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Determination of plasma cocaine and ethylcocaine (cocaethylene) in mice using gas chromatography–mass spectrometry and deuterated internal standards

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Abstract

A gas chromatographic–mass spectrometric method is described for the determination of cocaine and ethylcocaine (cocaethylene) from mouse plasma microsamples (50 μl). [$^2H_3$]Cocaine and [$^2H_5$]ethylcocaine served as internal standards, analytical separations were performed on a (5% phenyl)methylpolysiloxane capillary column, and detection was by selected-ion monitoring of electron-impact generated fragment ions [M − CO_2Ph]. Pilot study plasma concentrations of ethylcocaine following coadministration of cocaine and ethanol were less than 5% of the parent drug.

1. Introduction

Coadministration of cocaine and ethanol in humans [1,2], rats [3,4] and mice [5,6] leads to the metabolic formation of a pharmacologically active adduct of the two drugs, ethylcocaine (cocaethylene). Hepatic esterase(s), which hydrolyzes cocaine to benzoylecgonine in the absence of ethanol, appears to catalyze this intriguing transesterification pathway [6,7] (Fig. 1). A renal esterase has also been implicated in ethylcocaine formation [6].

While ethanol has been reported to inhibit cocaine metabolism, thereby elevating *in vitro* [4,8] and *in vivo* [1,2] cocaine concentrations, the extent to which the metabolite ethylcocaine contributes, *per se*, to the response associated with cocaine–ethanol dosing remains unclear. In studies using rats, cocaine and ethylcocaine exhibit similar potency in schedule-controlled responding [9], *in vitro* dopamine transporter blockade [10–13], transporter binding [11,14] and locomotor stimulating activity [10]. However, cocaine administered to mice has been reported to be more potent than ethylcocaine in inducing the locomotor response [15], but less lethal [15,16]. What pharmacological differences do exist between these two ester homologs may be as much pharmacokinetic as pharmacodynamic in nature.

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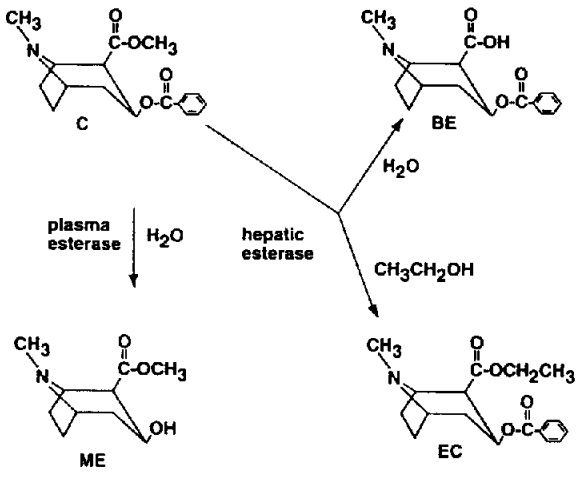

Fig. 1. Esteratic pathways of cocaine (C) to methylecgonine (ME), benzoylecgonine (BE) and ethylcocaine (EC).

Methods to detect or quantitate ethylcocaine from biological samples have utilized high-performance liquid chromatography [1,3–7,17–20] or gas chromatography (GC) with flame ionization [21,22], nitrogen–phosphorous [2,14,23] or mass spectrometric (MS) [4,7,11,14,22–25] detection. Of these methods, GC–MS offers the greatest molecular specificity and permits the incorporation of near ideal internal standards, *i.e.* isotopologs of the analyte(s) [26]. The present study describes the novel development and application of an electron impact (EI)–selected-ion monitoring GC–MS method for the analysis of cocaine and ethylcocaine from mouse plasma microsamples (50 μl) benefitting from the incorporation of both [$^2H_3$]cocaine and [$^2H_5$]ethylcocaine as internal standards (Fig. 2).

