Rapid Confirmation/Quantitation of Ecgonine Methyl Ester, Benzoylecgonine, and Cocaine in Urine Using On-Line Extraction Coupled with Fast HPLC and Tandem Mass Spectrometry

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Abstract

A rapid, rugged, and highly specific assay for the quantitation of cocaine (COC) and especially its primary metabolites benzoylecgonine (BZE) and ecgonine methyl ester (EME) in human urine has been established. Here, we investigated the use of on-line sample extraction coupled to rapid chromatography systems for tandem mass analysis of COC, EME, and BZE in human urine. Using this method, sample preparation consisted of a sole centrifugation step. Combined extraction and chromatographic run times were < 3.5 min. The lower limits of detection were 0.5 ng/mL, 2.0 ng/mL, and 0.5 ng/mL for EME, BZE, and COC, respectively. Linear calibration curves ranging from 7.5 ng/mL to 1000 ng/mL were produced for the test analytes. Within-day and between-day precision and accuracy of the assay were determined using human urine quality-control specimens at 5, 10, or 15; 150; and 1000 ng/mL. The analyses were performed over the course of five days, rendering %CVs < 10% for EME, BZE, and COC. Percent mean accuracy for the three analytes of 97 to 113% were obtained. Our data suggest that on-line sample extraction coupled with rapid high-performance liquid chromatography–tandem mass spectrometry may be a viable alternative for EME, BZE, and COC analyses in human urine.

Introduction

Cocaine (COC) the major alkaloid of the Erythroxylum coca plant is a potent brain stimulant and one of the most vigorously addictive drugs. The rewarding chemical effects produced by COC are thought to result from potentiation of dopamine re-uptake transporters in the limbic forebrain, specifically in the nucleus accumbens (NAc) (1).

Via distinct mechanisms, COC is metabolized in vivo to benzoylecgonine (BZE) and ecgonine methyl ester (EME). Both compounds remain detectable 4–6 times longer than COC in urine and plasma. Studying the causative factors, which alter the pharmacological and toxicological effects of COC, is of significant clinical importance. A sensitive and specific biochemical assay would provide insight into the in vivo disposition of COC.

Currently, quantitative methodology for the determination of EME, BZE, and COC in biological matrices includes high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), and liquid chromatography–mass spectrometry (HPLC–MS) (2–4). Additionally, a variety of immunoassay screening procedures are used to provide semiquantitative analysis of biological samples (5–7). Traditional quantitative methodologies based on HPLC, and to a lesser degree on HPLC–MS, necessitate a substantial amount of sample cleanup in order to attain desired levels of detection. A major disadvantage of quantitative GC–MS methodology is the requirement for extensive sample cleanup, followed by derivatization of the extracts to appropriate volatile analogues prior to analysis. Replacement of these established analytical techniques with emerging HPLC–MS strategies has met considerable resistance. Many current investigations continue to employ traditional GC–MS methodologies (8–10).

The use of automated solid-phase extraction procedures (SPE) is an alternative that minimizes the amount of time spent by technical staff preparing samples for analysis by traditional methods. There are a number of commercially available...
robotic systems for the analysis of drugs, and a few publications have demonstrated their utility for analyzing drugs of abuse (11–13). Although the precision and accuracy data obtained from automated procedures has been shown to be equal to or superior to that of manual extractions, a major drawback is that SPE remains compound specific. For that reason, development of a generic procedure for extraction of multiple “drugs of forensic interest” from biological matrices may be unachievable. Inevitably, sample preparation requirements make HPLC forensic interest” from biological matrices may be unachievable. Inevitably, sample preparation requirements make HPLC

HPLC—MS is an alternative approach that can be implemented to reduce off-line sample preparation because it allows direct analysis of thermally labile and non-volatile compounds (14–16). Furthermore, the utility of atmospheric pressure ionization mass spectrometry (API-MS) has gained widespread popularity as an analytical tool for the quantitative determination and structural characterization of pharmacologically active compounds a time-consuming process that cannot be circumvented with automated extraction procedures.

We report here an assay for the simultaneous analysis of EME, BZE, and COC in human urine with low nanogram-per-milliliter detection levels. This method is based upon on-line extraction—rapid HPLC—ESI—MS—MS, and was used to determine concentrations of EME, BZE, and COC in urine of COC users.

