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Determination of cocaine and its metabolites in serum microsamples by high-performance liquid chromatography and its application to pharmacokinetics in rats

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Abstract

A single-solvent extraction step high-performance liquid chromatographic method is described for quantitating cocaine and its three metabolites in rat serum microsamples (50 μl). The separation used a 2.1-mm I.D. reversed-phase Brownlee C_{18} column with an isocratic mobile phase consisting of methanol–acetonitrile–25.8 m*M* sodium acetate buffer, pH 2.2, containing $1.29 \cdot 10^{-4}$ *M* tetrabutylammonium phosphate (12.5:10:77.5, v/v/v). The detection limit was 2.5 ng/ml for all the compounds using an ultraviolet detector operated at 235 nm. The method was used to study the pharmacokinetics of cocaine after an intravenous (i.v.) bolus dose (4 mg/kg).

Keywords: Cocaine; Norcocaine; Benzoylecgonine; Benzoylnorecgonine

1. Introduction

Cocaine is widely abused by humans [1]. Research from various disciplines is being conducted to better understand its reinforcing, addictive and psychomotor stimulant effects. If one is interested in studying the effects of cocaine on behavior, it is advantageous to assess the parallel pharmacokinetics. In order to implement this approach, it is necessary to use a sensitive and precise analytical method to determine the concentration of cocaine and its metabolites for studying the pharmacokinetics. Many analytical techniques can be used to determine cocaine, such as high-performance liquid chromatography (HPLC) [2–6], radioimmunoassay [7], gas chromatography–mass spectrometry (GC–MS) [8,9], and others. Of these methods, HPLC is the most attractive and versatile technique for the determination of drugs in biological samples since it provides the possibility of adjusting the selectivity of the resolution over a wide range. Moreover, due to its milder working conditions, HPLC is the most suitable technique for the analysis of thermally labile compounds.

This paper describes a rapid and sensitive HPLC method capable of determining cocaine and its metabolites in small samples (50 μl). Sample size is critical when the animal species used is small, especially when repeated blood sampling is necessary to trace the temporal changes in drug levels in individual animals. The convenience of our method is facilitated by its use of a single-solvent extraction procedure and the commercially available 2 mm I.D. column. An added advantage of using the 2 mm I.D. column is a reduction in solvent consumption by up

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to 80%, compared to the 4.6 mm I.D. column. This method is hereby applied to evaluate the pharmacokinetics of cocaine after i.v. bolus cocaine administration.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Perkin-Elmer 200 LC pump coupled to an autosampler ISS-200 (Norwalk, CT, USA), and a 785A programmable absorbance UV detector, operated at 235 nm (Applied Biosystems, Foster City, CA, USA). The separation was performed on a Brownlee C_{18} column, 100 mm×2.1 mm I.D., 5 μm particle size (Applied Biosystems) with a 2-μm Rheodyne precolumn filter (Cotati, CA, USA). The data were collected using a PE Nelson 900 series interface, Turbochrom 4.1 software (Perkin-Elmer) and an IBM-type pentium microcomputer workstation.

2.2. Reagents and standards

(−)-Cocaine hydrochloride, norcocaine, benzoylecgonine and benzoylnorecgonine hydrochloride were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). HPLC-grade methanol, acetonitrile, chloroform and sodium acetate were purchased from Fisher Scientific (Springfield, NJ, USA) and HPLC-grade ethyl alcohol was from Sigma–Aldrich (St. Louis, MO, USA). 3-Isobutyl-1-methylxanthine was purchased from Sigma (St. Louis, MO, USA). The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared by the method of de Silva and Puglisi [10]. All other chemicals were of reagent grade.

Cocaine hydrochloride, norcocaine, benzoylecgonine, benzoylnorecgonine hydrochloride and 3-isobutyl-1-methylxanthine were dissolved in methanol individually to make 1 mg/ml stock base solutions. Dilutions of the 1 mg/ml standards, cocaine and its metabolites, were used to make the working standards (0.2, 0.5 and 1.0 μg/ml) containing the four compounds. The internal standard, 3-isobutyl-1-methylxanthine, was diluted and used at a concentration of 4 μg/ml.

HPLC analyses were performed using an isocratic mobile phase consisting of methanol–acetonitrile–25.8 m*M* sodium acetate buffer (adjusted to pH 2.2 with 40% phosphoric acid) containing $1.29 \cdot 10^{-4}$ *M* tetrabutylammonium phosphate (12.5:10:77.5, v/v/v). Mobile phases were degassed and filtered through a Solvent Filtration Apparatus (Alltech Associates, Deerfield, IL, USA). The flow-rate was set at 0.3 ml/min and was normally operated at a pressure of 104 bar (1500 p.s.i.).

