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Simultaneous determination of cocaine and its metabolites with caffeine in rat serum microsamples by high-performance liquid chromatography

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ABSTRACT

A single, isocratic high-performance liquid chromatographic method is described for the determination of cocaine and three of its metabolites along with caffeine in serum microsamples (50 μl). The small sample size permits the tracking of pharmacokinetic data over time in individual, small animals. The method also was used to demonstrate that cocaine, benzoylecgonine and norcocaine in rat serum samples were stable for at least a month without the presence of sodium fluoride.

INTRODUCTION

Cocaine is a psychomotor stimulant and is subject to widespread abuse [1]. Caffeine is the psychoactive agent most widely self-administered by humans [2]. Biological fluid samples relating to cocaine abuse are rarely pure and often contain other substances such as caffeine. Behavioral interactions between cocaine and caffeine have been reported in animals [3–5]. For studying a drug–drug interaction, both the kinetic and dynamic aspects need to be considered. A change in the concentration of one or both drugs in the body may influence the magnitude of their actions. It is important to develop a method which will enable the determination of cocaine and caffeine simultaneously.

For routine quantitative and kinetic purposes, high-performance liquid chromatography (HPLC) offers the most attractive technique for the determination of cocaine and caffeine. HPLC methods are available for measuring cocaine [6–13] and caffeine [14–23] alone, but no method was found that evaluated simultaneous determination. We report a simple HPLC method capable of quantitative analysis of cocaine and three of its metabolites with caffeine in small samples (50 μl). The conditions permitting stability of cocaine in serum samples need to be considered [24–26], while caffeine stability has not presented a problem [21]. Sample size is a critical consideration when the animal species used is small, especially when repeated blood sampling is necessary to trace the temporal changes in drug levels for individual animals. The convenience of our method is facilitated by its use of a single-extraction procedure and the commercially available 2 mm I.D.

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column. An added advantage of using the 2 mm I.D. column is a reduction in solvent concentration by up to 80%, compared to the 4.6 mm I.D. column.

EXPERIMENTAL

Instrumentation

All analyses were performed on a Spectra-Physics (San Jose, CA, U.S.A.) SP 8800 ternary solvent delivery system, equipped with a Rheodyne (Cotati, CA, U.S.A.) Model 7010 sample injection valve with a 20-μl loop, and a Spectra-Physics SP 8450 variable-wavelength detector. Absorbance at 230 nm was monitored on a Perkin-Elmer (Norwalk, CT, U.S.A.) LCI-100 integrator. We used a 300 mm × 2.0 mm I.D. column of μBondapak C_{18}, 10 μm particle size, from Waters Assoc. (Milford, MA, U.S.A.). A 2-μm precolumn filter (Rheodyne) was also used.

Reagents and standards

Methanol, acetonitrile and chloroform were all HPLC grade and purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Ethanol and tetrabutylammonium phosphate were also HPLC grade and purchased from Aldrich (Milwaukee, WI, U.S.A.) and Eastman Kodak (Rochester, NY, U.S.A.), respectively. All other chemicals were reagent grade. The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared as described by De Silva and Puglisi [27].

(−)-Cocaine hydrochloride, caffeine and lidocaine were purchased from Sigma (St. Louis, MO, U.S.A.). Norcocaine, benzoylecgonine and benzoylnorecgonine hydrochloride were obtained from the National Institute on Drug Abuse (Rockville, MD, U.S.A.). Separate aqueous stock solutions of cocaine, benzoylecgonine, benzoylnorecgonine and caffeine were prepared at a concentration of 1.0 mg (free base) per ml. Norcocaine (1.0 mg/ml) was prepared in 0.012 *M* hydrochloric acid. Working standards (0.05, 0.1, 0.25, 0.5 and 1.0 μg/ml) containing the five compouds were prepared by appropriate dilutions of the stock solutions with drug-free rat serum. These spiked serum standards were divided into 50-μl subsamples and stored at −20°C until analysis with no thawing or refreezing. The internal standard, lidocaine, was made up in 0.004 *M* hydrochloric acid and used at a concentration of 25 μg/ml.

