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Determination of cocaethylene, cocaine and their metabolites in rat serum microsamples by high-performance liquid chromatography, and its application to pharmacokinetic studies in rodents

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ABSTRACT

A single-solvent extraction step high-performance liquid chromatographic method is described for quantitating cocaethylene in rat serum microsamples (50 μl), a substance formed *in vivo* when cocaine and ethanol are present concurrently. The separation used a 2 mm I.D. reversed-phase Nova-Pak C_{18} column with a mobile phase of acetonitrile–phosphate buffer containing an ion-pairing reagent. With an ultraviolet detector operated at 230 nm, a linear response was observed from 0.05 to 2.0 μg/ml with a detection limit of 5 ng/ml for cocaethylene, cocaine and norcocaine. The method showed a longer half-life for cocaethylene than for cocaine in rat.

INTRODUCTION

Recent reports have identified alcohol in combination with cocaine as a common drug use pattern in the USA [1,2]. Cocaethylene is an ethyl homologue metabolite of cocaine formed in the liver in the presence of ethanol [3–7] as shown in Fig. 1. It is also known as ethylcocaine, benzoylecgonine ethyl ester or ethylbenzoylecgonine. Cocaethylene is a pharmacologically active compound that has neurochemical and behavioral effects similar to those of the parent compound, cocaine, but is possibly more addictive and lethal than cocaine [1,8–13]. There are increasing needs to analyze biological samples simultaneously for cocaine and cocaethylene and their active metab-

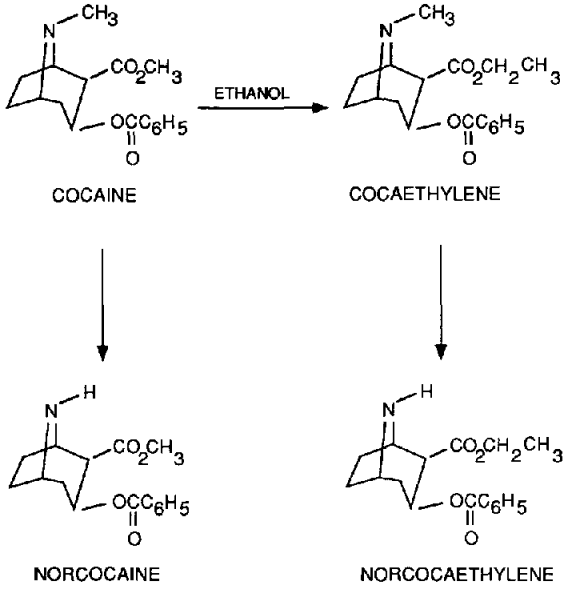

Fig. 1. Structures of cocaine, norcocaine, cocaethylene and norcocaethylene.

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olites, norcocaine and norcocaethylene, both for forensic purposes and for investigations of drug metabolism and disposition.

Thin-layer chromatography [3] and gas chromatography mass spectrometry (GC–MS) [3,4, 7,14,15] have been used to determine cocaine, cocaethylene and some of their metabolites. High-performancee liquid chromatography (HPLC) is the most attractive and versatile technique for the determination of cocaine, benzoylecgonine and cocaethylene [6,16,17] and simpler than GC–MS. We report a simple HPLC method capable of the simultaneous determination of cocaine and cocaethylene and their active metabolites, norcocaine and norcocaethylene, in small samples (50 μl). 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-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 to 80%, compared to the 4.6 mm I.D. column. This method enabled us to identify the presence of cocaethylene and norcocaethylene which were formed *in vivo* in an animal which had orally self-administered ethanol and was subsequently injected intraperitoneally with a dose of cocaine · HCl (22.5 mg/kg).

EXPERIMENTAL

Instrumentation

All analyses were performed with a Model 510 dual-piston pump (Waters Assoc., Milford, MA, USA), a Model 70-10 sample injection valve, a Model 70-11 loop filler port equipped with a 20-μl loop (Rheodyne, Cotati, CA, USA) and a Model 163 variable-wavelength detector (Beckmann Instruments, San Ramon, CA, USA). The separation was performed on a 150 mm × 2.0 mm I.D. column of Nova-Pak C_{18}, 4 μm particle size, from Waters Assoc. A 2-μm precolumn filter (Rheodyne) was also used. Absorbance at 230 nm was monitored with a PE Nelson 900 series interface on a Turbochrom 3 chromatography workstation (Norwalk, CT, USA).

