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# Quantitation of cocaine and cocaethylene in canine serum by high-performance liquid chromatography

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**Abstract**

A reversed-phase high-performance liquid chromatographic procedure for the determination of cocaine and cocaethylene in canine serum has been developed. The compounds were extracted from 1 ml of alkalinized canine serum with hexane. Chromatographic separation was achieved with a cyanopropyl column (250 × 4.6 mm I.D., 5 μm) using a mobile phase of acetonitrile and phosphate buffer, pH 7.40 (38:62, v/v) flowing at 1 ml/min. Eluate was monitored by a variable-wavelength UV detector set to 230 nm. The extraction procedure yields an average recovery of 99 and 96% for cocaine and cocaethylene, respectively. The between-day coefficients of variation, at 2400 ng/ml, for cocaine and cocaethylene were both 8.6% and the within-day coefficients of variation, at 400 ng/ml, for cocaine and cocaethylene were 7.3 and 8.0%, respectively. A concentration–time profile resulting from administration of 3 mg/kg cocaine and cocaethylene to the dog revealed a similar disposition between cocaine and cocaethylene, with a clearance and volume of distribution at steady-state values of 72.8 and 61.0 ml/min/kg and 2.6 and 2.7 l/kg, respectively.

*Keywords:* Cocaine; Cocaethylene

## 1. Introduction

Cocaethylene is a metabolite of cocaine, formed in the presence of ethanol in humans. Cocaine is converted to cocaethylene via transesterification catalyzed by a hepatic esterase [1,2]. The structure of cocaethylene differs from cocaine in that the ester group is an ethyl, rather than a methyl, group (see Fig. 1). The similarity in structure between these two compounds has lead to speculation that cocaethylene may retain pharmacological activity. Indeed, studies in animals demonstrate that cocaethylene, like cocaine, increases heart rate and blood pressure [3]. In addition, cocaethylene is a more potent sodium-channel blocker than cocaine [4,5], suggesting that it may be a more potent arrhythmogenic. Furthermore, in postmortem human blood samples, cocaethylene concentrations have been reported to exceed cocaine concentrations [6]. The extent to which cocaethylene may contribute to toxicity in cocaine users is extremely important, as 80% of cocaine users co-ingest ethanol [7].

Because of the growing interest in cocaethylene as a potential contributor to cocaine toxicity, the necessity for analytical techniques to measure both compounds arises. Several methods exist that employ high-performance liquid chromatography (HPLC) to quantitate both cocaine and cocaethylene. This paper

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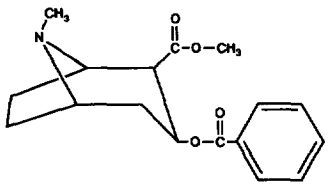
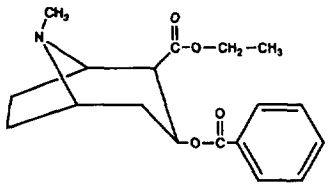


Cocaine



Cocaethylene

Fig. 1. The chemical structures of cocaine and cocaethylene.

describes a simple, isocratic, reversed-phase separation with a two-step liquid–liquid extraction of cocaine and cocaethylene. The mobile phase requires no additives for ion-pairing and provides complete separation of the compounds of interest.

## 2. Experimental

### 2.1. Materials and equipment

Methanol stock solutions of cocaine hydrochloride (Sigma, St. Louis, MO, USA), cocaethylene hydrochloride (Research Biochemicals, Natick, MA, USA), norcocaine hydrochloride (Research Biochemicals) and lidocaine hydrochloride (Sigma) at a concentration of 100 ng base/$\mu$l were prepared and stored at −70°C. HPLC-grade acetonitrile (Burdick and Jackson, Muskegon, MI, USA) and hexane (J.T. Baker, Phillipsburg, NJ, USA) were used.

A Shimadzu autosampler (Model SIL-10A, Shimadzu, Kyoto, Japan) with a sample cooler was employed to inject samples onto the analytical column. The eluate was monitored by a variable-wavelength UV detector (LDC Spectromonitor III, Milton Roy, Riviera Beach, FL, USA) set to a wavelength of 230 nm. The detector output was monitored and analyzed by a chromatography data acquisition system (Axxiom Chromatography, Model 727, Calabasas, CA, USA).