The mobile phase for concurrent determination of cocaine and its metabolites with caffeine was a mixture of solvent A and solvent B (72:28, v/v). Solvent A was 0.031 *M* sodium acetate buffer (adjusted to pH 5.1 with 40% phosphoric acid) containing $1.5 \cdot 10^{-4}$ *M* tetrabutylammonium phosphate. Solvent B consisted of methanol and acetonitrile (42.9:57.1, v/v). If caffeine was not present in the serum, the mobile phase composition of solvent A and solvent B was changed to 65:35 (v/v) for more rapid separation of cocaine and its metabolites. The flow-rate was set at 0.3 ml/min and normally operated at a pressure of 138 bar (2000 p.s.i.).

Sample preparation

Standards and serum samples were prepared as previously described [28]. A 10-μl volume of the internal standard (lidocaine, 25 μg/ml) and 50 μl of a working standard were mixed in a 15-ml conical centrifuge tube. Borate buffer (1 *M*, 100 μl) and 1 ml of chloroform–ethanol (82.5:17.5, v/v) were added, mixed and centrifuged. The organic layer was evaporated to dryness and the residue was resuspended in 50 μl of the mobile phase. Samples for serum drug analysis were prepared identically.

Extraction recovery

The assay recovery of caffeine and cocaine and its metabolites was assessed at concentrations of 0.25, 0.5 and 1.0 μg/ml. Six replicates of each concentration, containing the five compounds, extracted according to the method described above, were injected into the column. Six replicates of each concentration containing the five compounds prepared in mobile phase were directly injected. The assay recovery for each compound at each concentration was computed using the following equation: recovery = (peak height extract)/(mean peak height direct injection) × 100%.

Stability of cocaine and its metabolites in rat serum

Cocaine hydrochloride was dissolved in nanopure water and administered to one male, Holtzman albino rat intraperitoneally (15 mg/kg). The rat was decapitated by guillotine at 30 min post-injection. Blood was collected and centrifuged. Saturated sodium fluoride (50 μl, 5.6%) was added to 1 ml of the serum sample, while 50 μl of nanopure water were added to another 1 ml of the serum sample which served as a control. These two types of sample were divided into subsamples (50 μl) and stored at −20°C for later analysis. Values from these subsamples were determined in triplicate for cocaine and its metabolites on days 1, 2, 5, 9, 13, 23 and 33.

RESULTS

Fig. 1 shows the chromatogram of a serum blank with internal standard (A), a spiked serum sample containing 0.5 μg/ml working standard and internal standard (B) and a rat serum sample (tail tip) obtained 15 min after concurrent intraperitoneal injections of 15 mg/kg cocaine hydrochloride and 2.5 mg/kg caffeine (C). Our method simultaneously identifies caffeine and cocaine and three of its metabolites: benzoylnorecgonine, benzoylecgonine and norcocaine. This method can be easily modified to determine only cocaine and its metabolites if caffeine is not present in the serum sample. By changing the solvent proportion of A and B from 72:28 to 65:35, the retention times of cocaine and its metabolites are decreased and the peak heights are increased as shown in Fig. 2B. A serum sample at 1 h post-injection at a dose of 30 mg/kg cocaine is shown in Fig. 2C.

