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Quantitation of cocaine and cocaethylene in small volumes of rat whole blood using gas chromatography-mass spectrometry

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Abstract

A simple solid phase extraction (SPE) technique combined with gas chromatography-mass spectrometry (GC/MS) operated in selected ion monitoring (SIM) mode is described for quantitation of cocaine and cocaethylene in small samples (250 μl) of rat whole blood. Use of (*N*-[^2H_3C])-cocaine and (*N*-[^2H_3C])-cocaethylene internal standards resulted in high sensitivity and selectivity for this analytical method. Analysis was performed using a Hewlett-Packard 5890 GC equipped with a 7673A Automatic Liquid Sampler linked to a Hewlett-Packard 5972 Mass Selective Detector. Separation of analytes was accomplished on a cross-linked methyl silicone gum capillary column (Ultra 1: 12m × 0.2mm (i.d.) × 0.33 μm). Linearity was established over a wide range of concentrations (5.0–2000.0 ng ml^{-1}) with good precision. Limits of detection (LOD) were 1.0 and 2.0 ng ml^{-1} for cocaine and cocaethylene, respectively. This analytical method was designed for use in pharmacokinetic experiments studying the formation of cocaethylene following ethanol pretreatment in rats administered cocaine. © 1997 Elsevier Science B.V.

Keywords: Cocaine metabolism; Cocaethylene; Carboxylesterases; Gas chromatography/mass spectrometry; Selected ion monitoring; Solid phase extraction

1. Introduction

Cocaethylene has been implicated as a mediator of many pharmacologic and toxicologic effects resulting from combined use of ethanol and cocaine. Cocaethylene has been shown to possess similar potency as cocaine on many neurochemical systems [1–5] and is more potent in mediating lethality [6]. This metabolite has been recovered in mice [7,8], rats, [9,10] and humans [12–14] following the co-administration of cocaine and ethanol. In vitro experiments have shown that a certain non-specific carboxylesterase fraction in liver [15] and kidney [8] is able to catalyze the formation of cocaethylene from cocaine and ethanol, most likely via transesterification [8].

This research was conducted to study the formation and elimination rates of cocaethylene following combined administration of ethanol and

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cocaine using stable isotopes in the Wistar rat model. In pharmacokinetic studies utilizing small animal models, it is necessary to maximize analytical sensitivity since a sufficient number of blood samples is needed for adequate description of the drug concentration versus time curves. A variety of analytical methods have been used to measure cocaine and cocaethylene in different biological matrices including HPLC [7–11,16], GC [5,13,17–19] and GC-MS [2,5,10,18–21]. Many methods require large amounts of sample (ml) to gain sufficient sensitivity for kinetic analysis. Currently, GC-MS is the preferred method of analysis due to the increased sensitivity and selectivity attained when using deuterated internal standards for quantitative analysis [22].

This paper describes a sensitive and selective GC/MS SIM method to quantitate cocaine and cocaethylene using internal standards that have been deuterium-labeled at the *N*-methyl position for each analyte (i.e. (*N*-[^2H_3C])-cocaine and (*N*-[^2H_3C])-cocaethylene) from a single whole blood extract. This method can be used to quantitate these analytes in small volumes of whole blood as needed for kinetic analysis.

2. Materials and methods

2.1. Drug standards and chemicals

All analytical standards were purchased from Radian Corp. (Austin, TX) and included cocaine (1.0 mg ml^{-1}), cocaethylene (1.0 mg ml^{-1}), (*N*-[^2H_3C])-cocaine (100 μg ml^{-1}) and (*N*-[^2H_3C])-cocaethylene (100 μg ml^{-1}).

All Optima® grade solvents were obtained from Fisher Scientific (Fairlawn, NJ) and included ammonium hydroxide, isopropanol, methanol, methylene chloride and toluene. Potassium oxalate was also from Fisher Scientific (Fairlawn, NJ). Sodium fluoride was from Sigma (St. Louis, MO).

Potassium hydroxide was from Mallinkrodt Chemical (Paris, KY). Helium and nitrogen (each 99.9999%) were purchased from Jackson Welding Supply (Pittsburgh, PA). Bond Elut Certify® SPE tubes were obtained from Varian Sample preparation products (Harbor City, CA), as was the SPE 20-port vacuum manifold.

