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High-performance liquid chromatographic determination of cocaine and its metabolites in serum microsamples with fluorimetric detection and its application to pharmacokinetics in rats

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Abstract

A sensitive, selective and simple HPLC method with fluorimetric detection is described for quantitating cocaine and its three metabolites in rat serum microsamples (50 μl). Chromatographic separation is achieved on a Hypersil BDS C_{18} column (100×2.1 mm, 5 μm) with an isocratic mobile phase consisting of methanol–acetonitrile–25.8 m*M* sodium acetate buffer, pH 2.6, containing $1.0 \cdot 10^{-4}$ *M* tetrabutylammonium phosphate (14:10:76, v/v/v). The detection limit (0.5 ng/ml) for all the compounds, using direct fluorometric detection operated at excitation and emission wavelengths of 230 and 315 nm, respectively, was approximately five-times lower than that of using a UV detector operated at 235 nm. The effects of ratio of 2-propanol to chloroform in extraction solvents on the recovery and precision for cocaine and its metabolites were systematically examined. The method was used to study the pharmacokinetics of cocaine after administration of intravenous 2 mg/kg and oral 20 mg/kg doses. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cocaine; Benzoylecgonine; Benzoylnorecgonine; Cocaethylene

1. Introduction

Cocaine is a potent psychomotor stimulant and a major drug of abuse in the USA [1]. Methods for analyzing cocaine and its metabolites are of increasing importance for the purposes of defining the level of toxicity and lethality, or screening for cocaine use. Cocaine is rapidly distributed, deactivated by extensive metabolism, and then excreted (Fig. 1). Benzoylecgonine, one of the main metabolites, is formed by hepatic carboxylesterase or spontaneous hydrolysis [2]. Although benzoylecgonine is not a pharmacologically active metabolite, it is of great forensic and analytical interest because of its long half-life. Norcocaine is formed by *N*-demethylation of cocaine and is the only cocaine metabolite found to have in vivo pharmacological activity in animals and humans [3,4]. Benzoylnorecgonine is the hydrolysis product of norcocaine.

It is essential to determine the concentration of a drug and its active metabolites with precision before investigating concentration–effect relations to parti-

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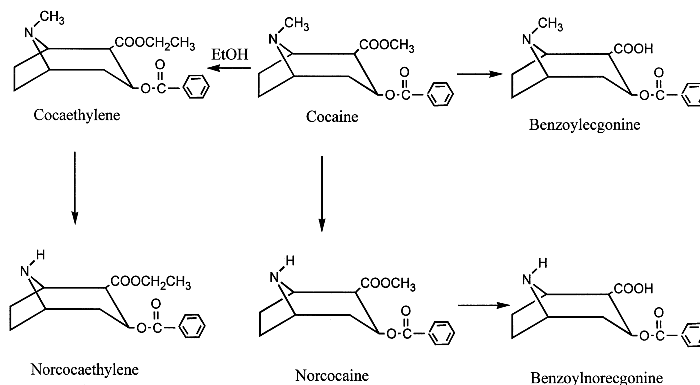

Fig. 1. The chemical structures of cocaine and its metabolites.

tion drug effects into pharmacokinetic and pharmacodynamic components [5–7]. High-performance liquid chromatography (HPLC) has been used extensively for the determination of cocaine and its metabolites in biological samples [8–17]. Most HPLC assays for cocaine with 4.6 mm I.D. columns required a large sample size (e.g., 0.2–2 ml) to attain a desired sensitivity [8–13]; these methods are more suitable for clinical evaluation, where the sample volume is not a limiting factor. However, in research with small laboratory animals (e.g., rats), sample size is a critical consideration, especially in pharmacokinetic studies where repeated blood sampling is necessary to trace temporal changes in blood levels of the drug and its metabolites. In past research, to determine cocaine and its metabolites in rats [14–16], we developed the most simple and sensitive HPLC–UV methods possible for serum microsamples (50 μl) to accommodate repeated sampling using commercially available 2 mm I.D. columns. These smaller bore columns enable one to not only increase sensitivity, but also reduce solvent consumption by up to 80%. The first aim of the present study was to increase detection sensitivity further for cocaine and its metabolites by modifying our previous HPLC–UV method [16] with a fluorescence detector. The fluorimetric detection based on the weak native fluorescence of the benzene ring present in the molecules of cocaine and its metabolites has been successfully used for determining cocaine concentrations in human plasma and hair samples [9].