Experimental

Materials

Cocaine (free base), EME, EME-d₃, benzoylecgonine hydrate (BZE), and COC-d₃ were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). A weighing of each analyte was made from neat material, and standard stock solutions (300,000 ng/mL) of these compounds were prepared in HPLC-grade dimethyl sulfoxide obtained from J.T. Baker Co. (Phillipsburg, NJ) and stored at 4°C. Primary dilutions of the standard stock solutions were made to provide secondary stock solutions (50,000 ng/mL) in 90% HPLC grade water: 10% HPLC-grade acetonitrile, both obtained from the J.T. Baker Co. Working standards were prepared by appropriate dilution of the secondary stock standards into sterile control human urine obtained from Rockland Immunochemicals (Gilbertsville, PA). Aliquots of the EME, BZE and COC working standards were combined into a single set, and serially diluted to six different concentrations (7.5, 15, 30, 75, 150, 500, and 1000 ng/mL). A dilution of the EME-d₃/COC-d₃ stock internal standard solution was made into a 90:10 mixture of HPLC-grade H₂O and HPLC-grade methanol (obtained from the J.T. Baker Co.) to give a working concentration of 300 ng/mL. All specimens were spiked with 200 µL of internal standard before analysis to give a working concentration of ~150 ng/mL. EME, BZE, and COC quality-control specimens were prepared using sterile control human urine obtained from Rockland Immunochemicals fortified at 5-, 10-, or 15-; 150-; and 1000-ng/mL concentrations.

The extraction mobile phase consisted of 4% 2-propanol (J.T. Baker Co.) in 10mM ammonium formate (Spectrum Chemical Manufacturing Corp., Gardena, CA) adjusted to pH 10.4 with ammonium hydroxide (J.T. Baker Co.). The aqueous component of the analytical mobile phase consisted of 50mM formic acid (Acros, Geel, Belgium) and 100mM ammonium formate (Spectrum Chemical Manufacturing Corp.), and the organic component consisted of 60% HPLC-grade acetonitrile/40% acetone (J.T. Baker Co.). All HPLC—MS—MS mobile phases were filtered through a 0.45-µm TF (PTFE®) membrane filter (Gelman Sciences Inc., Ann Arbor, MI).

Mass spectrometry and sample introduction

ESI—MS—MS experiments were conducted using a Finnigan MAT (San Jose, CA) Triple Stage Quadrupole (TSQ) 7000 MS with a Finnigan API source. The data system consisted of a Compaq (Houston, TX) Pentium® III AP400 Professional Workstation operating Finnigan XCALIBUR Rev. 1.0 system software, Gilson (Middleton, WI) #215 Multiprobe Autosampler with a #819 injection valve actuator, three Jasco Inc. (Tokyo, Japan) model PU-980 HPLC pumps and model DG-980-50 HPLC degasser, and a Rhodyne LabPro (Rohnert Park, CA) model PRT750-100-02 six-port column switching valve. Extraction chromatography was performed on a 20-mm x 2.0-mm i.d. Micro BioTrap® 500 MS column (Analytical Sales and Service, Mahwah, NJ). Analytical chromatography was performed on a 30-mm x 2.1-mm i.d. column with 5.0-µm packing Allure Basix® HPLC column (Restek Corp., Bellefonte, PA).

Analytical procedure

Ten-microliter injections of human urine specimens, control blanks, human urine QCs, and standards were made onto the extraction column at room temperature (~25°C). During this initial period, the switching valve was in the divert position, averting presentation of unwanted endogenous material to the ion source. At 1.21 min, the valve was switched in-line with the API ion source and delivered analytical mobile phase to the extraction column and subsequently to the analytical column. The output of the HPLC column flowed into the electrospray interface of the MS. The valve was held in this elution position for 2.0 min. At 3.2 min, the switching valve was reset and the system was restored to initial conditions. The heated capillary was operated at a temperature of 250°C. The positive ion mode was used for all acquisitions. Conditions for product ion scans and MRM transitions were as follows: ESI electrode voltage of 5500, sheath gas pressure at 50 psi, and auxiliary gas pressure at 30 psi. Collision-induced dissociation (CID) studies were performed using argon with a collision cell gas pressure of 2.0 mtorr and varying collision energies (CE). The detector was operated at 1440 volts, and each of the following MRM transitions was monitored for 300 ms: EME: m/z 199.9 → m/z 181.9, CE = 25 eV; EME-d₃: m/z 202.9 → m/z 184.9, CE = 25 eV; BZE: m/z
Three replicate injections of each blank, standard, QC, and sample were made. Quantitative results represent the average of these measurements.