### 2.2. Dosing and sample collection

To test the applicability of this assay procedure, a mongrel dog was given a 3 mg/kg dose of cocaine and cocaethylene simultaneously, a 3 mg/kg dose of cocaine alone and a 3 mg/kg dose of cocaethylene alone, on three separate study days as 5-min intravenous infusions into a foreleg vein. Arterial blood samples were collected from an in-dwelling catheter in the internal carotid artery. Blood was drawn into a 5-ml plastic syringe connected via a stopcock to the catheter, immediately transferred to a 7-ml Vacutainer® tube (gray top) containing 30 mg of sodium fluoride (to inhibit degradation of cocaine by plasma esterases), gently mixed and then put on ice. The blood was stored in ice for 15 to 30 min, then centrifuged at 1315 *g* for 5 min and the serum was transferred to a plastic screw-top cryogenic storage vial. Samples were stored at −70°C until assayed.

### 2.3. Extraction

The buffer for alkalinization of serum samples, prior to extraction, was made by adding 24 ml of 0.1 *M* $Na_2CO_3$ to 176 ml of 0.1 *M* $NaHCO_3$, yielding 200 ml of carbonate buffer with a pH of 10.7. One ml of serum was transferred to a glass, acid-washed screw-top test tube. A 50-$\mu$l volume of a methanolic solution containing 10 ng/$\mu$l of lidocaine (the internal standard) was added to the serum and the sample was vortex-mixed for 5 s. Carbonate buffer (1 ml) with a pH of 10.7 was added to the serum and the sample was again vortex-mixed (5 s). Hexane (5 ml) was added and the sample shaken for 3 min on an oscillating shaker (American Optical). The sample was then centrifuged for 3 min at 1200 *g*, and the lower aqueous layer was frozen by placing the test tube in an acetone bath cooled with dry ice, in a Dewar flask. The hexane was poured into a disposable borosilicate test tube, evaporated to dryness

under a stream of nitrogen and the residue was reconstituted in 200 μl of the mobile phase.

### 2.4. Separation

The reconstituted sample was transferred to a 250-μl polypropylene vial (Sun Brokers, Wilmington, NC, USA) that fits inside the autosampler vial. Samples were loaded into the autosampler and maintained at a temperature of 2°C to inhibit spontaneous hydrolysis of cocaine and cocaethylene. Separation was achieved using a Supelcosil LC-CN (25 cm × 4.6 mm I.D., 5 μm) analytical column (Supelco, Bellefonte, PA, USA). The mobile phase was acetonitrile–phosphate buffer pH 7.4 (38:62, v/v) with a mobile phase flow-rate of 1 ml/min.

### 2.5. Percentage recovery and variability

An eight-point standard curve, with a concentration range for cocaine and cocaethylene of 25 to 3200 ng/ml, was run daily. The quantitation of cocaine and cocaethylene was based on a weighted ($1/y$) least-squares regression of peak-height ratios to the known concentration standards. The percentage recovery was determined by extracting 22–24 samples containing 100, 400 and 3200 ng/ml of cocaine and cocaethylene per ml of serum. The resulting peak heights for the extracted samples were compared to peak heights of direct injections to determine the percentage recovery.

Within-day variability was determined by extracting 18 to 24 1-ml serum samples containing 100, 400 and 3200 ng/ml of cocaine and cocaethylene. For between-day variability, 50 ml of serum was spiked with 6 μg of cocaine and cocaethylene to achieve a concentration of 120 ng/ml and another 50 ml of serum was spiked with 120 μg of cocaine and cocaethylene to achieve a concentration of 2400 ng/ml serum. This pool of spiked serum was divided into 1-ml aliquots and frozen at −70°C. From two to four between-day variability samples were quantitated with each standard curve.

### 2.6. Pharmacokinetic analysis

The half-life was estimated using linear least-squares regression of the terminal concentrations. The area under the curve (AUC) and the area under the moment curve (AUMC) were estimated by the trapezoidal rule, with the area from the last time point to infinity (*Tail*) estimated by:

$$Tail_{AUC} = \frac{C_{LAST}}{k}$$

$$Tail_{AUMC} = \frac{t \times C_{LAST}}{k} + \frac{C_{LAST}}{k^2}$$

Where $C_{LAST}$ is the last quantifiable concentration time point, $t$ is the time relative to the start of the infusion, and $k$ is the estimated terminal elimination rate constant. Clearance was estimated by dividing the dose by the AUC, and the volume of distribution at steady-state ($V_{ss}$) was estimated by statistical moment theory using the following equation [8].