2.3. Sample preparation

Standards and serum samples were prepared as previously described [3,11]. Briefly, a 25-μl volume of the internal standard (3-isobutyl-1-methylxanthine, 4 μg/ml) and 50 μl of working serum standard were added to a 15-ml conical centrifuge tube. Borate buffer (1 *M*, pH 9.0, 100 μl) was added and the solution was mixed well. A 1-ml volume of chloroform–ethanol (87.5:12.5, v/v) was added and the sample mixture was vortex-mixed for 1 min and centrifuged for 5 min at 1100 *g*. The 1.15-ml sample mixture rose to 2 cm below the rim of the 15-ml conical centrifuge tube during vortex-mixing, a procedure which ensured vigorous mixing for the extraction of alkalized cocaine and its metabolites to the organic solvent. The organic layer was carefully transferred to a 5-ml conical centrifuge tube and evaporated to dryness in an evaporator (Pierce, Rockford, IL, USA) at 40°C under nitrogen. The residue was resuspended in 50 μl of the mobile phase, and 20 μl was injected onto the column by the autosampler. Samples for serum drug analysis were prepared in an identical manner, except that standards were not added.

2.4. Extraction recovery

The assay recoveries of cocaine and its metabolites were assessed at concentrations of 0.2, 0.5 and 1.0 μg/ml. Six replicates of each concentration, containing the four compounds, were extracted according to the method described above. Six replicates of each concentration were computed using the following equation:

$$\text{Recovery} = \frac{\text{(peak height extract)}}{\text{(mean peak height direct injection)}} \times 100\%$$

2.5. Cocaine administration and serum sampling

One male, albino, Sprague–Dawley rat from HSD (Indianapolis, IN, USA), held to 80% of his normal, adult starting weight, 382 g, was used. Right jugular vein cannulation and blood sampling have been described previously [12]. The catheter was flushed with 0.9% saline containing 20 units of heparin per ml and was sealed with fishing line when not in use. Animals were allowed to recover for two days and then received an i.v. bolus of 4 mg/kg cocaine hydrochloride. Injections were given in a volume of 1 ml/kg. Blood samples (100 μl) from the jugular catheter were collected at 5, 15, 30, 45, 60, 90 and 120 min and were centrifuged for 10 min at 13 700 *g* and stored frozen until analysis. Previously, we have found that cocaine and its metabolites were stable in rat serum samples for at least a month without the presence of sodium fluoride (a cholinesterase inhibitor) [3]; thus, sodium fluoride was not used in the present study. Experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publ. No. 85-23, revised 1985).

2.6. Pharmacokinetic analysis

Pharmacokinetic analysis was performed using SAAM II (SAAM Institute, Seattle, WA, 1994) [13]. The data were described by an open two-compartment model for cocaine and fit to the following equation:

$$C_p = A\,e^{\alpha t} + B\,e^{\beta t}$$

where C_p is the total serum drug concentration at time t, the terms A and B are the extrapolated zero intercepts, and α and β represent the apparent first-order distribution and elimination rate constants, respectively. The half-life ($t_{1/2}$) for the distribution or elimination phase and the volume of distribution for the central compartment (V_c) were calculated by the following equations: $t_{1/2} = 0.693/\alpha$ or β and $V_c = \text{dose}/(A+B)$. The area under the serum drug concentration–time curve ($AUC_{0-\infty}$) was calculated by the following equation: $AUC_{0-\infty} = A/\alpha + B/\beta$. Total clearance (Cl) was then defined as Dose/$AUC_{0-\infty}$. Cocaine dose was calculated in terms of the base, to estimate the pharmacokinetic parameters.

3. Results and discussion

3.1. Method evaluation

Fig. 1 shows chromatograms of (A) a serum blank with internal standard containing no interfering peaks, (B) a spiked serum sample containing a working standard (1.0 μg/ml), which was extracted by the liquid–liquid extraction procedure, and (C) a representative rat serum sample (50 μl) obtained 5 min following i.v. 4 mg/kg cocaine hydrochloride administration.

The internal standard method was used in the calibration and evaluation of the unknown samples. Table 1 shows the within-day and between-day precisions of cocaine and its metabolites, which were established at three different concentrations (0.2, 0.5 and 1.0 μg/ml) by adding these compounds to blank serum. Both within-day and between-day precisions for cocaine and its metabolites were high, as indicated by the coefficients of variation (C.V.) which ranged from 1.22–6.1% and from 2.98–10.87%, respectively.