**Sample preparation**

The Chemical Forensic Laboratories, School of Pharmacy, Medical Science Campus, University of Puerto Rico (San Juan, PR), provided human urine samples that had been previously screened and tested positive for BZE. The specimens were collected in 1.0-mL Eppendorf® polypolyene microcentrifuge tubes from Brinkman Instruments, Inc. (Westbury, NY). Aliquots (500 μL) of human urine specimens, human urine QCs, and standard curves were transferred into 96 deep-well plates (Marsh Biomedical Products, Inc., Rochester, NY) and fortificled with 200 g/L of internal standard solution. The deep-well plate was placed on a SORVALL® RC 3C PLUS centrifuge (Sorvall Instruments, Newton, CT) and spun at 4500 rpm for 15 min prior to analysis.

**Data analysis**

Calibration curves ranged from 7.5 to 1000 ng/mL for EME, BZE, and COC. For each curve, seven different concentrations were distributed throughout the range of the curves were used. Peak-area ratios between EME and EME-d₃, BZE and EME-d₃, and COC and COC-d₃ were calculated for each concentration using Finnigan XCALIBUR LCQuan Rev. 1.0 system software. The data were fit to a linear least squares regression curve with a weighting index of 1/x. Human urine blank samples fortified with internal standard were analyzed with each calibration curve.

**Accuracy and precision**

QC samples at three different concentrations for each analyte were examined on five separate days to validate the method. Five replicates for each QC sample concentration were processed and analyzed together with each seven-point standard curve sample. Method accuracy was assessed by comparing the means of EME, BZE, and COC with the theoretical concentrations in the QC samples, and expressed as percentages. Intra-assay precision was determined by calculating the percent ratio between the relative standard deviation (%RSD) of the five replicates and the mean at each concentration within the same validation run. Interassay precision was expressed as the %RSD of three different validation runs, each performed four times.

![Figure 1](image-url)
Results and Discussion

On-line sample extraction–LC–positive ion electrospray–MS–MS

The positive ion ESI daughter-ion mass spectra and proposed fragmentation pathways of the protonated molecules for EME, BZE, and COC are shown in Figure 1. These decompositions have been investigated and reported elsewhere (15,21). Although [M+H]+ ions were observed for EME, BZE, and COC, the decomposition of EME and COC to ecgonidine methyl ester and BZE to ecgonidine represent the base peak of each spectrum. Adducts derived from addition of ammonium salts and ammonium hydroxide, along with cluster ions, were not observed. To enhance method sensitivity, the capillary, tube lens, axis offset, and L11 voltages were fine-tuned to allow maximal transmission of these analytes. The retention times for EME, BZE, and COC were 1.55, 1.50, and 1.64 min, respectively. The tri-deuterated internal standards, EME-d3 and COC-d3, co-eluted with their structural analogues. Assay selectivity was confirmed by the absence of interfering peaks at the retention times for EME, EME-d3, BZE, COC, and COC-d3. The specific MRM transition and corresponding retention time were used to identify the analytes. CACM standard MRM ion chromatograms for a blank urine specimen and 7.5-ng/mL standard are depicted in Figure 2.

Mobile-phase additives are often used to improve chromatographic separations, increase analyte solubility, enhance ESI performance, and heighten ESI response of analytes (22). The volatile mobile-phase additives employed in this study are commonly used reagents for reversed-phase liquid chromatography (RPLC)–ESI–MS analyses. We recently reported on the effects of mobile-phase additives and eluents on the positive ion responses of EME, BZE, and COC (23). The results showed that the sensitivity for the test analytes was greatest in a mobile phase consisting of a 1:1 mixture of 60% acetonitrile/40% acetone and 100mM ammonium acetate. Our current analytical method was based on this information.

High-throughput HPLC–MS assays tend to relax chromatographic considerations and rely on the resolving ability of the MS to provide specificity. However, it is important to ensure that endogenous interferences from the matrix do not impede the analysis (24,25). For example, there may be endogenous

![Figure 2](image-url)
compounds in the biological matrix that give rise to ions identical to those of the analytes. Additionally, it is possible that interferences in "real-world" drug abuse specimens may not be observed in the control human urine. To eliminate these potential problems, we developed a high-throughput analytical method that exploits the positive attributes of on-line SPE, rapid chromatography with a new retentive stationary phase, and quantitative quadrupole tandem MS.