In a previous study [14], we found 1 ml of extraction solvent [ethanol–chloroform (17.5:82.5, v/v)] was sufficient for recovery of cocaine and its metabolites from serum samples. The ratio of ethanol to chloroform was known to be critical in determining the extent of recovery for benzoylecgonine and benzoylnorecgonine, which are insoluble in chloroform, but soluble in ethanol due to their amphoteric nature. Therefore, the second aim of the study was to systematically investigate the effects of the ratio of alcohol to chloroform in an extraction solvent on the recovery and precision for each of the four compounds, thus, facilitating the choice of an optimal solvent for extracting compounds of interest. This method was hereby applied to evaluate the pharmacokinetics of cocaine after intravenous (i.v.) and oral (p.o.) cocaine administration.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Perkin-Elmer (PE) 200 LC pump coupled to a Perkin-Elmer autosampler ISS-200 (Norwalk, CT, USA); both units were controlled by a PE Nelson 600 Series Link interface. A Hewlett-Packard HP 1100 fluorescence detector (Waldbronn, Germany) was operated at the excitation and the emission wavelengths of 230 and 315 nm, respectively. The separation was performed on a Hypersil BDS C_{18} column, 100 mm×2.1 mm I.D., 5 μm particle size (Hypersil, MA, USA) with a 2-μm Rheodyne pre-column filter

(Cotati, CA, USA). The data were collected using a PE Nelson 900 series interface, Turbochrom 6.0 software (Perkin-Elmer) and an IBM-type pentium microcomputer workstation.

2.2. Reagents and standards

(−)-Cocaine hydrochloride, norcocaine, benzoylecgonine, benzoylnorecgonine hydrochloride and cocaethylene fumarate were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). HPLC-grade methanol, acetonitrile, chloroform, 2-propanol and sodium acetate were purchased from Fisher Scientific (Springfield, NJ, USA). Tetrabutylammonium phosphate (HPLC grade) was purchased from Eastman Kodak (Rochester, NY, USA). All other chemicals were reagent grade. The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared by the method of de Silva and Puglisi [18].

Cocaine hydrochloride, norcocaine, benzoylecgonine, benzolynorecgonine hydrochloride and cocaethylene fumarate were dissolved in methanol individually to make 1 mg/ml stock base solution. Dilutions of the 1 mg/ml standards (cocaine and its metabolites), were used to make the working standards (0.05, 0.25, 0.5 and 1.0 μg/ml) containing the four compounds. The internal standard, cocaethylene, was diluted and used at concentration of 1 μg/ml.

The HPLC analyses were performed using an isocratic mobile phase consisting of methanol–acetonitrile–25.8 m*M* sodium acetate buffer (adjusted to pH 2.6 with 40% phosphoric acid) containing $1.0 \cdot 10^{-4}$ *M* tetrabutylammonium phosphate (14:10:76, 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 0.4 ml/min and normally operated at a pressure of 125 bar (1800 p.s.i.).

2.3. Sample preparation

Standards and serum samples were prepared as described previously [14,15]. In order to investigate the effects of extraction solvents on the recovery and precision of cocaine and its three metabolites, we used three extraction solvents, each with a different ratio of 2-propanol to chloroform (v/v): solvent A (0:100), solvent B (15:85) and solvent C (40:60). Briefly, a 25-μl volume of the internal standard (cocaethylene, 1 μg/ml) and 50 μl 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 extraction solvent 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 identically except that standards were not added.