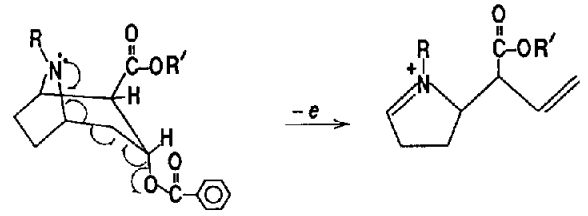

Fig. 2. Structures of cocaine (R = R′ = CH_3), ethylcocaine (R = CH_3; R′ = CH_2CH_3), [$^2H_3$]cocaine (R = C[$^2H_3$]; R′ = CH_3), [$^2H_5$]ethylcocaine (R = CH_3; R′ = C[$^2H_2$]C[$^2H_3$]) and the corresponding EI generated ions selected for monitoring by GC–MS.

2. Experimental

2.1. Chemicals

The cocaine-HCl used for the 60 mg/kg dosing was from Sigma (St. Louis, MO, USA). The cocaine-HCl (batch No. 6907-1022-167C, purity >95%) used for the 40 mg/kg dosing and the [$^2H_3$]cocaine (batch No. 3995-6-B, purity >98%, isotopic purity: 99.77% [$^2H_3$], 0.23% [$^2H_2$]) were obtained from the National Institute of Drug Abuse (Rockville, MD, USA). Ethylcocaine-HCl 2.5 H_2O was from Research Biochemicals (Natick, MA, USA). [$^2H_5$]Ethanol was from Aldrich (Milwaukee, WI, USA). Benzoylecgonine was synthesized from cocaine-HCl by the method of Jane *et al.* [27]. Ethanol was from Pharmaco (Bayonne, NJ, USA). Sodium fluoride was from Baker (Phillipsburg, NJ, USA). Sodium borate was from Mallinckrodt (Paris, KY, USA). The solvents were of HPLC grade. Acetonitrile was from Burdick and Jackson (Muskegon, MI, USA). Isopropanol was from Curtin Matheson (Houston, TX, USA). *n*-Pentane, methylene chloride, perchloric acid, sulfuric acid, sodium carbonate and sodium sulfate were from Fisher (Fairlawn, NJ, USA). Horse serum was from Sigma.

2.2. Synthesis of [$^2H_5$]ethylcocaine

[$^2H_5$]Ethylcocaine was prepared using a modification of a related synthesis [28]. Benzoylecgonine (11 mg) was heated with [$^2H_5$]ethanol (0.5 ml) and sulfuric acid (0.05 ml) in a derivatization vial at 85°C for 2 h. After cooling to room temperature, water (2 ml) was added and the pH was adjusted to 9–10 with saturated aqueous sodium carbonate. The [$^2H_5$]ethylcocaine was extracted with methylene chloride, treated with sodium sulfate, evaporated to dryness, then used without further purification as a solution in acetonitrile (50 ng/μl), Fig. 3: GC–

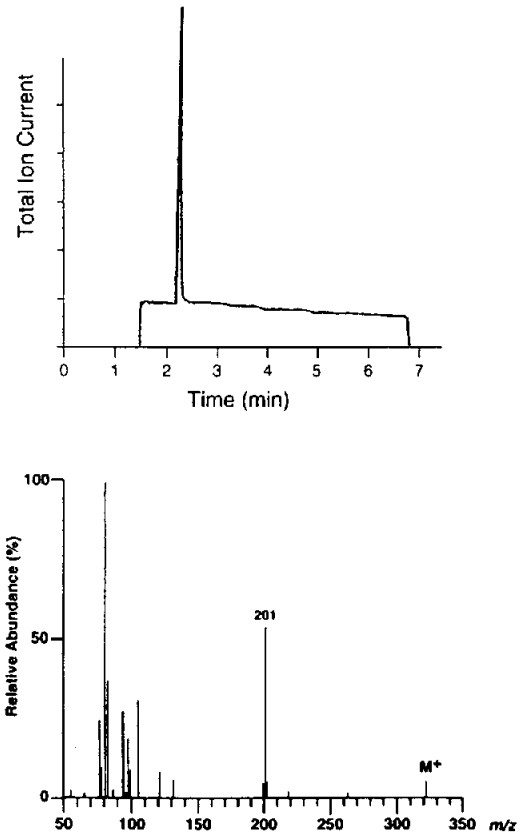

Fig. 3. Total ion chromatogram and EIMS of [$^2H_5$]ethylcocaine. The *m/z* 201 fragment-ion was monitored for this internal standard.