2.2. Preparation of working standard and internal standard solutions

Cocaine and cocaethylene standards were purchased in concentrations of 1.0 mg ml^{-1} in *N*,*N*-dimethylformamide and acetonitrile, respectively. In order to prepare standards over the wide concentration ranges used in this analysis working internal standards for cocaine and cocaethylene were prepared identically. Working standards were (10.0 µg ml^{-1}) prepared by adding each 100 µl of stock drug solution (1.0 mg ml^{-1}) to a separate 10 ml volumetric flask, bringing each to volume with methanol. Working standards (1.0 µg ml^{-1}) were then prepared by pipetting 1.0 ml of the 10.0 μg ml^{-1} standard into a 10.0 ml volumetric flask, bringing each to volume with methanol. These solutions were used to prepare various concentrations of whole blood standards for extraction and analysis. (*N*-[^2H_3C])-Cocaine and (*N*-[^2H_3C])-cocaethylene (internal standards) were purchased in 100.0 µg ml^{-1} solutions in acetonitrile. Working internal standard solutions (10.0 µg ml^{-1}) were prepared by diluting 1.0 ml of (*N*[^2H_3C])-analyte to 10.0 ml with methanol. All solutions were sealed and covered with aluminum foil and stored at −4°C. Stability of these internal standards was assessed by determining the extent of back-exchange of deuterium for hydrogen in the preparations. No apparent exchange was noted based on the lack of analyte detection in the extracted blank samples that contained deuterated internal standards.

2.3. Analytical parameters

GC/MS analysis in these experiments was performed with a Hewlett-Packard 5890 Series II Plus GC equipped with a Hewlett-Packard 7673 A Automatic Liquid Sampler linked to a Hewlett-Packard 5972 Mass Selective Detector (MSD). The GC was equipped with a cross-linked methyl silicone gum capillary column [Ultra-1®; 12 m × 0.2 mm (i.d.) × 0.33 μm film thickness(Hewlett-Packard)] and the injection port fitted with a 4.0

mm i.d. silanized borosilicate liner with a silanized glass wool plug. Injections were made in the splitless mode with a purge off time of 1.0 min. Ultra high purity helium was used as the carrier gas at a flow rate of 1.0 ml min^{-1}. The injector port and transfer line temperatures were maintained at 250 and 280°C, respectively. The initial oven temperature was 100°C for 1 min; programmed to 275 at 25°C min^{-1} and maintained for 2 min for a total run time of 12 min. The MSD was operated in the SIM mode using the following molecular ions for quantitation: (*N*-[^2H_3C])-cocaine (m/z 306.0), cocaine (m/z 303.0), (*N*-[^2H_3C])-cocaethylene (m/z 320.0), and cocaethylene (m/z 317.0) and using a pre-programmed 20 ms dwell time for acquisition. Instrument parameters and data acquisition was accomplished using Hewlett-Packard G1034C MS ChemStation software. Quantitation of each analyte was based on the comparison of peak area ratios (peak area of analyte/peak area of deuterated internal standard analog) for prepared standards versus unknown samples.

2.4. Experimental procedure—calibration curves for cocaine and cocaethylene

Whole blood drug standards and experimental samples were collected and stored in tubes containing 0.25% w/v sodium fluoride and 0.20% w/v potassium oxalate in 1.0 ml water with addition of (*N*-[^2H_3C])-cocaine and (*N*-[^2H_3C])-cocaethylene internal standards (100.0 ng ml^{-1}). Brogan et al. [23] have shown that 0.25% sodium fluoride contained in grey-top Vacutainer tubes effectively inhibits cocaine hydrolysis. Extraction required further dilution of these samples with 3.0 ml of phosphate buffer at pH 6.0. Dilution was a necessary step to allow unimpeded flow through the SPE cartridge. The solution was mixed for 1 min and was centrifuged at 2500 × *g* for 15 min. A pellet of cells was evident after centrifugation. The supernatants were poured onto the cartridge during sample loading. The extraction cartridges were conditioned which included pre-washing with 3.0 ml methanol, 2.0 ml deionized water and 2.0 ml phosphate buffer (pH 6.0) prior to sample loading. The cartridges were not permitted to dry between conditioning and sample application. Whole blood samples in buffer (pH 6.0) were then added to the cartridges, followed by 2.0 ml deionized water and 2.0 ml of 0.1 mol l^{-1} hydrochloric acid in water. Methanol, 3 ml, were added to the cartridges and aspirated to dryness for 5 min. The analytes were collected in 13 × 100 mm disposable glass culture tubes following elution with 2.0 ml of an 80:20 v/v solution of methylene chloride/isopropanol with 2% ammonium hydroxide. Extracts were placed in a 40°C water bath and dried under a stream of nitrogen. Extracts were reconstituted with 25 μl of toluene and 1 μl was injected for GC/MS analysis. Freshly prepared standards in 250 μl of whole blood were used to construct daily calibration curves which included injection of 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 1000.0 and 2000.0 ng ml^{-1} concentrations of cocaine and cocaethylene.