2.4. Extraction recovery

The assay recoveries of cocaine and its metabolites were assessed at concentrations of 0.05, 0.25 and 1.0 μg/ml. Six replicates of each concentration, containing the four compounds, were extracted according to the method described above using solvents A and B, respectively. Six replicates of each concentration were computed using the following equation:

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

2.5. Cocaine administration and serum sampling

Five male, albino, Sprague–Dawley rats from HSD (Indianapolis, IN, USA) with a mean initial body mass of 384 g (380–388 g) were used. They were reduced to 80% of their initial, adult free-feeding body masses, and held at these masses for the duration of the experiment. Right jugular vein cannulation and blood sampling have been described previously [19]. The catheter was flushed with 0.9% saline containing 50 units of heparin per ml and

sealed with fishing line when not in use. The animals were allowed to recover for at least 2 days from the jugular vein catheterization prior to the drug administration series. Animals received an i.v. bolus dose of cocaine (2 mg/kg) followed by a p.o. dose (20 mg/kg). Drug doses were separated by 3–5 days. All injections were given in a volume of 1 ml/kg. Serial blood samples (100 μl) from the jugular catheter were collected following each cocaine administration at 2.5, 5, 10, 15, 20, 30, 45, 60, 90, 120 and 180 min and were centrifuged for 10 min at 13 700 *g* and stored frozen until analysis. Previously, we found cocaine and its metabolites in rat serum samples were stable for at least a month without the presence of sodium fluoride (a cholinesterase inhibitor) [14]; 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 software system (SAAM Institute, Seattle, WA, USA, 1994) [20]. A two-compartment disposition model was most suitable for describing serum cocaine concentration-time profiles for the i.v. dose and fit to the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

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 volume of distribution for the central compartment (V_c) were calculated by the following equations: $t_{1/2} = 0.693/\alpha$ or β and $V_c = \mathrm{Dose}/(A+B)$. The area under the serum drug concentration–time curve (i.e., $\mathrm{AUC}_{0-\infty}$) was calculated by the following equation: $\mathrm{AUC}_{0-\infty} = A/\alpha + B/\beta$. Total clearance (Cl) was then defined as Dose/$\mathrm{AUC}_{0-\infty}$. An absorption compartment was added following oral administration to assess the absorption rate constant (k_a) and absolute oral bioavailability (F). Cocaine dose was calculated in terms of the base to estimate the pharmacokinetic parameters.

3. Results and discussion

3.1. Method evaluation

During method development, a Brownlee C_{18} column, 100 mm×2.1 mm I.D., 5 μm particle size (Applied Biosystems, Foster City, CA, USA) employed in our previous HPLC method [16] was used to compare the sensitivity of fluorimetric and UV detection for cocaine and its metabolite. Fig. 2A and B showed peak height for the four compounds increased markedly with fluorescence detector compared to UV detector operated at 235 nm. The performance of the Hypersil column described in the Experimental section for the compounds in terms of peak symmetry and peak height was also compared with that of the Brownlee column. A better peak symmetry with minimal tailing was attained for cocaine and norcocaine eluted with the Hypersil column than that with the Brownlee column; consequently, a better resolution was obtained between

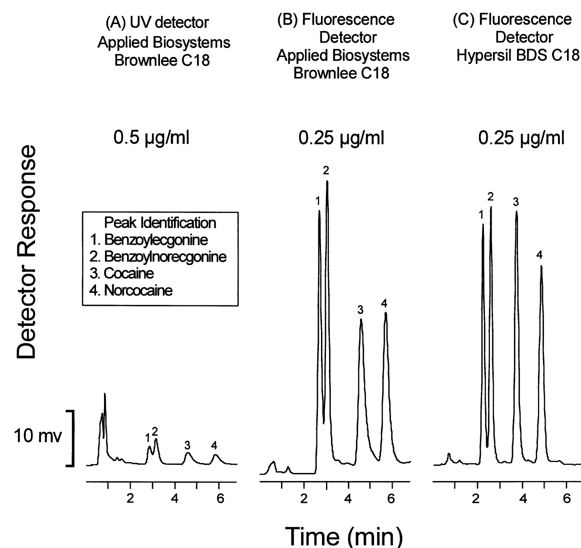

Fig. 2. Chromatograms of standards of cocaine and its metabolite with (A) UV detection using the Brownlee column; (B) fluorimetric detection using the Brownlee column; (C) fluorimetric detection using the Hypersil column.

cocaine and norcocaine, resulting in higher peak heights for the two compounds (Fig. 2B and C). Although the peak heights of benzoylecgonine and benzoylnorecgonine were similar between the two columns, the two compounds were resolved better with the Hypersil column. Thus, the Hypersil column was selected for this study.

