subjected to analysis via GC-MS or LC-MS.

Each SPE separation utilized an individual cartridge. Control solutions, containing either blank urine or urine from a victim who tested positive for cocaine, but not ethanol (obtained from the OCME of West Virginia), were subjected to similar SPE and LC-MS analysis.

Standard Curve Generation

A six point linear standard curve ($r^2 = 0.999$) was generated on LC-MS (Waters, Micromass) by injecting authentic AEEE (in ethanol) in a concentration range of 0.6 ng to 19 ng on column ($k' = 6.8$ min). The ion peak corresponding to $MH^+ = 196$ was integrated by MassLynx 3.5 software (Waters) and the resulting peak areas were quantified linearly by regression analysis (Sigma Plot 2001).

Results

AEEE was identified in a urine sample that also contained cocaine, cocaethylene and AEME. By GC-MS, the peak eluting at 4.67 min corresponded to AEEE. The other compounds identified exhibited the following retention times (min): AEME (4.28), cocaine (8.79), cocaethylene (9.04). A comparison of peak heights from 0 to 12.5 min showed relative concentrations of AEME, cocaine and cocaethylene. The relative peak height ratio of AEME:AEEE was 1.5:1. In addition, the relative peak height ratio of cocaine:cocaethylene was 1.7:1, whereas the ratios of cocaine or cocaethylene to there related breakdown product, AEME or AEEE, was 2.8:1 and 2.4:1, respectively. The possibility that AEEE is an artifact produced in the GC injector port by thermal decomposition of cocaethylene was not ruled out.

To address the possible thermal breakdown of cocaethylene on GC-MS, an analytical process was applied for the detection of AEEE that would have less potential for producing AEEE as an artifact. Urine from the same overdose victim was analyzed by SPE followed by LC-MS. Reconstituted urine SPE extract (40 μL) was injected and the corresponding compounds (with retention times in minutes) were detected: AEME (6.63), AEEE (6.83), cocaine (7.33) and cocaethylene (8.13). By comparison with a standard curve, the approximate concentration of AEEE in the urine sample was 50 ng/mL. Relative peak height ratios were as follows: cocaine:cocaethylene (1.6:1); AEME:AEEE (3.5:1); cocaine:AEME (1381:1); and cocaethylene:AEEE (3812:1).

Authentic AEEE (10 μg/mL in acetonitrile) was injected directly into an ion trap ESI-MS and the mass fragmentation was recorded. Confirmed AEEE in a similar concentration was injected (20 μL) onto the LC-MS (Shimadzu/Thermoquest system) and MS/MS analysis on the parent mass, $MH^+ = 196$, with collision energy of 20%, was performed up to 15 min. In the urine sample, a low intensity ion peak at a similar retention time to standard, ca. 8.44 min, was detected for the following MS/MS ions: 168, 150, 122, and 91, confirming the presence of AEEE in the sample. An additional ion peak eluting at 10.42 min was assigned as cocaethylene based on an analysis of its mass spectrum. It is interesting to note the presence of a cocaethylene fragment ion at the same m/z value as the AEEE MH^+ ion (m/z 196) indicating that the ESI ionization process produces the same product as does thermal degradation. Thus, separation of AEEE from cocaethylene by LC or other chromatographic process is necessary to give an accurate identification of AEEE.

Two different control urine samples were analyzed under the same experimental conditions as the case sample. LC-MS (Waters/Micromass system) on a 1 mL blank urine sample showed no trace of cocaine, cocaethylene, or pyrolysis products. Another urine sample obtained from the OCME was analyzed, and the compounds identified included cocaine and AEME. This sample contained no detectable levels of either cocaethylene or AEEE.

Discussion

Free-base ("crack") cocaine smoking, in particular, remains a popular method of cocaine abuse due to numerous factors, including rapid onset of action (<10 sec), high bioavailability of aerosolized particles, relatively inexpensive street value and lower risk of HIV transmission (29). Anhydroecgonine methyl ester (AEME) and related isomers were reported in 1985 as thermolytic breakdown products of cocaine in simulated in vitro pyrolysis experiments (3). Numerous studies thereafter have identified AEME in vivo as a pyrolysis product of smoked cocaine (5,7,9,30). Experimenters were able to recover up to a maximum of 5% AEME from a 30 mg freebase cocaine dose after a pyrolysis experiment utilizing a Bunsen burner and model crack pipe (4). Chemical and/or enzymatic hydrolysis of AEME forms anhydroecgonine (AE), or ecgonidine (20), which can also be used as an indicator of smoked cocaine use (31,32).

