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Determination of cocaine, norcocaine, benzoylecgonine and ecgonine methyl ester in rat plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

An isocratic high-performance liquid chromatographic method with ultraviolet detection at 235 nm is described for the determination of cocaine and its metabolites benzoylecgonine, norcocaine and ecgonine methyl ester in rat plasma, collected during toxicity studies. Following simultaneous solid-phase extraction of all analytes and the internal standard tropacocaine, cocaine, benzoylecgonine and norcocaine were separated on a C_{18} column. Ecgonine methyl ester and cocaine were separated on coupled cyanopropyl and silica columns, following derivatization of ecgonine methyl ester to *p*-fluorococaine. The extraction efficiencies of these compounds from plasma ranged from 78 to 87%, while the limits of detection ranged from 35 to 90 ng/ml. The assay was linear from 300 to 5000 ng/ml, and the within-day precision 2 to 8% over this concentration range.

Keywords: Cocaine; Norcocaine; Benzoylecgonine; Ecgonine methyl esters

1. Introduction

Cocaine abuse remains a major health problem in the USA [1]. Methods for analyzing cocaine and its metabolites are of increasing importance as it is realized that cocaine metabolites contribute to its toxicity; norcocaine (NOR) is a hepatotoxin [2], and benzoylecgonine (BE) and ecgonine methyl ester (EME) may provoke coronary spasm [3]. In addition, because of the short half-life of cocaine [4], plasma concentrations of metabolites such as BE and EME are increasingly being used to monitor cocaine abuse [5,6].

Cocaine (benzoylecgonine methyl ester) is (i) demethylated to norcocaine [7] by the hepatic cytochrome P450 system, (ii) hydrolyzed, by removal of the methoxy group to BE through poorly defined non-enzymatic pathways [8], and (iii) converted to EME, by removal of the benzoyl group by plasma cholinesterase and distinct liver esterases in man [9].

Several analytical methods based largely upon gas chromatography–mass spectrometry (GC–MS), are presently available to analyze cocaine and its metabolites. GC–MS is regarded as the

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method of choice because of its sensitivity and specificity but requires derivatization of both BE and EME to produce relatively intense high mass molecular ions [10,11]. In addition, deuterated cocaine, BE and EME are needed as internal standards [12] as other internal standards yield questionable results. This has been found to be the case particularly for the analysis of BE and EME [13]. Liquid chromatography–atmospheric pressure chemical ionization-mass spectrometry (LC–APCI-MS) has also been used, thus avoiding the need for derivatization and deuterated internal standards [14]. Unfortunately, GC–MS or LC–MS equipment requires a substantial investment and is not readily available in many laboratories.

High-performance liquid chromatography (HPLC) is more affordable and has been used extensively for cocaine and cocaine metabolite determination [15,16]. However, very few HPLC methods have been described for the simultaneous determination of EME with other cocaine metabolites [17,18]. This is because EME is not suitable for UV detection without derivatization and to date attempted derivatization techniques lack reproducibility [19].

We describe a sensitive and reproducible isocratic HPLC method using UV detection for the simultaneous determination of cocaine, BE, norcocaine and EME in rat plasma using tropacocaine as the internal standard. The method is based on solid-phase extraction of cocaine and its metabolites from plasma and derivatization of EME to *p*-fluorococaine. This analytical procedure was found to be suitable for determination of cocaine and its metabolites in rats receiving relatively high doses of these compounds for toxicity studies performed in our laboratory.

2. Experimental

2.1. Materials

Acetonitrile, chloroform, methanol, methylene chloride, 2-propanol, and triethylamine (all HPLC grade), as well as anhydrous sodium carbonate and dibasic sodium phosphate, citric acid monohydrate (all certified A.C.S), anhydrous monobasic sodium phosphate (enzyme grade), ammonium hydroxide (reagent A.C.S), and 1 *M* and 0.1 *M* hydrochloric acid (certified) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Benzene (thiophene free, reagent grade) was obtained from J.T. Baker (Phillipsburg, NJ, USA), pyridine (A.C.S., Chempure brand) from Curtin Matheson (Houston, TX, USA), and phosphoric acid 85% (analytical reagent) from Mallinckrodt (Paris, KY, USA). Tropacocaine (hydrochloride) and *p*-fluorobenzoyl chloride were purchased from Sigma (St. Louis, MO, USA). Helium and nitrogen (each 99.9999%) were obtained from Curtin Matheson (Morris Plains, NJ, USA). Cocaine hydrochloride, benzoylecgonine, norcocaine, ecgonine methyl ester hydrochloride and benzoylnorecgonine hydrochloride were donated by the National Institute on Drug Abuse (Rockville, MD, USA). Drug-free male Sprague–Dawley rat plasma was purchased from Rockland (Gilbertsville, PA, USA).

