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Short Communication

Determination of cocaine, benzoylecgonine and ecgonine methyl ester in plasma by reversed-phase high-performance liquid chromatography

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ABSTRACT

A combined assay is described for cocaine and its major metabolites, benzoylecgonine and ecgonine methyl ester. The method uses electrochemical and ultraviolet detectors in series. A non-silica column is used with high-pH mobile phase. The three compounds are completely separated from other cocaine metabolites. The assay has been suitable for pharmacokinetic studies of cocaine disposition in animal studies.

INTRODUCTION

Cocaine has become one of the most prominently abused drugs. There are increasing needs to analyze biological samples for the presence of cocaine and its metabolites for both forensic purposes and for investigations of drug metabolism and disposition. The analysis of cocaine by various high-performance liquid chromatographic (HPLC) and gas chromatographic methods has been described [1–4]. The major metabolites of cocaine are benzoylecgonine and ecgonine methyl ester [5]; however, these metabolites are also found in relatively high concentrations in plasma [6]. Benzoylecgonine, which is widely measured in urine by homogeneous enzyme immunoassay technique (EMIT, Syva, Palo Alto, CA, USA), has been co-analyzed with cocaine by HPLC and other methods [1–3]. HPLC methods for the simultaneous determination of cocaine, norcocaine, benzoylecgonine and norbenzoylecgonine have also been recently described [7–9]. Routine

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methods for analysis of ecgonine methyl ester, which lacks UV absorbance, have been established with gas chromatography–mass spectrometry [10–14]. We developed an HPLC method for combined analysis of cocaine, benzoylecgonine, and ecgonine methyl ester in plasma over a broad concentration range from the same sample following simple extraction and serial UV and electrochemical detection.

EXPERIMENTAL

Apparatus

Chromatography was performed with an automated HPLC system consisting of an IBM (IBM Instruments, Wellingford, CT, USA) Model 9533 pump fitted with an IBM 9505 automatic sampler and a 100-μl sample loop, A 150 mm × 4.6 mm, 10 μm particle size, Hamilton PRP-1 poly(styrene–divinylbenzene) column (Alltech, Deerfield, IL, USA) was used. An IBM Model 9523 variable-wavelength UV detector was operated at 235 nm. An ESA Model 5100A Coulochem detector (ESA, Bedford, MA, USA) was connected in series with the UV detector using a guard cell set at 0.3 V and a 5011 analytical cell set at 1.0 V. An IBM CS9000 laboratory computer was used to control the pump and injector and to record, store and analyze chromatograms.

Reagents and chemicals

Analytical-grade dibasic potassium phosphate, phosphoric acid and ammonium hydroxide, HPLC-grade acetonitrile, methanol, and ethanol were obtained from Fisher Scientific (Fairlawn, NJ, USA). Water was processed through a Milli-Q water system (Millipore, Bedford, MA, USA). Cocaine and benzoylecgonine were obtained from Sigma (St. Louis, MO, USA) while norcocaine, ecgonine methyl ester, norbenzoylecgonine and ecgonine were gifts from the National Institute of Drug Abuse (Bethesda, MD, USA).

Stock solutions containing 1.0 mg/ml cocaine and benzoylecgonine as the free bases in methanol were prepared. Ecgonine methyl ester was dissolved in methanol–water (50:50) to yield a concentration of 1 mg/ml while norcocaine and norbenzoylecgonine (1 mg/ml) were prepared fresh in acetonitrile–water (50:50).

An internal standard, ethylcocaine, was synthesized as previously described [1]. Briefly, benzoylecgonine, 20 mg, was added to a freshly prepared solution of 1.0 ml acetyl chloride (Aldrich, Milwaukee, WI. USA) in 20 ml of anhydrous ethanol. The mixture was slowly refluxed for 4 h, then evaporated to dryness. The residue was dissolved in water, made basic with dibasic potassium phosphate and extracted with hexane containing 2% isoamyl alcohol. The ethylcocaine was then back-extracted into 1.0 ml of 0.1 *M* hydrochloric acid. The hydrochloric acid solution was made basic with dibasic potassium phosphate, re-extracted with isoamyl alcohol in hexane, then evaporated with a gentle stream of nitrogen. The internal standard was reconstituted in ethanol to a final concentration of 0.1 μg/μl and stored at −15°C. A 1 mg/ml working stock solution was prepared by

dissolving ethylcocaine in methanol–water (50:50). Chromatography demonstrated a single peak and an absence of peaks corresponding to benzoylecgonine, cocaine or other known cocaine metabolites.