EIMS (% relative abundance): *m/z* 322 (M^+,9), 201(56), 105(33), 94(29), 82(100).

2.3. Pilot drug treatments

Four adult female C57BL/6J mice were injected i.p. (0.02 ml/g body weight) with an aqueous solution of ethanol (2.5 g/kg) and cocaine-HCl (40 mg/kg, base). The plasma samples (50 μl) were collected via the infraorbital sinus 20 min later, treated with sodium fluoride and stored at −70°C (see ref. 29).

Five adult female C57BL/6J mice were intubated with ethanol (5.8 g/kg) using volumes adjusted to 0.03 ml/g body weight. The mice were then injected s.c. with cocaine-HCl (60 mg/kg, base) in 0.9% NaCl (0.02 ml/g body weight) 10 min later, and plasma (50 μl) was collected as above after another 60 min.

2.4. Extraction and analysis

Sample extraction and GC–MS were conducted by a modification of a method recently detailed for cocaine determinations [29]. Briefly, 1% sodium fluoride (0.25 ml) containing [$^2H_3$]cocaine (100 ng) and [$^2H_5$]ethylcocaine (50 ng) was added to each 50 μl biological sample, followed by alkalinization (sodium borate), extraction with 3 ml of pentane–isopropanol (97:3, v/v), and evaporation to dryness under nitrogen. Calibration standards used horse serum (50 μl), which was found to be substitutable for mouse plasma, spiked with cocaine-HCl in methanol (25 ng/μl) to provide 0, 0.2, 0.5, 1, 2, 3, and 4 μg/ml (base) concentrations and with ethylcocaine-HCl (in ethanol, 1 or 10 ng/μl) to provide 0, 0.02, 0.05, 0.1, 0.2, 0.3, and 0.4 μg/ml (base) concentrations.

For all cocaine determinations and for ethylcocaine determinations in calibration standards greater than 0.2 μg/ml, samples were reconstituted in acetonitrile (20 μl), then concentrated to approximately 10 μl under a stream of nitrogen just prior to injection. For samples containing 0.2 μg/ml or less ethylcocaine, *i.e.* the lower calibration standards and the pilot unknowns, a second injection of each was required after further reducing the acetonitrile volume to approximately 4 μl. This provided adequate ethylcocaine sensitivity; however, with these more concentrated injections the cocaine and [$^2H_3$]cocaine ion-currents typically saturated the detector (> 100% detector response, see Fig. 4) under the instrumental conditions used (see below).

All analyses utilized a Finnigan Model 9610 GC–Model 4000 MS interfaced to an IBM-AT computer using a Teknivent Vector/One data system and software (St. Louis, MO, USA). The injector port was adapted to capillary bore using a 17.8-cm conversion sleeve and a reducing union (Supelco, Bellafonte, PA, USA). The MS

was calibrated with perfluorotributylamine (FC-43) using the m/z 219 and 220 ions underresolved to gain sensitivity. Splitless injections of 0.15 μl were made onto a (5% phenyl)methylpolysiloxane fused-silica column, 30 m × 0.32 mm I.D., 0.25 μm film thickness (DB-5, J and W Scientific, Folsom, CA, USA). The column oven was maintained at 240°C and the injector port and interface oven at 250°C. The helium carrier gas head pressure was 83 kPa and the linear velocity was 55 cm/s. Under these conditions, cocaine, [$^2H_3$]cocaine, ethylcocaine and [$^2H_5$]ethylcocaine eluted 2.05, 2.02, 2.25, and 2.23 min after injection, respectively (Fig. 4). The filament was powered 1.5 min after each injection. Detection was by selected-ion monitoring (Fig. 2) with EI (70 eV) ionization. The preamp was set at 10^{-8} A/V and the electron multiplier at 1925 V. The data system acquired 4 channels of fragment-ion current: cocaine m/z 182 (59% relative abundance), [$^2H_3$]cocaine m/z 185 (56), ethylcocaine m/z 196 (58), and [$^2H_5$]ethylcocaine m/z 201 (56).