2.2. Methods

2.2.1. Preparation of standards and reagents

Calibration standards, for cocaine and norcocaine at 1, 10 and 100 ng/μl were prepared in methanol from 1 mg/ml stock solutions and stored at −15°C. Benzoylnorecgonine, EME and BE stock solutions (1 mg/ml) were prepared in water, then diluted to obtain calibration standards at 1, 10 and 100 ng/μl. The diluted solutions were aliquoted in glass autosampler vials, sealed and stored at −15°C. The working stock solution of the internal standard, tropacocaine, was prepared by successive dilutions in water to give a final concentration of 20 ng/μl and was stored in a refrigerator.

2.2.2. Sample collection

Blood samples were collected in cryovials that had been treated with saturated NaF solution (50 μl) and were immediately stored at −80°C until analyzed.

The enzymatic hydrolisis of cocaine to both benzoylecgonine and ecgonine methyl ester is

inhibited in vitro by NaF, while the non-enzymatic degradation of cocaine at low temperature is insignificant [20–22].

To determine the stability of various analytes during storage, we reanalyzed a number of samples within a period of one month and observed no significant loss in their concentration.

2.2.3. Extraction and derivatization

Solid-phase extraction of cocaine and its metabolites was performed using 300-mg Bond Elut Certify columns (Varian, Harbor City, CA, USA) and a Vac-Elut manifold. The extraction columns were conditioned by successive addition of 6 ml methanol, 3 ml water and 5 ml of 0.01 *M* monobasic sodium phosphate buffer, pH 2.0, without drying the columns. To aliquots of 1 ml plasma samples 100 μl of internal standard solution (2 μg of tropacocaine) and 3 ml of the same buffer were added. After brief vortex-mixing, the samples were applied to the pre-conditioned extraction columns and drawn slowly through the columns (ca. 0.5 ml/min). The columns were dried for 1–2 min under full vacuum (2 kPA) and were then sequentially washed with 3 ml water and 3 ml 0.1 *M* HCl at a flow-rate of 1 ml/min, dried each time for 3 min, washed rapidly with 6 ml methanol and again dried for 5 min under full vacuum. Elution of cocaine and metabolites was performed with 6 ml methylene chloride–2-propanol (4:1, v/v) containing 2% ammonium hydroxide, without vacuum application. The eluents were evaporated to dryness at 50°C under a gentle stream of nitrogen and reconstituted in 2 ml mixture of methylene chloride–2-propanol (4:1). The resultant solutions were divided into two equal parts and dried under nitrogen. One set of dry residue was reconstituted in 300 μl of mobile phase, mixed for 30 s and an aliquot was applied to the HPLC column for the analysis of cocaine, BE and norcocaine. The second part was mixed with 1 ml of benzene, 200 μl pyridine and 100 μl *p*-fluorobenzoyl chloride for derivatization of EME to *p*-fluorococaine, using the method of Isenschmid et al. [13] with minor modifications. This mixture was heated in a water bath for 1 h at 85°C. Following derivatization, the samples were allowed to cool to room temperature. The contents were vortex-mixed with 3 ml 1 *M* HCl for 5 min. Following centrifugation at 800 *g* for 10 min, the phases were separated. The upper organic layer was discarded by aspiration and the lower aqueous layer was transferred into a clean snap-cap conical glass centrifuge tube and alkalinized with 2.5 ml of 2 *M* Na_2CO_3. The samples were then treated with 1 ml chloroform, vortex-mixed and centrifuged as above. The upper aqueous layer was discarded and the chloroform layer transferred into a clean glass tube and evaporated to dryness at 50°C under nitrogen. The residue was redissolved in 300 μl mobile phase and 100 μl was injected onto the HPLC system for EME analysis.