On-line sample extraction was performed with a 20-mm x 2.0-mm i.d. Micro BioTrap® 500 MS bio-extraction column. This column was designed to permit continuous direct injections and extraction of plasma, serum, urine, milk, and other biological matrices. The solid support of the column is coated with a-1 acid glycoprotein, and the interior channels are lined with a hydrophilic polymer material. This protein is stable in the pH range of 2-11 and is tolerant of high concentrations of organic modifier in the mobile phase. When an injection from a biological matrix is made onto the column, proteins and other endogenous material from the matrix are too large to penetrate the pores of the particle channel and are washed off the column to waste. In turn, the analytes become trapped to the hydrophilic polymer until the column is backflushed with analytical mobile phase. When analyzing basic drugs, the extraction mobile phase needs to be adjusted to at least a pH of 10. Traces of extraction mobile phase at this elevated pH could have serious effects on a typical silica-based analytical column. Therefore, the analytical mobile phase was adjusted to 50mM formic acid to eliminate the possibility of adversely affecting the analytical column. The addition of formic acid to the mobile phase also prevents the hydrolysis of EME, BZE, and COC. More than 500 injections of human urine specimens were made onto this column without an observed increase in column backpressure or decrease in extraction performance.

As previously noted, EME is extremely polar and elutes with relatively short retention times on a variety of stationary phases. Needham et al. (26) recently described the use of a pentfluorophenylpropyl (PFPP) stationary phase for the ESI-MS-MS analysis of EME and COC in urine. The PFPP stationary phase was shown to retain EME and COC with 90% acetonitrile in the mobile phase, whereas a C18 stationary phase only required 12% acetonitrile in the mobile phase. We evaluated the use of C1, C6, C8, C18, Supelcosil ABZ + Plus, and Allure Basix columns for the analytical separation. Only the Allure Basix column met our requirements for asymmetry and retention of COC and EME. The Allure Basix is a cyanopropyl-based column designed for the analysis of basic drugs, and exhibits retention characteristics similar to the PFPP stationary phase. This column was chosen because it gave excellent peak shapes for EME, BZE, and COC.

### Accuracy and Precision

**Accuracy and precision.** Human urine blanks showed no interfering signals at the retention times corresponding to EME, BZE, and COC (as depicted in Figure 2). Acceptable accuracy and precision were set at ±15% for the human urine QC specimens. The intrarun and inter-run accuracy and precision (%RSD) for the three analytes ranged from 97 to 113% and 0.7 to 9.3%. These data are summarized in Table I.

**System repeatability.** The system repeatability, given as percent coefficient of variation (%CV) of mean peak-area ratios, was assessed using 50 injections of a positive human urine specimen, #BDW11 12605 (see Table II). The analyses were performed over the course of five days, rendering %CVs of 0.73, 1.2, and 0.58 for EME, BZE, and COC, respectively.

**Limits of quantitation and detection.** The lower limit of detection (LOD) was defined as the lowest concentration of the calibration standards fortified in "pooled" control human urine yielding a signal-to-noise (S/N) ratio of at least 3:1. The limit of quantitation (LOQ) was defined as the lowest concentration of the calibration standards fortified in control urine with a precision of ±15%. The limits of detection were as follows: EME = 0.5 ng/mL, BZE = 2.5 ng/mL, and COC = 0.25 ng/mL. The LOQs for EME, BZE, and COC were 5.0 ng/mL, 7.5 ng/mL, and 2.5 ng/mL, respectively, with total coefficients of variation less than 10%. These data suggest that a quantitative assessment of EME, BZE, and COC in "real-world" positive urine specimens can be made with satisfactory assurance.

**Positive human urine samples.** The validated assay was used to determine levels of EME, BZE, and COC in human urine samples previously identified as positive by a certified drug-testing laboratory (CDTL). As shown in Table II, our data for BZE are in close agreement with the GC–MS values determined by the CDTL. Data above the upper limits of the calibration curves were reported as exceeding the linear range for the measurement. Additionally, our methodology provided data as previously noted, EME is extremely polar and elutes with relatively short retention times on a variety of stationary phases. Needham et al. (26) recently described the use of a pentfluorophenylpropyl (PFPP) stationary phase for the ESI-MS-MS analysis of EME and COC in urine. The PFPP stationary phase was shown to retain EME and COC with 90% acetonitrile in the mobile phase, whereas a C18 stationary phase only required 12% acetonitrile in the mobile phase. We evaluated the use of C1, C6, C8, C18, Supelcosil ABZ + Plus, and Allure Basix columns for the analytical separation. Only the Allure Basix column met our requirements for asymmetry and retention of COC and EME. The Allure Basix is a cyanopropyl-based column designed for the analysis of basic drugs, and exhibits retention characteristics similar to the PFPP stationary phase. This column was chosen because it gave excellent peak shapes for EME, BZE, and COC.