Calibration curves for cocaine and its three metabolites are linear within the ranges (0.2–1.0 μg/ml) examined. For each of the six regression lines, the correlation coefficients are larger than 0.998. The coefficients of variation of the slopes ($n=6$) of the regression lines ranged from 5.84 to 7.63, with intercepts all being close to zero (Table 2). The detection limit was 2.5 ng/ml for all the compounds, with a signal-to-noise ratio of 4. The column used in this study provides a higher sensitivity, lower operating pressure and faster separation of cocaine and its metabolites when compared to the one used in the previous study [3].

The extraction recoveries (mean±SD) for cocaine, benzoylecgonine, and norcocaine at the three concentrations (0.2, 0.5 and 1.0 μg/ml) were in the range of 69.29–82.82%, whereas those for benzoylnorecgonine were markedly lower, 34.97–

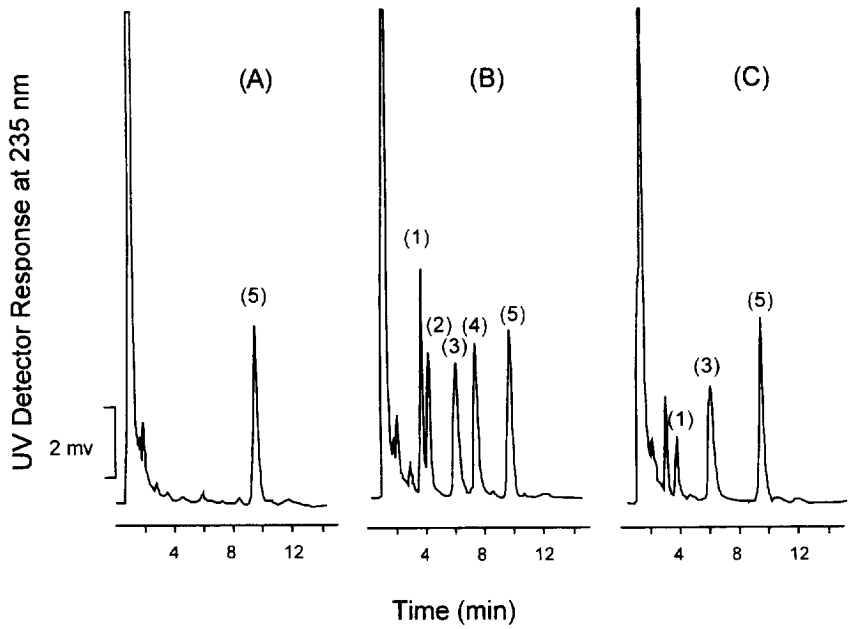

Fig. 1. Chromatograms of (A) rat serum blank, (B) rat serum containing 1.0 μg/ml benzoylecgonine, benzoylnorecgonine, cocaine, norcocaine and 3-isobutyl-1- methylxanthine, (C) a 50-μl rat serum sample obtained 5 min after i.v. administration of 4 mg/kg cocaine. Peaks: 1 = benzoylecgonine; 2 = benzoylnorecgonine; 3 = cocaine; 4 = norcocaine; 5 = 3-isobutyl-1-methylxanthine.

40.46% (Table 3). We have found a small volume of extraction solvent, 1 ml of a chloroform–ethanol mixture, is sufficient for recovery of these compounds from biological fluids, but the ratio of ethanol to chloroform is the determinant for the recovery of benzoylecgonine and benzoylnorecgonine, since these two compounds are insoluble in chloroform, but are soluble in ethanol due to their

Table 1
Precision data for cocaine and its metabolites in serum

Compound	Within-day ($n=6$)		Between-day ($n=6$)	
	Concentration (mean±SD) (μg/ml)	C.V. (%)	Concentration (mean±SD) (μg/ml)	C.V. (%)
Cocaine	0.1994±0.012	6.10	0.1969±0.015	7.38
	0.4976±0.029	5.83	0.5051±0.037	7.29
	1.0043±0.041	4.04	1.0129±0.063	6.22
Norcocaine	0.2032±0.009	4.31	0.2014±0.018	8.97
	0.5035±0.026	5.19	0.4940±0.054	10.87
	1.0002±0.046	4.63	0.9968±0.068	6.79
Benzoylecgonine	0.2025±0.007	3.41	0.1992±0.006	2.98
	0.4973±0.006	1.22	0.4997±0.018	3.57
	1.0002±0.015	1.52	0.9989±0.040	3.95
Benzoylnorecgonine	0.2006±0.008	4.05	0.1997±0.006	2.98
	0.4924±0.025	4.99	0.4958±0.032	6.37
	0.9825±0.054	5.51	0.9884±0.043	4.30

Table 2
Mean of calibration equations for cocaine and its metabolites over the concentration range 0.2–1.0 μg/ml