2.2.4. Chromatographic conditions

Plasma cocaine and its metabolites were quantitated using an HPLC system consisting of a Spectra Series P100 isocratic pump (Spectra-Physics Analytical, Fremont, CA, USA), a Rheodyne Model 7125 NS injector with a 100-μl sample loop and a Spectro Monitor 3200 variable-wavelength detector (Thermo Separation Products, Riviera Beach, FL, USA) connected to a Spectra-Physics Chrom Jet integrator. Separation of cocaine, benzoylecgonine, norcocaine and the internal standard was accomplished on a Nucleosil C_{18} column (Sigma-Aldrich, 250 × 4.6 mm I.D., 5 μm particle size), preceded by a Brownlee C_8 guard cartridge (30 × 4.6 mm I.D., 5 μm particle size, Rainin Instrument, Emeryville, CA, USA). The mobile phase consisting of 0.05 *M* citric acid–0.1 *M* dibasic sodium phosphate (4:1, v/v) buffer, pH 3.0, 18% (v/v) acetonitrile and 0.3% triethylamine was used at a flow-rate of 1.5 ml/min. A 100-μl sample was injected manually and the elution of various analytes was monitored for 28 min (total run time) at 235 nm, with the detector range set at 0.01 AUFS.

The separation of the EME derivative was achieved on a tandem of a Bakerbond Cyanopropyl (J.T. Baker, Phillipsburg, NJ, USA) column, 250 × 4.6 mm I.D., 5 μm particle size, and a Microsorb Silica (Rainin) column, 150 × 4.6 mm

I.D., 5 μm particle size. The mobile phase consisting of 0.01 *M* monobasic sodium phosphate buffer, pH 3.0, and 30% (v/v) acetonitrile was passed through the columns at a flow-rate of 1.1 ml/min. The elution profile of analytes was monitored at 235 nm, with the detector range set at 0.005 AUFS. The injection volume and the total run time were the same as above.

2.2.5. Preparation of standard curves

Standard curves were prepared by spiking drug-free rat plasma (aliquots of 1 ml) with calibration standards to achieve cocaine and metabolite concentrations ranging from 0.3 to 5 μg/ml. A 100-μl volume of internal standard (2 μg tropococaine) was also added to each sample. In the buffered calibration samples (see Section 2.2.3), the concentration of methanol, used to prepare standard solutions of cocaine and norcocaine, did not exceed 3%. Extraction, derivatization and chromatography were performed as described above. Peak-height ratio of each analyte to the internal standard was plotted versus analyte concentration by linear regression. In every batch of samples, four standards were analyzed with appropriate plasma control (blank) included. All calibration samples were prepared on the day of assay.

3. Results and discussion

Fig. 1 shows the typical elution profile of extracted blank rat plasma (A) and calibration standard (B) on C_{18} column, before derivatization. Cocaine, norcocaine, benzoylecgonine and tropacocaine were distinctly separated from one another, with retention times of 17.6, 19.3, 6.8 and 12.8 min, respectively. A typical elution profile of the analytes after derivatization, on coupled cyano and silica columns, is shown in Fig. 2. EME, COC (which was unaffected by the derivatization reaction) and the internal standard were eluted with retention times of 20.5, 19.5 and 16.0 min, respectively.

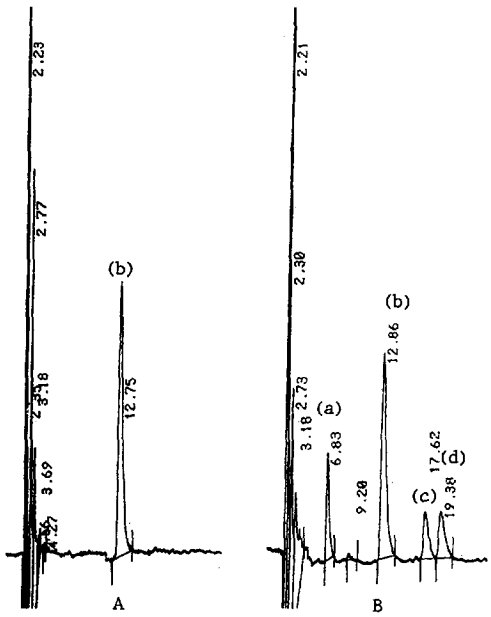

Fig. 1. Elution profile of cocaine and its metabolites on C_{18} column. (A) Blank (drug-free) rat plasma; (B) 600 ng/ml calibration sample; the peaks correspond to benzoylecgonine (a), internal standard, tropacocaine (b), cocaine (c) and norcocaine (d).

The use of two HPLC columns in series not only achieved a good resolution of EME, COC and internal standard, but it also provided a method to avoid further purification of various analytes from the contaminating derivatization byproducts (eluted within 12 min before the analytes) and also offered a system for the separation of two structurally very similar compounds, namely *p*-fluorococaine (EME derivative) and cocaine.