2.5. Recovery, within run and between run precision and accuracy, limit of detection (LOD) and limit of quantitation (LOQ)

Extraction recovery was performed by comparing peak area ratios of the molecular ion for each analyte extracted from whole blood with peak area ratios of the molecular ion from the same concentration of analyte in organic solvent. Deuterated internal standards were added to whole blood extracts following the solid phase extraction of analyte. Extraction recovery was expressed as the percent of cocaine and cocaethylene recovered from whole blood following solid phase extraction. Extraction recovery of cocaine and cocaethylene from whole blood was expressed as the mean percentage recovery ± S.D. of five determinations using 100.0 ng ml^{-1} standards.

Within-run and between-run precision and accuracy was determined on three separate days. A geometric progression of target concentrations of cocaine and cocaethylene, including 10.0, 100.0, and 1000.0 ng ml^{-1}, were extracted from whole blood in triplicate. Results were expressed as relative accuracy of the measured concentration compared with the target value and precision as percentage relative standard deviation (%RSD) of

triplicate measurements of the same concentration.

LOQ was determined by demonstrating linearity at a 10:1 signal to noise ratio. LOD was assessed by preparing standards of lesser concentration than the determined LOQ and demonstrating detection at a 3:1 signal to noise ratio.

3. Results

Cocaine, cocaethylene and both deuterated internal standards were extracted from whole blood using a simple one-step SPE method. Additional clean-up procedures, such as precipitation steps, were unnecessary in this extraction procedure. Chromatograms of cocaine and cocaethylene following SPE exhibited little background noise with no interfering substances evident. Fig. 1 represents the total ion chromatogram and selected ion chromatograms following injection of an extracted 500.0 ng ml^{-1} whole blood standard. Cocaine (and (N-[^2H_3C])-cocaine) eluted in 6.49 min while cocaethylene (and (N-[^2H_3C])-cocaethylene) were retained an additional 0.20 min, demonstrating appropriate resolution of these analytes. All peaks were baseline-resolved allowing for accurate and precise peak integration.

Whole blood samples, with cocaine and cocaethylene added, served as standards in the construction of calibration curves used to determine the concentration of drug in experimental whole blood samples. The concentration ranges of 5.0–2000.0 ng $m^{-1}l$ for both analytes were selected based on cocaine concentrations determined in range finding experiments where rats were administered 25.0 mg kg^{-1} cocaine intraperitoneally and whole blood collected at time intervals over a 4 h period. A least-squares linear regression equation was applied to these data and linearity was demonstrated over the entire range of concentrations for both cocaine and cocaethylene. The correlation coefficients obtained from cocaine and cocaethylene regressions were 0.998 and 1.000, respectively.

The efficiencies of the extraction procedure were determined as extraction recovery tested at 100.0 ng ml^{-1} concentrations of cocaine and cocaethylene. Recoveries were evaluated by comparing concentrations of cocaine and cocaethylene extracted from whole blood to concentrations of each drug added to extraction solvent and are given as mean $\pm$ S.D. of five determinations per group. Elution of analytes with methylene chloride/2-propanol (80:20) with 2% ammonium hydroxide resulted in recoveries of $82.0 \pm 1.8\%$ and $86.0 \pm 5.0\%$ for cocaine and cocaethylene, respectively.

Relative accuracy and precision of the extraction of cocaine and cocaethylene from whole blood are shown in Table 1. Within-run and between-run relative accuracies were determined on samples containing either 10.0, 100.0, or 1000.0 ng ml^{-1} concentrations of cocaine and cocaethylene by comparing the measured concentrations to the target concentrations. All relative accuracies were $> 90\%$ except for cocaethylene, at 1000.0 ng ml^{-1} concentration, where within-run and between-run accuracies were 89.6 and 86.8%, respectively. Within-run and between-run percent RSD determined on triplicate analysis at each concentration and conducted on three separate experimental days were $< 10\%$ for all analytes except for the between-run percent RSD for cocaethylene at 100.0 ng ml^{-1}, which was 11.0%.

Analytical sensitivity was evaluated in this extraction by determining the LOD and LOQ for this procedure. LOD was defined as the lowest concentration detected at a signal to noise ratio of 3:1. LOD for cocaine and cocaethylene in whole blood was 1.0 and 2.0 ng ml^{-1}, respectively. LOQ represented the lowest concentration quantified at a signal to noise ratio of 10:1 and was established at 5.0 ng ml^{-1} for cocaine and cocaethylene.