Reagents and standards

Acetonitrile and chloroform were HPLC grade and purchased from Fisher Scientific (Springfield, NJ, USA). Tetrabutylammonium phosphate was also HPLC grade and 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].

Cocaethylene fumarate, norcocaethylene fumarate, cocaine · HCl and norcocaine were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Mazindol was obtained from Sandoz Research Institute (East Hanover, NJ, USA). Separate aqueous stock solutions of cocaine, cocaethylene and norcocaethylene were prepared at a concentration of 1.0 mg/ml (free base). Norcocaine (1.0 mg/ml) was prepared in 0.012 *M* hydrochloric acid. Standards for calibration curves were prepared by spiking 1-ml aliquots of serum containing 5.6 mg sodium fluoride with diluted stock solutions of these four compounds ranging from 0.1 to 1 μg/ml. The internal standard, mazindol, was made up in 0.02 *M* hydrochloric acid, diluted with nanopure water and used at a concentration of 2.5 μg/ml.

The mobile phase consisted of acetonitrile–53 m*M* phosphate buffer pH 2.35 containing 0.35 m*M* tetrabutylammonium phosphate (TBAP) (15:85, v/v). The flow-rate was set at 0.3 ml/min and normally operated at a pressure of 104 bar (1500 p.s.i.).

Behavioral procedure

One adult male, albino rat was given a daily 3-h experimental session in a Plexiglas chamber (30 × 26 × 23 cm). The chamber was equipped with a stainless-steel food pellet receptacle and a drinking fluid reservoir contained a solution composed of 3.5% ethanol, 3.0% glucose, and 0.16% saccharin. For 3 h daily, a 45-mg food pellet (Bio Serv, Frenchtown, NJ, USA) was delivered automatically into the food receptacle ev-

ery 60 s. At the end of each session, fluid intakes were recorded and replaced with distilled water to drink and a food ration (Purina Lab Chow) sufficient to maintain 80% *ad libitum* body weight levels. In order to produce a large, oral intake of ethanol solution in daily experimental sessions the method of schedule-induced polydipsia was used. If an animal is food-limited and receives small food pellets intermittently there is a concurrent, excessive intake of fluid [19]. This behavioral method has been employed to produce a chronic, excessive intake of various drug solutions, including cocaine [20] and ethanol [21,22]. In the present experiment, to ensure a high intake of ethanol, the schedule induction method was used as well as a sweet solution for the ethanol vehicle. The experimental protocol was reviewed and approved by the Rutgers Animal Care and Facilities Committee.

Serum sampling

Cocaine · HCl was dissolved in saline. A 22.5 mg/kg cocaine dose calculated as the salt was administered to the animal intraperitoneally 15 min after a drinking session. Tail blood samples (100 μl) were collected in capillary tubes that had been treated with saturated sodium fluoride solution and were obtained at various times between 15 and 240 min after the cocaine injection as described previously [23,24]. Samples were frozen at −20°C until analyzed.

Sample preparation

Standards and serum samples were prepared as previously described [25]. A 10-μl volume of the internal standard (mazindol, 2.5 μg/ml) and 50 μl of a working standard were mixed in a 15-ml conical centrifuge tube. Borate buffer (1 *M*, pH 9.0, 100 μl) and 1 ml of chloroform were added to the sample, vortex-mixed for 40 s, followed by centrifuging at room temperature for 5 min at 1100 *g*. The chloroform layer was carefully transferred to a 5-ml conical centrifuge tube and evaporated in an evaporator (Pierce, Rockford, IL, USA) at 40°C under nitrogen. The residue was resuspended in 50 μl of the mobile phase and injected onto the column. Samples for serum drug analysis were prepared identically.

Extraction recovery

The assay recovery for these four compounds was assessed at concentrations of 0.1, 0.5 and 1 μg/ml. Six replicates of each concentration, containing the four compounds, extracted according to the method described above, were injected into the column. Six replicates of each concentration were computed using the following equation: recovery = (peak height extract) / (mean peak height direct injection) × 100%.