### Method validation

**Linearity.** The standard curves were plots of the ratios of analyte/internal standard responses (peak-area) as a function of analyte concentration. The concentrations of the standards ranged from 7.5 ng/mL to 1000 ng/mL. The data were fit to a linear least-squares regression curve with a weighting index of 1/x.

### Table I. Accuracy and Precision for the HPLC–MS–MS analysis of EME, BZE, and COC in Human Urine

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal concentration (ng/mL)</th>
<th>Accuracy (%)</th>
<th>Intra-assay precision (%)</th>
<th>Interassay precision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EME</td>
<td>10</td>
<td>102</td>
<td>3.1</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>105</td>
<td>2.6</td>
<td>8.6</td>
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<tr>
<td></td>
<td>1000</td>
<td>113</td>
<td>3.4</td>
<td>9.3</td>
</tr>
<tr>
<td>BZE</td>
<td>15</td>
<td>110</td>
<td>1.4</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>102</td>
<td>1.9</td>
<td>2.8</td>
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<tr>
<td></td>
<td>1000</td>
<td>101</td>
<td>1.2</td>
<td>2.2</td>
</tr>
<tr>
<td>COC</td>
<td>5.0</td>
<td>99</td>
<td>1.9</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>97</td>
<td>2.8</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>98</td>
<td>0.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Mean of three experiments each performed in quintuple.

* Mean of replicate analyses performed in quintuple, over the course of five days.
Table II. Comparison of GC-MS and On-Line Extraction-HPLC-MS-MS Data for 50 Urine Samples Previously Tested Positive for Cocaine

<table>
<thead>
<tr>
<th>Sample</th>
<th>GC-MS* (BZE ng/mL)</th>
<th>GC-MS* (ng/mL)</th>
<th>EME (ng/mL)</th>
<th>BZE (ng/mL)</th>
<th>COC (ng/mL)</th>
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<tbody>
<tr>
<td>TQ 62 23625</td>
<td>&gt; 1500</td>
<td>1500</td>
<td>&gt; 1000</td>
<td>1000</td>
<td>97</td>
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<tr>
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<td>&gt; 1000</td>
<td>502</td>
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<tr>
<td>CB84 19420</td>
<td>&gt; 1500</td>
<td>1500</td>
<td>530</td>
<td>&gt; 1000</td>
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<tr>
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<td>1500</td>
<td>&gt; 1000</td>
<td>1000</td>
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<tr>
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<td>1200</td>
<td>940</td>
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<td>11</td>
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<tr>
<td>IEG6 14363</td>
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<td>1500</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
</tr>
<tr>
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<td>1200</td>
<td>180</td>
<td>&gt; 1000</td>
<td>240</td>
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<td>950</td>
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<td>84</td>
</tr>
<tr>
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<td>1200</td>
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<td>1200</td>
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<td>940</td>
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<td>180</td>
<td>&gt; 1000</td>
<td>240</td>
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<tr>
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<td>&gt; 1000</td>
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<tr>
<td>Iw76 23284</td>
<td>1200</td>
<td>1200</td>
<td>1200</td>
<td>220</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* Data exceed the upper quantitation limit of 1500 ng/mL.

** Data exceed the upper quantitation limit of 1000 ng/mL.

Conclusions

We have shown that a combination of on-line sample extraction coupled to rapid chromatography and quadrupole tandem MS provides a sensitive, selective, and rugged system for the analysis of EME, BZE, and COC in urine specimens testing positive for COC. Our methodology offers a level of sensitivity and specificity unattainable with a traditional GC-MS-SIM measurement, suggesting its use for legal cases. With this method, reproducible retention times, along with high accuracy and precision, were obtained for EME, BZE, and COC. Total run time was 3.2 min, making it possible to analyze > 150 urine specimens in a single daily run. Sample preparation was held to a minimum and consisted of a sole centrifugation step. This method is an attractive alternative to traditional methodology for the quantitative and qualitative assessment of previously tested positive urine samples for COC.

Acknowledgments

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References

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