2.5. Calculations

The concentrations of cocaine and ethylcocaine in unknown samples were calculated from the slope and intercept of the associated standard curve, plotted as GC–MS peak-area ratios (cocaine/[$^2H_3$]cocaine or ethylcocaine/[$^2H_5$]ethylcocaine) *vs.* known cocaine or ethylcocaine concentrations.

3. Results and discussion

In addition to an analytical control function and potential carrier role [26], inclusion of both [$^2H_3$]cocaine and [$^2H_5$]ethylcocaine as internal standards assisted in confirming the identity of the analytes through comparison of retention times and peak shapes (Fig. 4). In spite of the well established chromatographic advantages of temperature programming, an isothermal mode was used to increase sample throughput. The cocaine metabolite methylecgonine, and a minor by-product of the [$^2H_5$]ethylcocaine synthesis, [$^2H_5$]ethylecgonine, gave rise to the ions m/z 182 and 201, respectively. However, these compounds eluted prior to powering the filament and thus did not interfere with any determinations.

The quality of the present analytical method is indicated in Table 1. Precision ranged from 22.2 to less than 3% coefficient of variation (C.V.) going from 0.02 to 0.4 μg/ml ethylcocaine calibration standards and from 17.8 to 1.7% in the 0.2–4.0 μg/ml cocaine standards. The mean correlation coefficients for the cocaine and ethylcocaine calibration plots were 0.9971 ± 0.0006 (S.E.) and 0.9968 ± 0.0016, respectively. For the lowest ethylcocaine calibration standard, 0.02 μg/ml using a 50-μl sample, the signal-to-noise ratio was approximately 4. With the exception of the detection of cocaine in the m/z 201 ion profile (0.5% relative abundance), the chromatograms were free of significant extraneous peaks. This is illustrated in Fig. 4, where plasma ethylcocaine was determined to be present at 0.06 μg/ml after the high-dose drug protocol (see Experimental). The chromatogram in Fig. 5 demonstrates the negligible biochemical noise observed from a blank mouse plasma extract

Table 1
Accuracy and precision of the analytical method

Concentration (μg/ml)		n	C.V.
Added	Detected (mean)		
Cocaine			
0.20	0.208	4	17.76
0.50	0.505	4	10.03
1.00	1.033	4	5.99
2.00	2.015	4	1.70
3.00	3.03	4	4.21
4.00	3.94	4	1.70
Ethylcocaine			
0.02	0.023	4	22.22
0.05	0.048	4	10.53
0.10	0.101	4	6.86
0.20	0.201	4	1.89
0.30	0.295	4	4.38
0.40	0.403	4	2.38

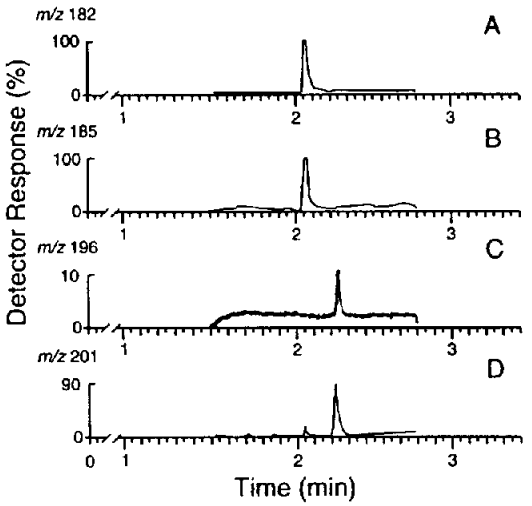

Fig. 4. Selected-ion chromatograms of cocaine (A), [$^2H_3$]cocaine (B, internal standard), ethylcocaine (C), and [$^2H_5$]ethylcocaine (D, internal standard) extracted from plasma (50 μl) of a mouse treated with the high-dose cocaine–ethanol regimen (see Experimental). Detector response is indicated as % full scale deflection.