$$V_{ss} = \frac{k_o \times T \times AUMC}{AUC^2} - \frac{k_o \times T^2}{2 \times AUC}$$

## 3. Results

Separation of cocaine and cocaethylene was achieved with a run time of 25 min (see Fig. 2). Benzoylecgonine and ecgonine methyl ester both elute in the solvent front under these conditions. Norcocaine elutes before cocaine and, under these conditions, achieves baseline separation from cocaine (see Fig. 2). Mean recoveries from spiked

Table 1
Quality control data demonstrating the recovery of cocaine and cocaethylene from canine serum at three different concentrations: 100, 400 and 3200 ng/ml

| Compound | $n$ | Amount (ng) | Recovery (%) |
|---|---|---|---|
| Cocaine | 23 | 100 | 97±11.5 |
| | 22 | 400 | 100±4.9 |
| | 24 | 3200 | 99±6.7 |
| Cocaethylene | 23 | 100 | 95±10.8 |
| | 22 | 400 | 99±5.2 |
| | 24 | 3200 | 95±6.4 |

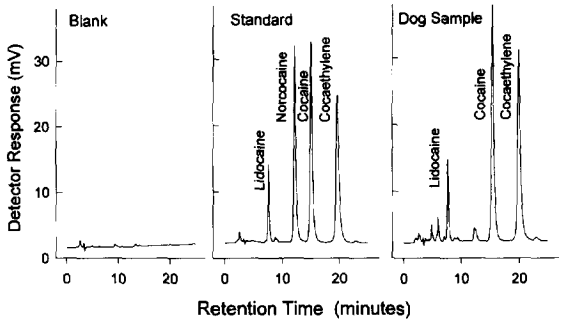



Fig. 2. Three separate chromatograms of canine serum; (left) Blank = extracted plasma sample from dog, (middle) standard = spiked with internal standard, 800 ng of norcocaine, cocaine and cocaethylene, and (right) dog sample = assay of serum concentration from dog after simultaneous administration of 3 mg/kg of cocaine and cocaethylene (result 911 ng/ml cocaine and 945 ng/ml cocaethylene). The retention times for lidocaine, norcocaine, cocaine and cocaethylene were 9.3, 13.6, 16.8 and 20.1 min, respectively.

serum for cocaine and cocaethylene ranged from 95 to 100% (see Table 1).

The accuracy and coefficients of variation (C.V.) are given in Table 2. The coefficient of variation (C.V.) for within-day and between-day quantitation remained within clinically acceptable values over the entire range of the standard curve.

Table 2
Quality control data demonstrating the within-day and between-day variability for cocaine and cocaethylene at 100, 400 and 3200 ng/ml

| Compound | $n$ | Concentration (ng/ml) | | C.V. (%) |
|---|---|---|---|---|
| | | Actual | Found | |
| *Between-day* | | | | |
| Cocaine | 18 | 100 | 110 | 9.7 |
| | 18 | 3200 | 2982 | 11.1 |
| Cocaethylene | 18 | 100 | 105 | 10.2 |
| | 18 | 3200 | 2919 | 11.6 |
| *Within-day* | | | | |
| Cocaine | 18 | 100 | 110 | 8.1 |
| | 24 | 400 | 381 | 7.3 |
| | 18 | 3200 | 2982 | 8.7 |
| Cocaethylene | 18 | 100 | 105 | 9.7 |
| | 24 | 400 | 409 | 8.0 |
| | 18 | 3200 | 2919 | 7.8 |

For between-day variability six samples were quantified each day, on three separate days.

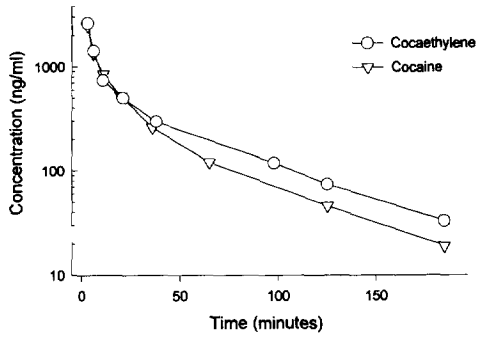



Fig. 3. The concentration–time profile of cocaine and cocaethylene following the administration of 3 mg/kg of cocaine and cocaethylene in the dog. Cocaine and cocaethylene were administered on separate days.