The EME derivative was confirmed as *p*-fluorococaine by GC–MS. To achieve this, initially, the derivatization product was analyzed by HPLC and the fraction corresponding to the EME derivative was collected. An aliquot of this fraction was then subjected to GC–MS analysis. The total ion scan of derivatized ecgonine methyl ester (*p*-fluorococaine) is shown in Fig. 3 and matches that found in the literature [13].

Tropacocaine was found to be a suitable inter-

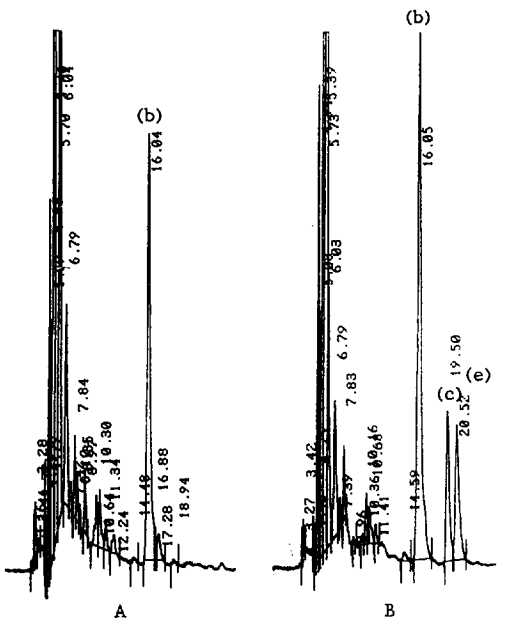

Fig. 2. Elution profile of cocaine and its metabolites on coupled cyano and silica columns, following derivatization. (A) Blank (drug-free) rat plasma; (B) 600 ng/ml calibration sample; the peaks correspond to internal standard (b), cocaine (c) and ecgonine methyl ester (e).

nal standard as it was structurally similar to the analytes and remained stable at 85°C, which was essential for the derivatization of EME to *p*-fluorococaine. Atropine, lidocaine, caffeine, cyclazine, and meperidine were found unsuitable for this purpose.

Calibration curves for cocaine and metabolites were consistently linear with good precision as noted in Table 1. The regression equations determined from 6 different experiments were as follows: $y = 0.00038\ x - 0.00225$ ($r^2 = 0.99962$) for cocaine on the C_{18} column and, respectively, $y = 0.00050\ x - 0.03647$ ($r^2 = 0.99848$) on the coupled columns; $y = 0.00038\ x - 0.01924$ ($r^2 = 0.99962$) for norcocaine; $y = 0.00084\ x + 0.00211$ ($r^2 = 0.99986$) for benzoylecgonine and $y = 0.00042\ x - 0.02611$ ($r^2 = 0.99917$) for EME.

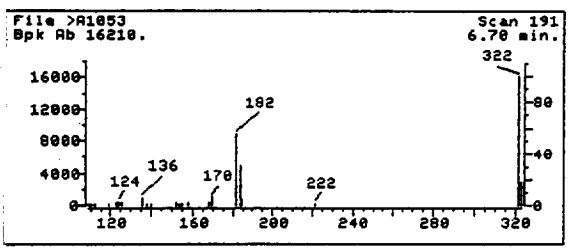

Fig. 3. Total ion scan of derivatized ecgonine methyl ester.

To determine the extraction efficiency, an aliquot of plasma spiked with 1 μg/ml of each analyte and internal standard was subjected to extraction and chromatography. The peak heights of the various metabolites extracted from plasma were compared with the peak heights of pure analytes injected in the chromatographic system. In the case of EME both the extracted and pure analyte were derivatized before injection. The extraction efficiency of cocaine and its metabolites varied from 78 to 86% as shown in Table 1. For the internal standard, the extraction efficiency was 87%.

Detection and quantitation limits for cocaine and its metabolites (Table 1) were determined according to the method described by Knoll [23].

The method described in this paper compares favorably to the one employing LC–APCI-MS described by Nishikawa et al. [14], with regard to extraction efficiency, detection limit and precision. We used this procedure in our laboratory to analyze over one hundred samples collected from rats during toxicity studies with cocaine and its metabolites. Fig. 4 shows the chromatograms of plasma samples drawn during intravenous cocaine (A) and ecgonine methyl ester (B) infusion in rats. The figure illustrates that even at high concentrations (over 50 μg/ml) of some analytes, the adjacent peaks of the other low concentration analytes were satisfactorily resolved. In a next run, such samples were further diluted to bring the high concentration analytes in the range of the calibration curves.