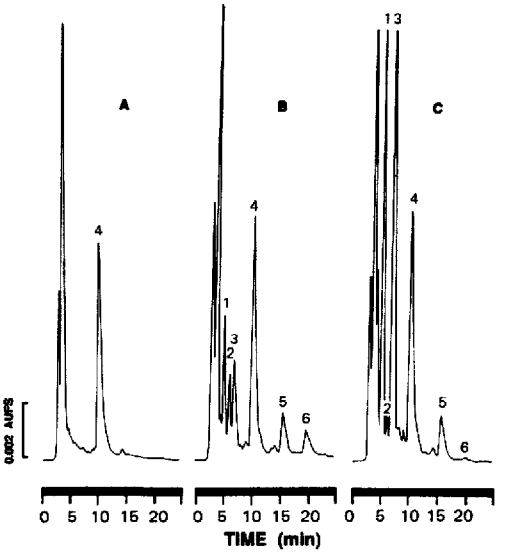

Fig. 1 Chromatograms of (A) rat serum blank with internal standard, (B) rat serum containing 0.5 μg/ml caffeine, cocaine and three of its metabolites taken through the extraction procedure and (C) a 50-μl rat serum sample obtained 15 min after concurrent intraperitoneal injections of 2 5 mg/kg caffeine and 15 mg/kg cocaine hydrochloride. Peaks: 1 = caffeine; 2 = benzoylnorecgonine; 3 = benzoylecgonine; 4 = lidocaine; 5 = cocaine, 6 = norcocaine

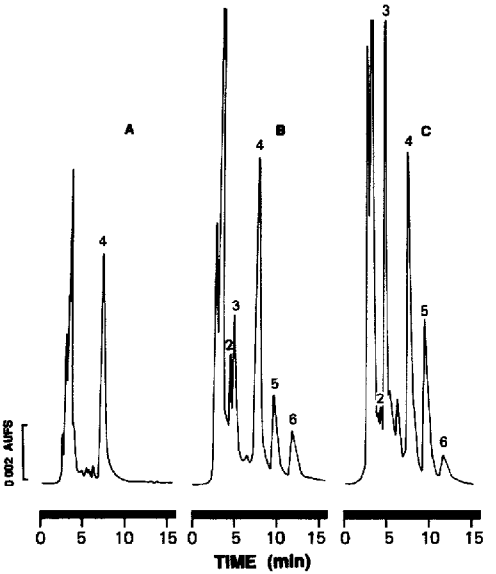

Fig 2. Chromatograms of (A) rat serum blank with internal standard, (B) rat serum containing 0 5 μg/ml cocaine and three of its metabolites taken through the extraction procedure and (C) a 50-μl rat serum sample obtained 1 h after a 30 mg/kg cocaine hydrochloride intraperitoneal injection. For peak identification, see Fig. 1.

TABLE I

PRECISION DATA FOR CAFFEINE AND COCAINE AND ITS METABOLITES IN SERUM

Compound	Within-day ($n=6$)		Between-day ($n=6$)	
	Concentration (mean ± S D) (μg/ml)	C V. (%)	Concentration (mean ± S D) (μg/ml)	C.V. (%)
Caffeine	0 2492 ± 0.0171	6.85	0 2539 ± 0.0124	4.90
	0 4981 ± 0 0349	7.00	0 5168 ± 0.0309	5.98
	0 9934 ± 0.0216	4.83	1 0363 ± 0.0750	7.23
BNE[a]	0.2522 ± 0 0221	8.78	0.2447 ± 0.0051	4.65
	0.5025 ± 0 0380	7.55	0 4965 ± 0 0467	9.40
	1.0032 ± 0 0212	4.72	1 0078 ± 0 0187	1 86
BE[a]	0.2483 ± 0.0071	2 84	0 2518 ± 0.0070	2 78
	0 4982 ± 0 0150	3 02	0.5015 ± 0 0150	2 98
	1 0035 ± 0.0352	3.51	1.0221 ± 0 0204	4.45
Cocaine	0 2519 ± 0.0154	6 11	0.2490 ± 0.0140	5 64
	0.5060 ± 0.0312	6 17	0 5006 ± 0.0462	9 24
	1.0203 ± 0.0352	3 45	0.9999 ± 0 0499	4 99
Norcocaine	0 2508 ± 0 0273	10.86	0 2475 ± 0 0146	5.89
	0 5000 ± 0 0215	4.31	0 5081 ± 0.0403	7.92
	0 9731 ± 0.0584	6.00	0 9990 ± 0.0365	3.65

[a] BNE = benzoylnorecgonine; BE = benzoylecgonine.