The chromatograms of rat serum standards [spiked serum sample containing a working standard (0.25 μg/ml)] extracted with three solvents (solvents A–C) are shown in Fig. 3. The peak heights for cocaine, norcocaine and internal standard (cocaethylene) remained similar across the three extraction solvents; however, the peak heights for benzoylecgonine and benzoylnorecgonine increased with the increasing proportion of 2-propanol in the extraction solvents. Fig. 3B is re-presented in Fig. 3C with a run time of 28 min for comparison with the chromatogram using extraction solvent C. The chromatogram of serum standard after extracting with solvent A contained only one unknown peak at 2.9 min (Fig. 3A); however, unknown endogenous peaks emerged as the ratio of 2-propanol to chloroform increased across the three solvents (Fig. 3A–D). The unknown peak(s) using solvents A and B did not interfere with the peaks of cocaine and its metabolites (Fig. 3A and B). Therefore, if one desires to determine cocaine and norcocaine concentrations, both solvents A and B can be used; however, if all of the four compounds are required to be characterized, solvent B is the solvent of choice. This is consistent with the results found that a proportion of alcohol (10–12.5%) is required to optimize the recoveries for cocaine and its metabolites [9,16]. Another major advantage of using solvents A and B over solvent C is the short analysis time (8 min) for cocaine and its metabolites without any interference (Fig. 3A–C); long lasting unknown endogenous peaks were eluted for 28 min with extraction solvent C (Fig. 3D).

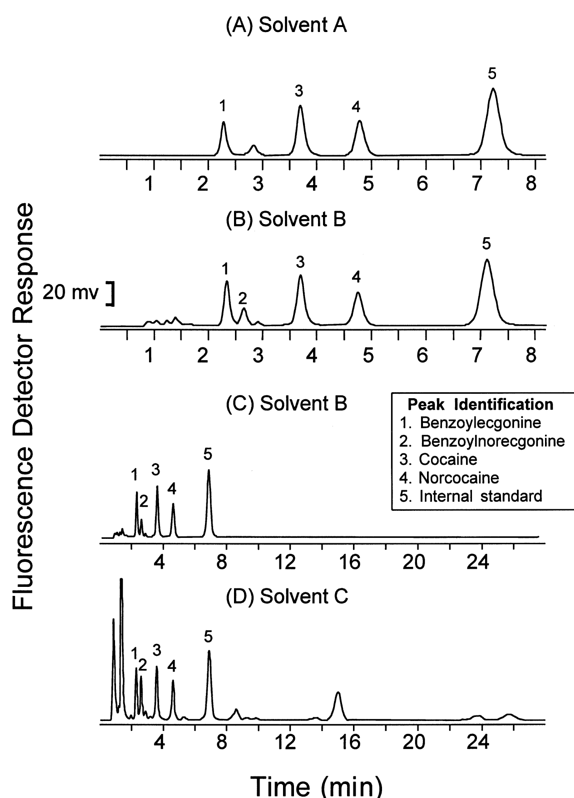

Fig. 3. Chromatograms of rat serum containing benzoylecgonine, benzoylnorecgonine, cocaine and norcocaine (0.25 μg/ml), as well as cocaethylene (0.5 μg/ml) extracted with (A) solvent A; (B) solvent B; (C) re-presentation of (B) with a run time of 28 min; and (D) solvent C.

The internal standard method was used in the calibration and evaluation of the unknown samples. Both within-day and between-day precision established at four (0.05, 0.25, 0.5 and 1.0 μg/ml) different concentrations for cocaine and its metabolites extracted using solvent B were high as indicated by the relative standard deviations (RSDs), which ranged from 1.55 to 7.85% and from 2.08 to 9.83%, respectively (Table 1). In our previous studies with UV detection, the precision data for cocaine and its metabolites can only be able to evaluate in concentration higher than 0.2 μg/ml [14,16]. In this study with fluorimetric detection, the within-day and between-day precision of 0.05 μg/ml was similar to those of 0.2 and 0.25 μg/ml reported previously, indicating that this method is at least five-times more sensitive than the previous studies.