RESULTS

Method evaluation

Fig. 2 shows the chromatogram of (A) a serum blank with no interfering peaks for the analysis, (B) a spiked serum sample containing a working standard and internal standard, (C) the rat serum sample (50 μl) obtained 45 min after a rat self-administered 7.1 g/kg ethanol in a 3-h schedule-induced drinking session and 30 min post-injection of a 22.5 mg/kg cocaine dose intraperitoneally and (D) as in (C) except a 100-μl rat serum sample was used 45 min post-injection of cocaine. Our method simultaneously identifies cocaine, norcocaine, cocaethylene and norcocaethylene. Norcocaethylene was not identified in the 50-μl sample but it was detectable in the 100-μl sample.

Within-day and between-day precisions were established with three different concentrations (0.1, 0.5 and 1 μg/ml) for cocaethylene, cocaine, norcocaine and norcocaethylene by adding these four compounds to blank serum. The coefficients of variation (C.V.) for these compounds ranged from 3.61 to 10.7% for within-day and 1.84 to 7.89% for between-day precision (Table I).

Mean ± S.D. recoveries (n = 18) of cocaethylene, cocaine, norcocaine and norcocaethylene were 98.3 ± 6.3, 86.0 ± 5.5, 89.7 ± 5.8 and 78.9 ± 3.5%, respectively. The detection limit, corresponding to a signal-to-noise ratio of 3, was 0.1 ng (5 ng/ml) for cocaine, norcocaine and cocaethylene, whereas for norcocaethylene it was 0.2 ng (10 ng/ml). Standard curves showed a linear relationship over the range 0.05–2 μg/ml for all the four compounds.