Within-day and between-day precisions were established for three different concentrations (0.25, 0.5 and 1.0 μg/ml) for caffeine and for cocaine and three of its metabolites by adding these five compounds to blank serum. The coefficients of variation (C.V.) for these compounds ranged from 2.84 to 10.86% for within-day and 1.86 to 9.40% for between-day precisions (Table I). Calibration curves for caffeine were linear within the range 0.05–10 μg/ml, whereas cocaine and its metabolites were linear within the range 0.05–5.0 μg/ml. For each of the five regression lines the correlation coefficients were all greater than 0.993. The coefficients of variation of the slopes ($n=5$) of the regression lines ranged from 3.0 to 8.74%, with intercepts all close to zero (Table II).

Mean recoveries of caffeine, benzoylnorecgonine, benzoylecgonine, cocaine and norcocaine were 94.6, 82.0, 87.0, 81.0 and 89.0%, respectively. The detection limit, corresponding to a signal-to-noise ratio of 4, was 0.25 ng (12.5 ng/ml) for caffeine and 0.1 ng (5 ng/ml) for cocaine and its metabolites.

To determine the specificity of the method, Table III shows the retention times of other local anaesthetic agents and drugs, at a concentration of 1 μg/ml, that might be co-administered with cocaine and caffeine. D-Amphetamine, benzodiazepines and the tricyclic antidepressant desipramine did not interfere with this

TABLE II

MEAN OF FIVE CALIBRATION EQUATIONS FOR CAFFEINE AND COCAINE AND THREE OF ITS METABOLITES OVER THE CONCENTRATION RANGE 0.05–1 00 μg/ml

y = peak-height ratio (compound/internal standard); x = concentration of each compound.

Compound	Equation	Correlation coefficient	C.V. of slope (%)
Caffeine	$y=0.4619(\pm 0\ 0404)x+0.0580(\pm 0.0137)$	0 994	8.74
BNE[a]	$y=0.2987(\pm 0\ 0090)x-0.0293(\pm 0\ 0062)$	0.996	3.00
BE[a]	$y=0.5367(\pm 0.0348)x-0.0189(\pm 0.0127)$	0 997	6.48
Cocaine	$y=0.3307(\pm 0\ 0161)x+0.0244(\pm 0\ 0077)$	0.998	4.87
Norcocaine	$y=0\ 2227(\pm 0\ 0129)x+0\ 0015(\pm 0\ 0071)$	0.993	5.77

[a] BNE = benzoylnorecgonine; BE = benzoylecgonine.

analysis. However, nicotine, its metabolite cotinine and procaine partially overlapped with caffeine and benzoylnorecgonine.

Cocaine, benzoylecgonine and norcocaine were identified 30 min after the rat was injected with 15 mg/kg cocaine hydrochloride intraperitoneally (Fig. 3). Benzoylnorecgonine was not identified at this specific time interval with this rat.

TABLE III

RELATIVE RETENTION TIMES (k') OF CAFFEINE, COCAINE, ITS METABOLITES, OTHER LOCAL ANAESTHETICS, CENTRAL NERVOUS SYSTEM STIMULANTS AND SOME OTHER COMMON DRUGS UNDER CHROMATOGRAPHIC CONDITIONS DESCRIBED IN TEXT

N D = peak not observed within 30 min

Compound	k'	Compound	k'
Nicotine	0 52	Pentobarbital	N D
Caffeine	0.81	Chlorpromazine	N D
Procaine	0.88	Clonazepam	N.D.
Cotinine	0.93	Midazolam	N D.
Benzoylnorecgonine	1.18	Oxazepam	N D.
Barbital	1.21	Chlordiazepoxide	N.D.
Benzoylecgonine	1.45	Haloperidol	N.D.
Lidocaine	2.68	Desmethyldiazepam	N.D.
Phenobarbital	4.13	Diazepam	N D.
Cocaine	4.57	Buspirone	N.D.
Norcocaine	6 08	Reserpine	N.D.
Flumazepil	6 52	Methadone	N.D.
Mazindol	7.72	*d*-Amphetamine	N.D
Hexobarbital	8 66	Desipramine	N.D.
		Tetracaine	N.D.