4. Discussion

This analytical procedure is a simple, one-step SPE method combined with GC/MS analysis to quantitate cocaine and cocaethylene in small volumes of whole blood needed for small animal blood sampling experiments. The technique does not require derivatization and use of deuterated internal standards greatly improves the sensitivity and selectivity of this method over other alterna-

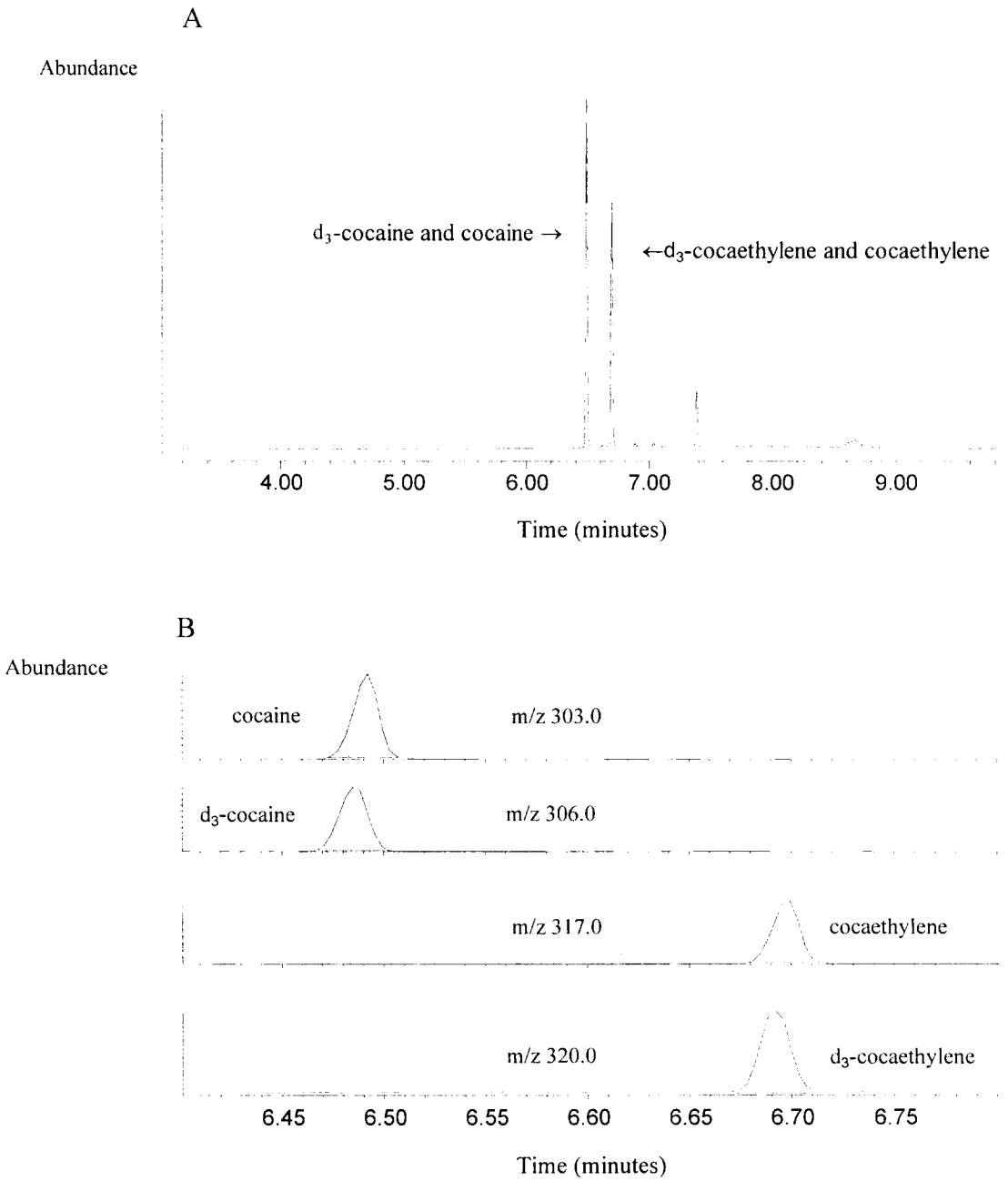

Fig. 1. Typical (A) total ion chromatogram and (B) selected ion chromatogram from whole blood extraction of a prepared 500.0 ng ml^{-1} standard.