Table 3
The pharmacokinetic parameter estimates determined from the concentration–time profiles shown in Fig. 3

| Parameter | Cocaine | Cocaethylene |
|---|---|---|
| $k$ ($min^{-1}$) | 0.0171 | 0.015 |
| $t_{1/2}$ (min) | 40.4 | 46.0 |
| Clearance (ml/min/kg) | 72.8 | 61.0 |
| $V_{ss}$ (l/kg) | 2.6 | 2.7 |

Cocaine and cocaethylene concentration–time profiles are shown in Fig. 3 and the corresponding pharmacokinetic parameters are given in Table 3. The estimates for half-life, $V_{ss}$ and clearance demonstrate the similarity in the pharmacokinetic profiles of these two compounds.

## 4. Discussion

The isocratic, reversed-phase separation and liquid–liquid extraction described in this paper provide a simpler assay method, with several advantages over previously published assays. The extraction procedure yields excellent recovery of the compounds of interest, is less expensive and laborious than solid-phase extraction, and produces a minimal amount of organic waste. Other methods described in the literature employ various techniques in extracting and chromatographically separating cocaine and cocaethylene. Dean et al. [1] used solid-phase car-

tridges to extract cocaine and cocaethylene from tissue samples and serum of rats. This solid-phase extraction procedure requires seven steps and many solutions to complete. The step before elution requires a rinse using 9 ml of methanol, which, when extracting numerous samples, produces a large amount of organic waste. Also, the eluting solution must be made fresh daily and requires the use of methylene chloride, a known carcinogen. Separation of the compounds of interest was achieved using a $C_{18}$ analytical column with an ion-pairing agent added to the mobile phase. Under these conditions, complete separation of norcocaine from cocaine was not achieved. Jatlow and Nadim [9] alkalinized the serum sample and extracted the un-ionized cocaine and cocaethylene into hexane, then back-extracted into dilute hydrochloric acid. Ion-pair chromatography was used with a $C_6$ analytical column heated to 45°C to separate cocaine and cocaethylene. Our separation procedure does not require an ion-pairing agent or column heating. Roberts et al. [10] extracted samples using methylene chloride as the primary extraction solvent and the entire extraction procedure was performed at 4°C. Lau [11] extracted the compounds of interest employing chloroform as the extraction solvent. Puopolo et al. [12] used a solution of 1 *M* sodium carbonate to alkalinize serum. Five ml of hexane were added to extract the compounds and then the sample was agitated for 2 min. The organic layer was transferred, without centrifugation, and evaporated to dryness with nitrogen, in a heated (35–40°C) water bath.

Our method differs from Puopolo's in that we use a carbonate buffer solution composed of sodium carbonate and sodium bicarbonate, to improve reproducibility of the pH used to alkalinize the serum, an increased shaking time to improve recovery, and centrifugation and freezing of the aqueous layer to facilitate maximum transfer of hexane and to eliminate the need for pipetting the hexane. Recovery of the compounds from our assay was 99 and 96%, on average, for cocaine and cocaethylene, respectively, compared to 83.6 and 78.1% reported by Roberts et al. [10], 98.3 and 86.0% reported by Lau [11] and 81% recovery for both compounds reported by Puopolo et al. [12].

The clearance of cocaine in our dog is comparable to previous data published by Wilkerson et al. [13], i.e. 72.8 versus 71.7 ml/min/kg. There are no previous reports of cocaine's $V_{SS}$ in the dog, but our estimate of 2.6 l/kg is similar to the volume of distribution reported in humans (2.0 l/kg) [14]. The elimination of cocaethylene appears to parallel that of cocaine. The clearance, half-life, and $V_{ss}$ are similar for cocaine and cocaethylene (see Table 3). These data suggest that the minor difference in structure between cocaine and cocaethylene does not significantly alter their distributional characteristics or their elimination.

This method allows the quantitation of canine serum concentrations from 25 to 3200 ng/ml. However, higher concentrations could be estimated by extracting less than 1 ml of serum and applying a dilution factor. The usefullness of the procedure is demonstrated by the concentration–time profiles and by the pharmacokinetic parameter estimates that are shown in Fig. 3 and Table 3, respectively. The method is sufficiently sensitive to be adapted for the analysis of human serum samples.

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