Standards for calibration graphs

Standards for calibration curves were prepared by spiking 0.5-ml aliquots of plasma with diluted stock solutions to make cocaine and metabolite standards ranging from 100 to 2400 ng/ml. The internal standard concentration was 1000 ng per sample. Calibration graphs of the recovered samples were prepared for each day of analysis. Peak-height ratio of each compound of interest to the internal standard was plotted against drug concentration and analyzed by linear regression using the LOTUS 1-2-3 microcomputer program (Lotus Development, Cambridge, MA, USA).

Sample collection and storage

Mature female ewes were catheterized as described elsewhere and administered various intravenous doses of cocaine for pharmacokinetic studies [15]. All blood samples for drug analysis were drawn into heparinized syringes, immediately centrifuged at 13 000 *g* for 30 s using a microcentrifuge (Fisher Scientific), and the plasma was transferred to a clean polypropylene tube. Each sample was then acidified with 40 μl of 20% (5 *M*) hydrochloric acid to yield pH 5 and frozen at −20°C until analyzed [16,17].

Extraction procedure

To 0.5 ml of plasma in a 1.8-ml polypropylene microcentrifuge tube, 1 μg (10 μl) of internal standard, 0.5 ml of acetonitrile and 0.5 ml of saturated solution of dibasic potassium phosphate were added. The tubes were stoppered and mixed for 30 s with a vortex touch mixer, then centrifuged at 13 000 *g* for 2 min. The upper organic layer was transferred to a clean polypropylene microcentrifuge tube and evaporated under nitrogen at 30°C. Samples were reconstituted with 100 μl of 0.1% hydrochloric acid, transferred to 300-μl autoinjector tubes, sealed with aluminium foil and loaded onto the automatic injector for HPLC elution.

Chromatographic conditions

Elution was performed at 2.0 ml/min beginning with a mobile phase consisting of 15 m*M* ammonium phosphate buffer containing 7.5% acetonitrile (pH 8.8). At 8.0 min, the mobile phase was automatically switched to 15 m*M* ammonium phosphate buffer containing 50% acetonitrile (pH 8.8). The electrochemical detector was auto-zeroed with a + 15-mV offset at the time of injection and again at 12 min, 3 min before elution of cocaine.

RESULTS

Cocaine, norcocaine, benzoylecgonine, ecgonine methyl ester and the internal standard, ethylcocaine, were separately eluted with retention times of 15, 14, 5.3, 5 and 17 min, respectively. Under the conditions described benzoylecgonine had no electrochemical activity and methylecgonine had no UV absorbance. Separation was achieved with a lack of interference from other peaks. Fig. 1 illustrates typical chromatograms of a methanolic stock solution, extracted blank sheep plasma, a calibration standard and plasma collected from a sheep after cocaine administration.

Calibration curves were consistently linear with correlation coefficients typically greater than 0.99. Within-day precision ($n = 6$) was 6% for cocaine, 5% for benzoylecgonine and 11% for ecgonine methyl ester at a concentration of 400 ng/ml. Between-day precision ($n = 9$) data collected over a three-month period at a concentration of 600 ng/ml was 14.3% for cocaine, 12.2% for benzoylecgonine and 9.5% for ecgonine methyl ester.

Extraction recovery from 0.5 μl of sheep serum at a concentration of 200 ng/ml ($n = 5$) was 62.3% for cocaine, 78.3% for benzoylecgonine, 80.0% for ecgonine methyl ester and 71.5% for ethylcocaine. The limits of detection for cocaine and its metabolites were 25 ng/ml.