We found that benzoylnorecgonine could be readily included in the assay by increasing the volume of elution mixture to 10 ml. However, the extraction efficiency was low, probably due to the high polarity of this cocaine metabolite.

Table 1
Validation results for the chromatographic assay

	Benzoylecgonine (%)	Cocaine (%)	Norcocaine (%)	Ecgonine methyl ester (%)
Within-day precision (n = 6)				
300 ng/ml	2.87	5.88 (8.16)	5.17	8.32
1000 ng/ml	1.84	2.56 (5.30)	2.71	6.53
2000 ng/ml	1.95	2.78 (4.81)	2.13	6.48
Between-day precision (n = 6)				
300 ng/ml	3.83	8.18 (9.02)	6.08	11.03
600 ng/ml	3.18	7.92 (7.58)	5.85	10.72
1000 ng/ml	3.77	3.84 (6.67)	3.48	8.58
5000 ng/ml	2.74	4.55 (5.89)	2.01	6.79
Extraction efficiency (at 1000 ng/ml)	87	86	83	78
Limit of detection (ng/ml)	35	75 (60)	80	90
Limit of quantitation (ng/ml)	100	220 (180)	230	260

The values for cocaine pertain to the assay on the C_{18} column and, in parentheses, on the coupled cyanopropyl and silica columns.

Yet, at high concentrations of this metabolite in plasma samples, it was detected (retention time of 4.58 min) without any modification of the described method (Fig. 4A).

At very high doses of the infused cocaine and norcocaine, an unknown peak (retention time of 10.58 min) appeared in the vicinity of peaks of interest (Fig. 4A). However, this peak did not interfere in our assay.

Previously, a procedure based on the fluorescence detection of various cocaine metabolites, with pre-column derivatization and post-column ion-pair formation, has been described by Roy et al. [17]. However, this procedure required elaborate instrumentation, which is not available in many laboratories. The combination of UV and electrochemical detectors to analyze cocaine and its metabolites has been described recently by Miller and DeVane [18]. This method uses a pH-stable polymeric HPLC column and a mobile phase of pH 8.8 to separate various analytes. Although resolution of various analytes was achieved, we encountered a few problems with this procedure, such as difficulty in benzoylecgonine extraction, the need for switching of the solvents during chromatographic run, and poor peak symmetry under the chromatographic conditions described.

4. Conclusions

We have shown that cocaine, norcocaine, benzoylecgonine and particularly ecgonine methyl ester can be analyzed simultaneously, based on UV detection only, using tropacocaine as internal standard and performing derivatization of EME. The demonstrated sensitivity and precision of this HPLC method make it particularly suitable for toxicity and pharmacokinetic studies of cocaine metabolites.

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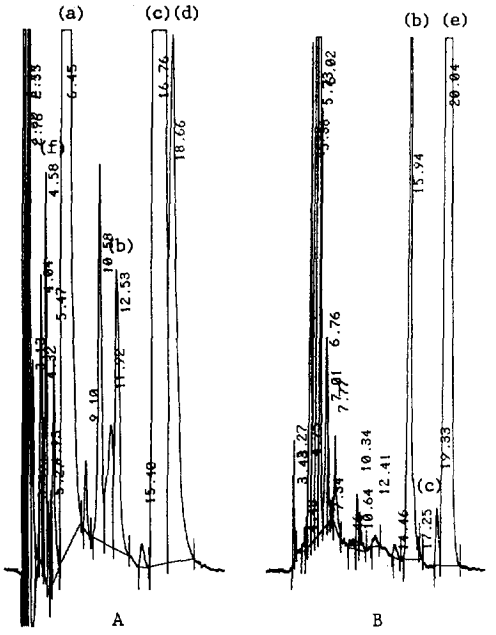

Fig. 4. Chromatograms of: (A) rat plasma sample collected during intravenous cocaine infusion at 8.83 μmol kg^{-1} min^{-1}, showing benzoylecgonine (a) 85 μg/ml, internal standard (b) 2 μg/ml, cocaine (c) 51 μg/ml and norcocaine (d) 6 μg/ml; (B) rat plasma sample drawn during intravenous ecgonine methyl ester infusion at 8.83 μmol kg^{-1} min^{-1}, showing ecgonine methyl ester (e) 51 μg/ml and cocaine (c) 1.2 μg/ml. The samples were diluted 1:1 and, respectively, 1:4, extracted and derivatized according to the method described. (A) Injected before derivatization, on C_{18} column, and (B) injected after derivatization, on coupled cyano and silica columns. In A the peak of benzoylnorecgonine (f) can be identified (retention time 4.58 min).

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