Compound	Equation	Correlation coefficient	C.V. of slope (%)
Cocaine	$y=3570\ (\pm219)x-30\ (\pm102)$	0.9984	6.13
Norcocaine	$y=4405\ (\pm284)x-152\ (\pm113)$	0.9987	6.45
Benzoylecgonine	$y=6267\ (\pm366)x-97\ (\pm148)$	0.9990	5.84
Benzoylnorecgonine	$y=3735\ (\pm285)x-116\ (\pm102)$	0.9993	7.63

y = peak height; x = concentration of each compound.

amphoteric nature [3]. In the present study, 87.5% chloroform was used in the extraction mixture to maximize the recoveries of the compounds of major interest, cocaine and norcocaine. Nevertheless, the lower recovery observed for benzoylnorecgonine can be improved by increasing the ethanol content of the extraction solvent.

Table 4 shows a series of compounds at a concentration of 1 μg/ml, which did not interfere with the determination of cocaine and its metabolites. It is interesting that the present method is capable of simultaneously determining not only cocaine and its three metabolites, but also cocaethylene and norcocaethylene, the metabolites of cocaine formed in the liver following the concurrent administration of ethanol. Thus, all five compounds can be determined simultaneously in a serum sample using a longer analysis time. Table 4 also shows that mazindol can be used as an internal standard rather than 3-isobutyl-1-methylxanthine for the determination of cocaine and its three metabolites.

Table 3
Recovery of cocaine and its metabolites

Compound	Recovery (mean±SD) (%)	C.V. (%)
Cocaine		
0.2 μg/ml	82.82±4.86	5.87
0.5 μg/ml	77.91±5.070	6.50
1.0 μg/ml	76.47±6.42	8.40
Norcocaine		
0.2 μg/ml	73.32±4.78	4.78
0.5 μg/ml	69.29±6.300	9.09
1.0 μg/ml	72.92±6.59	9.03
Benzoylecgonine		
0.2 μg/ml	80.61±2.99	3.71
0.5 μg/ml	74.89±3.24	4.32
1.0 μg/ml	77.57±4.60	5.93
Benzoylnorecgonine		
0.2 μg/ml	39.25±2.12	5.39
0.5 μg/ml	34.97±1.10	3.15
1.0 μg/ml	40.46±2.25	5.57

3.2. Pharmacokinetic results

Fig. 2 shows the serum cocaine and benzoylecgonine concentration–time profiles following i.v. administration of 4 mg/kg cocaine hydrochloride. Benzoylnorecgonine and norcocaine were not detectable by the i.v. route. The distribution and terminal elimination half-lives for cocaine were 8.6 and 34.7 min, respectively. Clearance and V_c for cocaine were 9.6 l/h/kg and 2.7 l/kg, respectively. The terminal elimination half-life of cocaine, as found in this study, was consonant with those values (18–38 min) reported for the i.v. route [14,15], but differed from those (0.9–1.8 h) of the i.p. and p.o. routes using tail-tip samples of the rat [16].

Table 4
Relative retention times (k') of other drugs for possible interference with cocaine and its three metabolites

Compound	k'
Caffeine	1.07
Lidocaine	1.64
Benzoylecgonine	2.22
Benzoylnorecgonine	2.69
Cocaine	4.37
Norcocaine	5.71
3-Isobutyl-1-methylxanthine	7.85
Cocaethylene	8.07
Norcocaethylene	10.04
Mazindol	11.04
Phenobarbital	13.05
Hexobarbital	24.56
Flurazepam	N.D.
Barbital	N.D.

N.D. = peak not observed within 30 min.

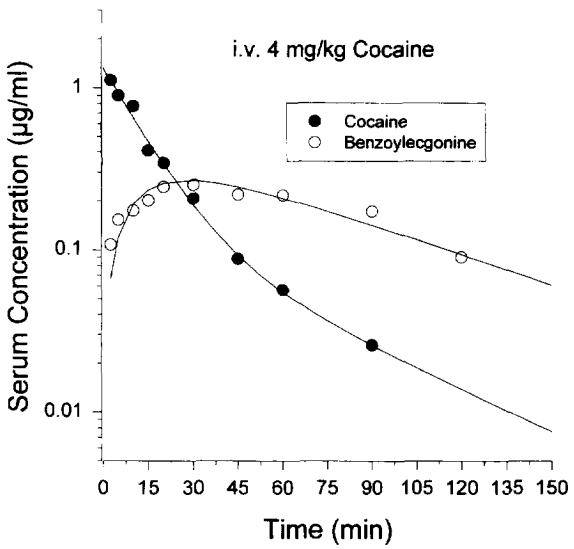

Fig. 2. Serum cocaine and benzoylecgonine concentration–time profiles following an i.v. bolus of 4 mg/kg cocaine hydrochloride administration.

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