A series of 23 compounds at a concentration of

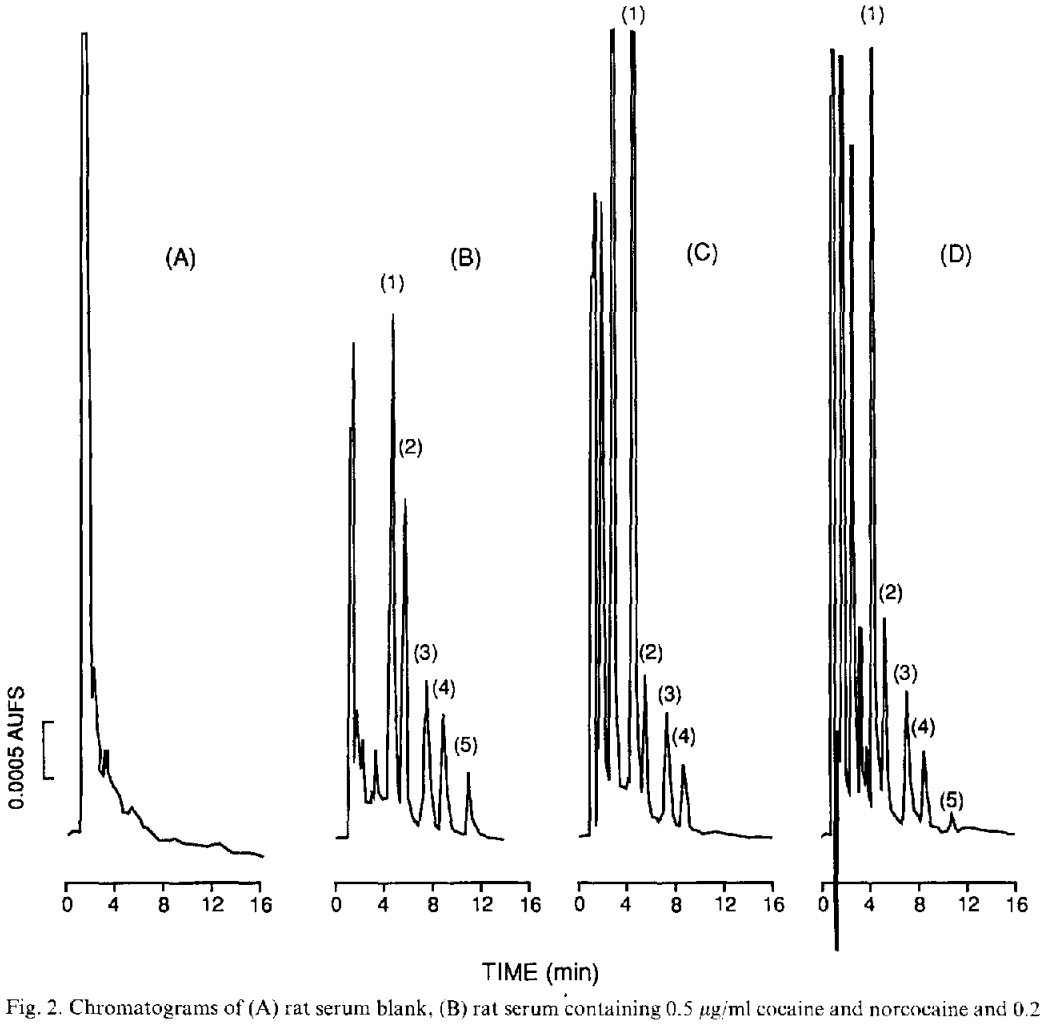

Fig. 2. Chromatograms of (A) rat serum blank, (B) rat serum containing 0.5 μg/ml cocaine and norcocaine and 0.2 μg/ml cocaethylene and norcocaethylene, (C) a 50-μl rat serum sample obtained 45 min after a 3-h ethanol oral self-administration and 30 min after intraperitoneal injection of 22.5 mg/kg cocaine dose and (D) a 100-μl rat serum sample obtained 60 min after a 3-h ethanol oral self-administration and 45 min after intraperitoneal injection of 22.5 mg/kg cocaine dose. Peaks: 1 = cocaine; 2 = norcocaine; 3 = mazindol; 4 = cocaethylene; 5 = norcocaethylene.

1 μg/ml were tested for possible interference with cocaethylene, cocaine, norcocaine or norcocaethylene detection (Table II). Caffeine and the two more polar cocaine metabolites, benzoylecgonine and benzoylnorecgonine, eluted much earlier under the described conditions. The relative retention times of the other drugs did not interfere with these analyses. However, haloperidol partially overlapped with cocaine; nicotine was insufficiently resolved from cocaethylene.

Pharmacokinetic results

Fig. 3 shows the serum concentration–time profiles of cocaine, norcocaine, and cocaethylene for an individual animal after oral self-administration of ethanol followed by a single intraperitoneal dose of 22.5 mg/kg cocaine as described in the method. The elimination rate constant was determined by the slope of the linear regression line for the log-linear portion of the curve. The half-lives were obtained from the elimination rate constants and the values were 36.8, 57.9 and 55.4

TABLE I

PRECISION DATA FOR COCAETHYLENE, COCAINE, NORCOCAINE AND NORCOCAETHYLENE

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. (%)
Cocaethylene	0.1022 ± 0.0109	10.70	0.1007 ± 0.0072	7.18
	0.5023 ± 0.0387	7.71	0.5071 ± 0.0245	4.83
	1.0172 ± 0.0736	7.24	0.9966 ± 0.026	2.61
Cocaine	0.1033 ± 0.0094	9.12	0.1025 ± 0.0043	4.18
	0.504 ± 0.0248	4.92	0.5059 ± 0.0201	3.97
	1.0175 ± 0.0904	8.88	1.0003 ± 0.0225	2.25
Norcocaine	0.1042 ± 0.0094	9.00	0.0997 ± 0.0055	5.47
	0.5158 ± 0.0371	7.20	0.5084 ± 0.0304	5.99
	1.0072 ± 0.0639	6.34	1.0013 ± 0.0184	1.84
Norcocaethylene	0.1007 ± 0.0047	4.69	0.1018 ± 0.0062	6.13
	0.5016 ± 0.042	8.37	0.5095 ± 0.0402	7.89
	1.0254 ± 0.037	3.61	0.9898 ± 0.0455	4.60

min for cocaine, norcocaine and cocaethylene, respectively.