A known, documented drug-drug interaction exists during the concomitant administration of cocaine and ethanol, forming ethyl cocaine, or cocaethylene (10,11,33). Cocaethylene contains similar reinforcing properties as cocaine, a longer elimination half-life and slower clearance, suggesting that it is a more toxic compound than cocaine (34,35). This methyl to ethyl replacement reaction, or transesterification, occurs between AEME and ethanol in rat liver microsomes, and is blocked by the non-specific esterase inhibitor, NaF (20).

AEME exhibited muscarinic activity when given intravenously (0.1–3.0 mg/kg) to sheep, and these effects were blocked by pretreatment with atropine methyl bromide (15 μg/kg), a known cholinergic antagonist (32). Pharmacological studies conflict on the chemical actions of AEME's transesterification product, AEEE. An intravenous dose of 3 mg AEEE was administered to rabbits, producing similar effects to AEME, such as hypotension and increased heart rate (36). Another study reported that AEEE exhibited only weak activity at muscarinic subtypes and showed no receptor

selectivity (24). The exact role of cocaine pyrolysis products (AEME, AEEE, AE) in the sequelae of cocaine related toxicity continues to be studied.

In this forensic investigation, we utilized a SPE method to separate cocaine and related metabolites from urine. We report the first documented identification, to our knowledge, of AEEE in a human subject who co-abused crack cocaine and ethanol. GC-MS was initially used to detect AEEE in a urine sample. However, the amount of AEEE in the urine quantified by standard curve (data not shown) on GC-MS was inflated due to pyrolysis of cocaethylene to AEEE in the heated GC injector port. A recent report documented the analogous generation of an artifact AEME peak from pyrolysis of cocaine during GC-MS analysis (28). Furthermore, artifact AEME production is linearly dependent on cocaine concentration and attenuated by the use of a clean insert liner (28). Identification of AEME by GC-MS analysis is equivocal because of the potential for artifactual production in heated injector ports of gas chromatographs. Analogously, application of GC-MS methods to the identification of AEEE in the presence of cocaethylene is also equivocal.

A more reliable alternative to the recently reported internal or external standardization method (28) is the use of a less thermolytically driven analytical step, such as LC-MS.

Our LC-MS assay detected AEEE in urine following SPE at a concentration of approximately 50 ng/mL. This extrapolated value estimates the relative amount of AEEE in the urine and further confirms its presence. The concentrations of cocaine and cocaethylene in the sample were substantially greater than their respective thermolytic products, but were about equally concentrated. The data suggests that the transesterification of cocaine to cocaethylene proceeds at a faster rate than the analogous reaction of AEME to AEEE. Furthermore, it may be likely, although not proven by our investigations, that cocaine is a better substrate than AEEE for hCE-1, the transesterification enzyme (12–14,34).

Our multistage mass spectrometry LC-MS analysis represents a third analytical approach for identifying AEEE in the urine sample. Our analysis found that the electrospray ionization process also fragments cocaethylene to yield an ion of the same molecular weight as protonated AEEE, but LC was capable of separating the ions. This points to the need for LC separation of cocaethylene from AEEE to correctly identify AEEE in future samples. Although GC-MS fragmentation pathways are more reliable and more commonly used in forensic laboratories, our concern for artifactual production of AEEE warrants confirmation by LC-MS ion trap analysis.

To summarize, we detected AEEE in the urine of a drug overdose victim with a history of drug abuse by GC-MS, LC-MS, and LC-MS/MS. AEEE is a potential additional forensic biomarker for the detection of crack cocaine abused concomitantly with ethanol. LC-MS provides the basis for a useful analytical method in forensic cases involving suspected free-base cocaine/ethanol use.

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