Calibration curves for cocaine and its three metabolites are linear within the range (0.01–1.0 μg/ml) examined. For each of the six regression lines, the coefficients of determination are larger than 0.996. The coefficients of variation of the slopes ($n=6$) of the regression lines ranged from 1.25 to 1.49% with intercepts all close to zero. The detection limit was 0.5 ng/ml for all the compounds with a signal-to-noise ratio of 4, which is five times lower than that

Table 1
Precision and accuracy data for cocaine and its metabolites in serum using extraction solvent B (2-propanol–chloroform, 15:85)

Compound	Concentration added (μg/ml)	Within-day ($n=6$)		Between-day ($n=6$)	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Cocaine	0.05	7.17	100.0	2.93	100.0
	0.25	1.55	100.2	3.90	99.9
	0.5	2.34	99.9	2.08	100.2
	1.0	2.71	100.4	2.35	100.2
Norcocaine	0.05	4.00	100.2	6.74	100.0
	0.25	4.86	99.8	4.22	99.9
	0.5	3.23	100.0	2.33	100.3
	1.0	3.15	100.3	3.40	99.6
Benzoylecgonine	0.05	5.44	101.0	5.40	101.0
	0.25	3.38	100.2	4.92	100.0
	0.5	2.38	100.4	3.38	100.2
	1.0	3.04	100.0	3.59	100.1
Benzoylnorecgonine	0.05	7.85	100.4	9.83	99.0
	0.25	3.02	100.0	3.42	99.9
	0.5	2.48	100.5	2.95	99.9
	1.0	2.94	100.5	3.28	100.8

reported previously [16], corresponding to the precision data discussed above.

The extraction recoveries (mean±SD) for cocaine and norcocaine at the three concentrations (0.05, 0.25 and 1.0 μg/ml) were in the range of 63.58–96.76% regardless of extraction solvents used (solvents A and B), whereas those for benzoylecgonine and benzoylnorecgonine were dependent on extraction solvent (Table 2). The recovery for benzoylnorecgonine was minimal when using solvent A (1.49–2.31%); however, the recoveries for both benzoylecgonine and benzoylnorecgonine increased markedly with the increasing proportion of 2-propanol from 0 to 15%. This is, to our best knowledge, the first study to systematically examine the effects of ratio of alcohol to chloroform in an extraction solvent on the recovery and precision for cocaine and its metabolites.

A total of 14 compounds (1 μg/ml) were tested for possible interference with cocaine and its metabolites (Table 3). Only three of the tested agents (clonazepam, zolpidem, and mazindol) may have partially interfered with the analysis; these three compounds are fluorescent due to the presence of a benzene ring in their molecular structures. It is relevant to note that the present method is capable of simultaneously determining not only cocaine and its three metabolites, but also cocaethylene and norcocaethylene, which are formed in the liver following the concurrent administration of cocaine with ethanol [10,12,15,17]. Thus, all six compounds can be determined simultaneously in a serum sample using a longer analysis time. However, the use of cocaethylene as an internal standard for forensic samples is not recommended, because this compound is potentially present in samples as a condensation product of cocaine with ethanol.

3.2. Pharmacokinetic results

Fig. 4 shows the mean serum cocaine, benzoylecgonine and norcocaine concentration–time profiles after bolus doses of i.v. 2 mg/kg and p.o. 20 mg/kg cocaine administration (using solvent A). Cocaine concentration decreased bi-exponentially following i.v. administration. One set of pharmacokinetic parameter values described cocaine concentration–time profiles well for both routes of administration (Table 4). The pharmacokinetic parameter values for cocaine herein reported were consonant with those

Table 2
Recovery of cocaine and its metabolites

Compound (μg/ml)	Solvent A		Solvent B	
	Recovery (%) (mean±SD)	RSD (%)	Recovery (%) (mean±SD)	RSD (%)
Cocaine				
0.05	71.08±5.80	8.16	96.76±3.34	3.46
0.25	71.72±5.72	7.98	82.99±6.23	7.50
1.0	73.84±2.29	3.11	85.07±5.80	6.82
Norcocaine				
0.05	66.49±6.52	9.80	78.44±5.94	7.57
0.25	70.48±5.34	7.57	71.70±5.84	8.15
1.0	63.58±4.00	6.29	75.10±6.41	8.53
Benzoylecgonine				
0.05	44.38±4.41	9.94	74.11±5.77	7.78
0.25	44.22±2.63	5.95	68.30±2.90	8.16
1.0	45.71±2.55	5.57	64.09±4.63	7.23
Benzoylnorecgonine				
0.05	2.31±0.15	7.07	43.46±3.29	7.56
0.25	1.58±1.28	8.12	37.89±2.05	5.42
1.0	1.49±0.64	4.31	37.96±1.51	3.97
Cocaethylene (internal standard)				
0.05	68.21±6.11	8.96	84.17±2.20	2.62
0.25	67.71±3.77	5.57	73.91±6.13	8.29
1.0	69.61±2.61	3.76	79.73±5.25	6.58