DISCUSSION

The results demonstrate that cocaine, norcocaine, cocaethylene and norcocaethylene can be quantitatied effectively in serum microsamples with a small volume of extraction solvent (1 ml) and a single-solvent extraction step by HPLC. Benzoylnorecgonine and benzoylecgonine were not resolvable with this column even with a lower acetonitrile concentration. Therefore, no attempts were made to extract these two amphoter-

TABLE II

RELATIVE RETENTION TIMES (k') OF OTHER DRUGS FOR POSSIBLE INTERFERENCE WITH COCAETHYLENE, COCAINE AND NORCOCAINE

Compound	k'	Compound	k'
Caffeine	0.99	Theobromine	N.D.
Benzoylecgonine	1.86	Theophylline	N.D.
Benzoylnorecgonine	1.89	Paraxanthine	N.D.
Haloperidol	3.02	Chlorpromazine	N.D.
Cocaine	3.35	Clonazepam	N.D.
Norcocaine	4.4	Diazepam	N.D.
Mazindol	6.2	Flurazepam	N.D.
Cocaethylene	7.97	Temazepam	N.D.
Nicotine	8.49	Methadone	N.D.
Norcocaethylene	10.03	Desipramine	N.D.
Phenobarbital	N.D.[a]	d-Amphetamine	N.D.
Pentobarbital	N.D.	Lidocaine	N.D.
Triazolam	N.D.	Flumazenil	N.D.
Alprazolam	N.D.	Procaine	N.D.

[a] N.D. = peak not detectable under assay conditions.

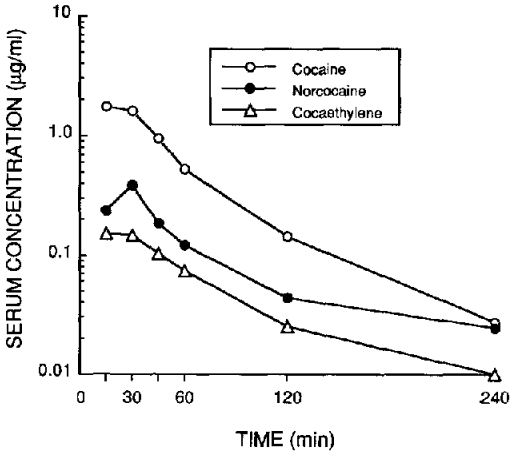

Fig. 3. Cocaethylene, cocaine and norcocaine serum concentration-time profiles for a rat following a single intraperitoneal injection of 22.5 mg/kg cocaine dose 15 min after a 3-h oral ethanol self-administration session.

ic metabolites from the serum samples using the appropriate solvents, *e.g.* a mixture of ethanol and chloroform [23,25]. However, our samples still partially extracted these compounds and had a peak at 2.8 min as shown in Fig. 2C and D.

The pH, buffer salt concentration, TBAP concentration and the percentage of acetonitrile were adjusted to result in the optimal separation of drug peaks that were free of interfering endogenous serum peaks. It is observed that the k' values of the five compounds increased as pH increased. Decreasing the KH_2PO_4 or TBAP concentration also increased the retention times of all the compounds and resulted in peak tailing of cocaethylene and norcocaethylene. This general increase in solute retention was insignificant at and above 53 m*M* phosphate buffer and 0.35 m*M* TBAP. Thus, at these mobile phase KH_2PO_4 and TBAP concentrations, the retention of these compounds is least sensitive to changes in either KH_2PO_4 or TBAP concentration and accordingly provides better reproducibility.

Mazindol, a dopamine agonist, is chemically different from cocaine. However, mazindol is completely resolved from the other compounds and chemically stable in our extraction procedure. We chose internal standard calibration (Table I), since the precision was better than with an external standard technique [26].

Although this procedure is intended for quantification of cocaethylene, cocaine and their metabolites, we also evaluated a series of 23 drugs and drug metabolites (Table II). The chromatographic conditions should be adjusted if haloperidol or nicotine is present in the samples.

Our results show that, in the rat, cocaethylene and norcocaethylene are formed *in vivo* with concurrent ethanol and cocaine administration. As in humans, the half-life of cocaethylene (55.4 min) is much longer than that of cocaine (36.8 min) in the rat. The half-lives for cocaethylene and cocaine in humans are approximately 2 h and 38–60 min, respectively [8]. The method also detected a trace amount of norcocaethylene in rat, the N-demethylated metabolite of cocaethylene, which was detected in human hair [14]. However, the level of this compound was not sufficient for half-life evaluation.

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