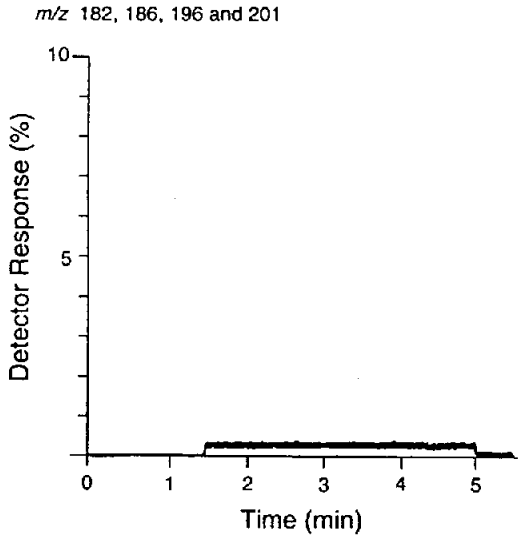

Fig. 5. Merged ion chromatogram (*m/z* 182, 185, 196 and 201) from an extracted blank mouse plasma sample where the internal standards have been omitted. Detector response is indicated as % full scale deflection.

(the 4 ions acquired are displayed in a merged fashion).

Under the 40 and 60 mg/kg cocaine treatment schedules used in the two pilot studies, the mean concentrations for cocaine were 1.32 ± 0.33 (S.E.) and 1.93 ± 0.15 μg/ml, respectively; the corresponding metabolically formed ethylcocaine concentrations were 0.06 ± 0.01 and 0.04 ± 0.01 μg/ml. These mean ethylcocaine concentrations represent only 4.5% that of the parent drug at the 40 mg/kg dose and 2% at the higher dose. The difference between these two percentages may be attributed to the dissimilar doses, routes of administration, and sacrifice times used in the two pilot experimental designs. Species-specific differences in esterases may be pertinent to the various ethylcocaine concentrations relative to cocaine concentrations reported in the literature (Table 2). Indeed, genotypic differences in esteratic rates find precedent in the fourfold difference in cholinesterase (which catalyzes the deesterification of cocaine to methylecgonine [30], Fig. 1) activities between various strains of mice [31].

The small sample size (50 μl), simplicity of workup, specificity of detection, and the analytical control offered by the incorporation of both [$^2H_3$]cocaine and [$^2H_5$]ethylcocaine distinguish the present method. The low plasma concentrations of ethylcocaine relative to cocaine found in these pilot studies are consistent with the low hepatic concentrations of ethylcocaine in cocaine–ethanol dosed mice reported by Boyer and Petersen [6] and suggests, at most, only a minor direct role for this metabolite in the pharmacological response to coadministration of these two drugs in female C57BL/6J mice under the described experimental conditions.

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Table 2
Ethylcocaine and cocaine concentrations reported in the literature

Investigation	Matrix	Species	Dose cocaine (mg/kg)	route	Dose ethanol (g/kg)	route	Sampling time (min after dose)		Mean concentrations (μg/ml or μg/g)	
							Ethanol	Cocaine	Cocaine	Ethylcocaine
Present study	Plasma	Mouse	60	s.c.	5.8	p.o.	70	60	1.93	0.04
			40	i.p.	2.5	i.p.	20	20	1.32	0.06
Lau [3]	Serum	Rat	20	i.p.	7.1	p.o.	75	60	0.5	0.07
Roberts *et al.* [5]	Liver	Mouse	50	i.p.	3	i.p.	120	60	1.5	0.25
McCance–Katz *et al.* [1]	Serum	Human	1.78	i.n.	1	i.p.	70	60	0.36	0.05
Perez and Jeffcoat [2]	Plasma	Human	1.11	i.n.	0.85	p.o.	75	60	0.14	0.02
			1.69		0.85		75	60	0.27	0.03
Dean *et al.* [7]	Liver	Rat	22.2	i.p.	2	i.p.	45	15	1.09	0.25
	Brain						45	15	2.09	0.19
	Serum						45	15	1.52	0.16
Boyer and Petersen	Liver	Mouse	44.7	i.p.	3	p.o.	15	3	74.54	4.98

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