DISCUSSION

The pharmacology and clinical effects of cocaine have been extensively studied [5,18,19]. HPLC has been used to identify cocaine and its metabolites in urine, but application to plasma samples has not been widespread due to a lack of available methods. Recently developed methods for assay of cocaine, norcocaine, benzoylecgonine and benzoylnorecgonine from sample volumes as small as 50 μl may be suitable for forensic investigations of human cocaine ingestion [7–9].

The assay described in this study was developed for determination of cocaine and its major metabolites in plasma obtained from pharmacokinetic studies in rodents and sheep [15,20]. Fig. 2 shows the plasma concentration *versus* time curve of cocaine, benzoylecgonine and ecgonine methyl ester in a pregnant sheep following a single intravenous 3.0 mg/kg dose of cocaine [15]. Concentrations were in the range chosen for reproducibility studies (400 and 600 ng/ml); however, the practical limit of sensitivity of this assay is 25 ng/ml. This compares favorably to other published HPLC assays [1–3] and it has proven adequate for pharmacokinetic studies in animals. Nevertheless, the present assay might not be suitable for human studies because the internal standard we used, the ethyl homologue of cocaine, may be formed in man when alcohol is coingested with cocaine.

Cocaine and benzoylecgonine possess UV-absorbing properties while the electrochemical activity of cocaine and ecgonine methyl ester has been described [21–24]. Therefore, we developed an HPLC method utilitizing combined electro-