tive forms of analysis. Cone et al. [14] have described the advantages of using copolymeric bonded-phase columns for extracting cocaine analytes from biological matrices in a rapid one step isolation procedure. A recent electron impact (EI) GC/MS method for analyzing cocaine and co-

Table 1
Within-run and between-run precision and accuracy for cocaine and cocaethylene analysis determined at three different concentrations

Analyte	Concentration, ng ml^{-1}		Relative accuracy, %	%RSD
	Target	Measured*		
Within-run				
Cocaine	10.0	10.2(0.6)	102.0	5.6
	100.0	100.7(4.4)	100.7	4.4
	1000.0	985.6(75.7)	98.6	7.7
Cocaethylene	10.0	9.8(0.1)	98.0	0.7
	100.0	92.7(7.2)	92.7	7.2
	1000.0	895.6(55.7)	89.6	6.2
Between-run				
Cocaine	10.0	10.5	105.0	8.7
	100.0	103.4	103.4	8.3
	1000.0	979.6	98.0	6.7
Cocaethylene	10.0	10.4	104.0	6.8
	100.0	93.1	93.1	11.0
	1000.0	867.5	86.8	6.1

[a] Data represent mean ± S.D. for triplicate determinations of each analyte.

caethylene from small samples (50 μl) of mouse plasma used liquid/liquid extraction and [$^2H_3$] cocaine and [$^2H_5$] cocaethylene internal standards for quantitation [21]. They reported precision at 17.8% for 0.20 μg ml^{-1} concentrations of cocaine and 22.2% for 0.02 μg ml^{-1} concentrations of cocaethylene. Signal to noise ratio of 4:1 was reported for the 0.02 μg ml^{-1} cocaethylene standards. These authors did not attempt to establish lower LOD for cocaine analysis based on their pilot studies for expected cocaine concentration in experimental samples. The present SPE extraction followed by EI GC/MS analysis using (*N*-[$^2H_3$C]) internal standards was able to quantitate 5.0 ng ml^{-1} cocaine and cocaethylene from 250 μl whole blood at a 10:1 S/N ratio. Extraction of multiple standards of cocaine and cocaethylene resulted in < 11% RSDs for all concentrations tested. LOD were 2.0 ng ml^{-1} for cocaethylene at a 3:1 S/N ratio. The improved precision and sensitivity may be related to the efficiency of the solid phase extraction procedure. The use of solid phase columns to extract whole blood samples represents several advantages including reducing sample manipulation, decreasing solvent volumes needed for extraction and minimizing matrix interferences compared with liquid/liquid extraction.

Solid phase extraction was sufficient in cleanup strategies for whole blood components and did not require precipitation. Dilution of whole blood samples with phosphate buffer allowed unimpeded flow of the sample through the SPE packing material under vacuum. The resultant extract resulted in chromatograms free of background noise and interference with adequate separation of the analytes of interest. This extraction procedure yielded recoveries for cocaine and cocaethylene of 82.0 and 86.0%, respectively, in whole blood, which are consistent with absolute recoveries using other solid phases and in other matrices. For example, Corburt et al. [24] extracted cocaine from postmortem blood using an Amberlite® XAD-2 resin with 84% recovery. De La Torre et al. [25] have recently reported absolute recoveries of cocaine and cocaethylene in urine as 87.5 ± 2% and 93.4 ± 7%, respectively, using Bond Elut Certify® cartridges and a different sample preparation procedure.

Precision and accuracy were evaluated for this method at three separate concentrations. Precision was consistent in these experiments at the

low, medium, and high concentration ranges (10, 100 and 1000 ng ml^{-1}) tested with percent RSDs ranging from 0.7–11.0%. Relative accuracies were > 95% for both cocaine and cocaethylene in this analysis and sensitivities of this method were demonstrated at 1.0 and 2.0 ng ml^{-1} for cocaine and cocaethylene, respectively.

5. Conclusions

Pharmacokinetic analysis requires a number of blood samples be taken over a sufficient period of time to allow for adequate description of the blood concentration versus time curves. Use of small animal models presents further difficulty for these experiments given the small volumes of blood obtained at each time point. The present analytical method is sensitive and specific and enables use of small sample volumes of whole blood for quantitation of cocaine and cocaethylene in a simple one step extraction procedure followed by conventional EI GC/MS analysis. This method resulted in low background interferences, provided sufficient sensitivity and was highly reproducible. It should be a valuable tool in future pharmacokinetic studies in rodents that investigate cocaine and cocaethylene metabolism.

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