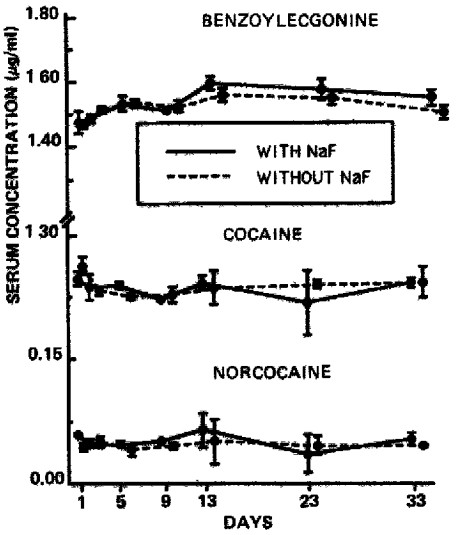

Fig. 3. Stability of cocaine, benzoylecgonine and norcocaine in serum samples stored with or without the addition of sodium fluoride.

These three compounds were detected after the *in vivo* metabolism of cocaine and were stable throughout sample storage (1–33 days), either with or without sodium fluoride (0.27%) in the serum.

DISCUSSION

The results demonstrate that cocaine and caffeine can be effectively quantitated in serum microsamples with a single extraction step by liquid chromatography. A small volume of extraction solvent, 1 ml of a chloroform–ethanol mixture, is sufficient for recovery of these compounds from biological fluids. However, the ratio of chloroform to ethanol is important for the recovery of benzoylnorecgonine and benzoylecgonine since these two compounds are insoluble in chloroform, but soluble in ethanol owing to their amphoteric nature. By increasing the ethanol concentration from 10% to 17.5% the recovery of these two compounds was approximately doubled.

Tetrabutylammonium phosphate in the mobile phase increased the peak height sensitivity and decreased the k' values of cocaine and norcocaine. The tetrabutylammonium cation blocks adsorption sites on the column for cocaine and norcocaine and effectively lowers their retention volumes. The pH of solvent A is optimum at 5.1 for the separation of cocaine and norcocaine. At higher pH values, cocaine and norcocaine lose their peak height sensitivities and significantly increased their k' values, whereas at lower pH values, the two peaks begin to

overlap. However, the separation of caffeine, benzoylnorecgonine and benzoylecgonine is affected neither by the presence of tetrabutylammonium phosphate nor by the pH of solvent A.

This method is capable of detecting some acidic, neutral and basic drugs as shown in Table III. Other compounds might interfere with these determinations.

Our results show that, in the rat, the concentrations of cocaine, benzoylecgonine and norcocaine in serum remain constant for at least one month when the samples were stored at −20°C. Stewart *et al.* [26] observed that the incubation of cocaine *in vitro* with either plasma or liver homogenates led to no detectable cocaine esterase activity. By contrast, cocaine is rapidly hydrolyzed by plasma and liver esterases to benzoylecgonine and ecgonine methyl ester in humans [24, 25]. The addition of esterase inhibitors (*e.g.* physostigmine or sodium fluoride) appears necessary for the storage of human serum samples containing cocaine, while our rat samples remained stable without such addition. Our stability data were based on a single rat. The results may be dependent on individual or species differences.

The difference in serum norcocaine concentration after two dosage treatments of cocaine, with and without caffeine (15 mg/kg cocaine with 2 5 mg/kg caffeine or 30 mg/kg cocaine), is shown for two animals in Fig. 1C and Fig. 2C. This difference is substantial and may be due to either between-subject variability or serum sampling at different times (15 min *versus* 1 h). It may also occur owing to dose-dependent cocaine kinetics as reported for humans [29,30]. The co-administration of caffeine with cocaine may play an important role in cocaine kinetics. The dose ranges for studying the behavioral response to the simultaneous administration of cocaine and caffeine were 1.25–32.0 mg/kg (cocaine) and 3.2–32 mg/kg (caffeine) in two studies [4,5]. We plan to use this method to investigate the pharmacokinetics and pharmacodynamics of cocaine and its metabolites, with and without the presence of caffeine, in rats.

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