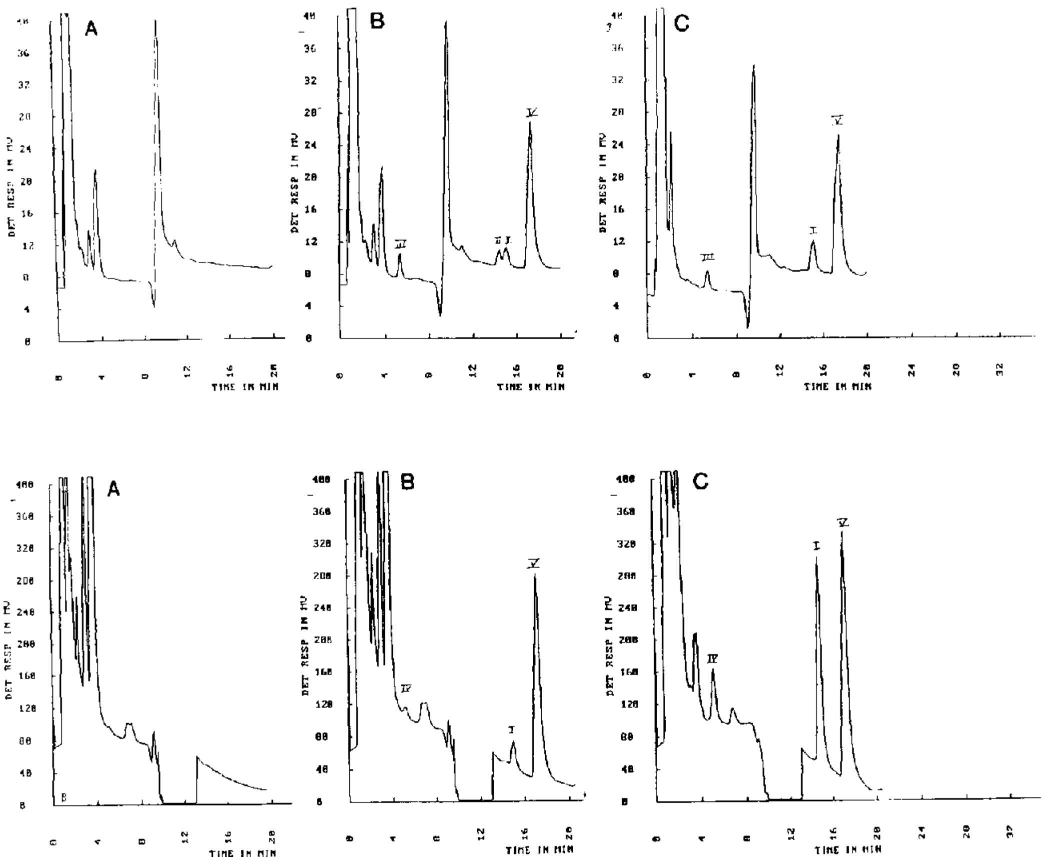

Fig. 1. Chromatograms of (A) extracted blank plasma, (B) a 100-ng plasma calibration standard and (C) extracted plasma from a sheep collected 1 min after a 2.0 mg/kg intravenous cocaine dose. Concentrations in C were 1448 ng/ml for cocaine (I), 44 ng/ml for benzoylecgonine (III) and 498 ng/ml for ecgonine methyl ester (IV). Norcocaine (II) does not appear as a cocaine metabolite in sheep. The internal standard, ethylecgonine (V), and cocaine (I) appear in both UV and electrochemically monitored chromatograms. Monitoring of UV absorbance is shown in the top of each chromatogram while electrochemical activity is shown on the bottom.

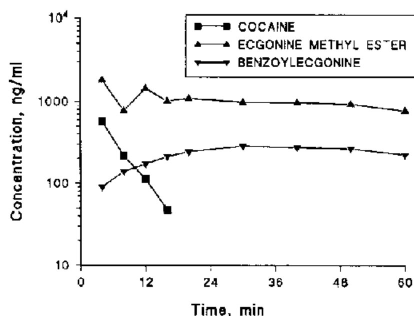

Fig. 2. Cocaine, benzoylecgonine and ecgonine methyl ester plasma concentrations *versus* time profiles in a sheep following a single 2 mg/kg intravenous dose (from ref. 15).

chemical and ultraviolet absorbance detection to quantitate cocaine, benzoylecgonine and ecgonine methyl ester in plasma following a single plasma extraction. The three compounds are completely separated from other cocaine metabolites: norcocaine, norbenzoylecgonine and ecgonine (Fig. 1).

The importance of ecgonine methyl ester in the disposition of cocaine may have been underestimated as methods for its measurement in plasma have not been widely available. In 1976, Misra *et al.* [6] reported only 0.1% of ecgonine methyl ester as [^{3}H]cocaine metabolite in dogs. In 1984, Matsubara *et al.* [14] found 6.6–27.1% ecgonine methyl ester as cocaine metabolite in dogs. Ambre *et al.* [11,12] identified and quantitated ecgonine methyl ester in human urine using gas chromatography–mass spectrometry.

The quantitation of benzoylecgonine has been hampered by the difficulty of extraction characterized by the need to use large volumes of chloroform [1]. The use of acetonitrile as an extracting solvent in the present method allows cocaine's major metabolites to be extracted and concentrated in a simple procedure.

Due to great differences in chromatographic characteristics of the metabolites compared with cocaine, solvent switching was used during chromatography. Gradient elution could likely be used as an alternative but this approach was not thoroughly investigated. Although use of LiChrosorb C_1 or CN as a stationary phase gave better peak symmetry, the pH necessary for electrochemical detection (pH $>$ 8.5) of ecgonine methyl ester resulted in rapid deterioration when columns were used with these packings. Using the poly(styrene–divinylbenzene) column, more than 500 injections could be obtained without serious column deterioration. Attempts were made to optimize the mobile phase. The pH, ionic concentration and percentage acetonitrile were adjusted to obtain a chromatogram free of interfering endogenous plasma peaks. Increasing the pH of the mobile phase increased the column retention of ecgonine methyl ester and cocaine but not that of benzoylecgonine. Ammonium phosphate enhanced while methanol inhibited electrochemical activity. Increasing the concentration of ammonium phosphate decreased the retention time of ecgonine methyl ester and cocaine but not benzoylecgonine.

In summary, the use of combined UV and electrochemical detection allow quantitation of cocaine, benzoylecgonine and ecgonine methyl ester in a single sample following a simple plasma extraction. Application to studies of cocaine pharmacokinetics in sheep has revealed measurable plasma concentrations of ecgonine methyl ester and benzoylecgonine persisting in plasma long after disappearance of cocaine. This method should be suitable to a wide range of studies of the disposition of cocaine and its major metabolites.

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