Table 3
Relative retention times of other drugs for possible interference with cocaine and its metabolites

Compound	Relative retention times
Caffeine	0.78
Benzoylecgonine	1.89
Benzoylnorecgonine	2.29
Clonazepam	3.42
Cocaine	3.60
Zolpidem	4.37
Norcocaine	4.95
Cocaethylene	7.90
Mazindol	8.05
Norcocaethylene	10.63
Alprazolam, amphetamine, Chorpromazine, clozapine, Diazepam, flunitrazepam, Flurazepam, lidocaine, Methaphetamine, midazolam	N.D.

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

Table 4
Pharmacokinetic parameters (RSD, %) for cocaine after bolus dose of i.v. (2 mg/kg) and p.o. (20 mg/kg) cocaine administration in rats ($n=5$)

V_c (l/kg)	1.57 (1.79)
Cl (l/h/kg)	8.52 (1.55)
α (min^{-1})	0.156 (5.46)
β (min^{-1})	0.021 (4.74)
$t_{1/2\alpha}$ (min)	4.44 (5.46)
$t_{1/2\beta}$ (min)	33.4 (4.74)
k_a (min^{-1})	0.099 (4.62)
F (%)	2.30 (2.04)
Cocaine (mg/kg)	$AUC_{(0-\infty)}$ (μg min/ml)
i.v. 2	12.4 (1.57)
p.o. 20	2.85 (2.00)

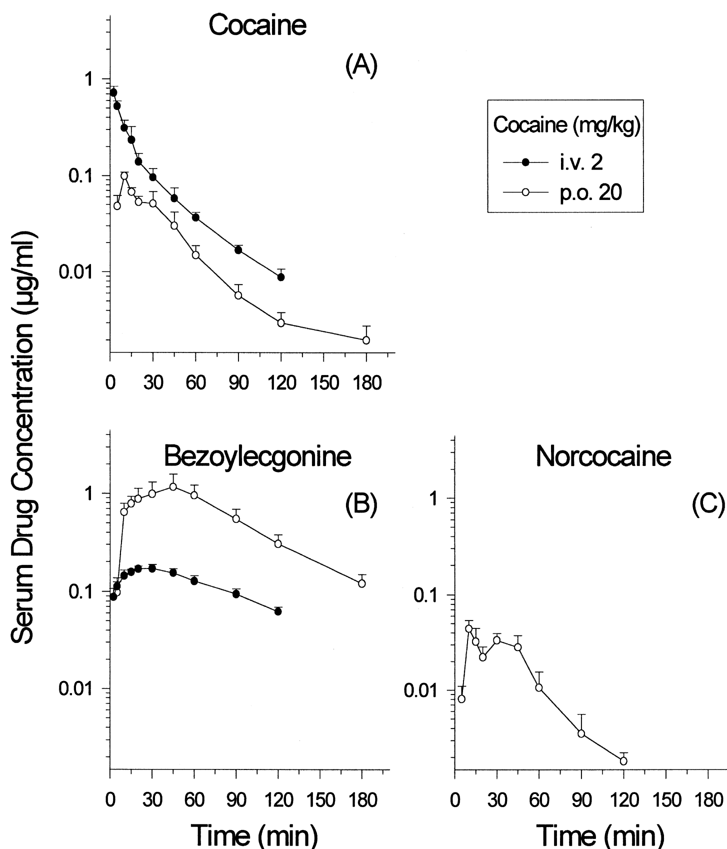

Fig. 4. Mean (±S.E.) serum concentration–time profiles of cocaine and its metabolites after bolus doses of i.v. 2 mg/kg and p.o. 20 mg/kg cocaine administration: (A) cocaine; (B) benzoylecgonine; and (C) norcocaine.

obtained in rats by previous HPLC method with UV detection [10,16,21].

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