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MICROASSAY FOR THE SIMULTANEOUS DETERMINATION OF COCAINE, NORCOCAINE, BENZOYLECGONINE AND BENZOYLNORECGONINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An improved method for the simultaneous determination of cocaine, norcocaine, benzoylecgonine and benzoylnorecgonine using reversed-phase high-performance liquid chromatography with ultraviolet detection is described. Following solid-phase extraction, chromatography was performed using a column containing an octadecylsilica-coated packing, eluted with 6% acetonitrile in phosphate buffer, pH 2.1, and detected at 233 nm. Using 80-μl samples, the detection limit is 18 ng/ml for benzoylecgonine and benzoylenorecgonine and 35 ng/ml for cocaine and norcocaine. The coefficients of variation range from 3.5% (benzoylecgonine) to 7.0% (norcocaine). The procedure has been applied to samples of guinea pig plasma, urine and amniotic fluid and human urine.

INTRODUCTION

Cocaine (COC) use among women of childbearing age has been a significant problem is recent years and the number of clinical reports suggesting increased morbidity and mortality of the infants of these substance abusing mothers has increased [1,2]. Detrimental effects to the fetus could be caused by the actions of COC itself and/or metabolites such as norcocaine (NOR), benzoylnorecgonine (BN) and benzoylecgonine (BE) [3]. BE is a major urinary metabolite [4] often used in toxicology screens to confirm COC abuse [5]. Animal studies have demonstrated that COC is also demethylated to NOR [6], a metabolite with adrenergic [3] and local anesthetic properties similar to COC [7]. The

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extent to which these metabolites are formed in the fetus is not known. In this laboratory, the guinea pig is used as an animal model to determine the pharmacokinetics of COC and its metabolites in pregnancy. The guinea pig is an appropriate model since its placental structure and permeability characteristics are similar to those of the human placenta [8].

Reversed-phase high-performance liquid chromatography (HPLC) is commonly used for the determination of polar, non-volatile compounds such as BE and related substances. Various assays exist that can detect COC [9], COC, BE and NOR [10] and COC, BE, NOR and BN [11,12]. However, most methods either require sample sizes too large to be compatable with repeated sampling from a small animal [11] or do not demonstrate sensitivity low enough to fully delineate the elimination curve of COC and metabolites in the guinea pig [12]. In addition, these methods use a solvent extraction procedure which is less convenient than the solid-phase extraction procedure proposed in this paper. The solid-phase method is similar to that used in this laboratory for the determination of morphine and metabolites morphine-3- and -6-glucuronides [13].

The assay described in this study was developed for the simultaneous determination of COC, NOR, BE and BN following solid-phase extraction of microsamples obtained from maternal and fetal guinea pigs. The solid-phase procedure was compared to solvent extraction. Validation of the assay for COC and BE was done by gas chromatographic–mass spectrometric (GC–MS) analysis.

EXPERIMENTAL

Chemicals

COC·HCl, NOR, BE, BN·HCl, methamphetamine·HCl, amphetamine ·HCl, morphine, morphine-3-glucuronide, ecgonine methyl ester (EME) and ecgonine were obtained from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) through the National Institute of Drug Abuse (Bethesda, MD, U.S.A.). The internal standard lidocaine (LID) was purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions (50 ng/μl) were made by dissolving COC, BE, BN and LID in water and NOR in ethanol. Aliquots of 1 ml were stored at $-15°C$ until used. Methanol, water and acetonitrile (Mallinckrodt, Paris, KY, U.S.A.) were HPLC grade. Tetrabutylammonium hydroxide (TBA-OH, Aldrich, Milwaukee, WI, U.S.A.) and all other chemicals were reagent grade.

Liquid chromatography

Mobile phase (100 μl) containing drugs was injected into a Model 334 liquid chromatographic system (Beckman Instruments, Berkeley, CA, U.S.A.) in line with a 100 mm$\times$3.2 mm I.D., 3 μm particle size, reversed-phase cartridge col-

umn (Velosep ODS, Applied Biosystems, Santa Clara, CA, U.S.A.) and a Model 783A programmable absorbance detector (Applied Biosystems, Ramsey, NJ, U.S.A.) set at 233 nm and 0.002 absorbance units full scale (a.u.f.s.). The detector response was integrated with a Model 5640A integrator (Hewlett-Packard, Los Angeles, CA, U.S.A.). The mobile phase consisted of a 0.01 *M* phosphate buffer, pH 2.1, and 0.0002 *M* TBA-OH with 6% (v/v) acetonitrile. The mobile phase was filtered through a 0.22-μm membrane filter (Rainin, Woburn, MA, U.S.A.) and run at a flow-rate of 1.5 ml/min.

Sample extraction

Solvent extraction. A 80-μl sample (plasma, urine or amniotic fluid) was added to 720 μl of 0.01 *M* phosphate buffer, pH 8.5, containing 450 ng of LID as the internal standard. A 5-ml mixture of chloroform–isopropanol (95:5, v/v) was added, vortex-mixed for 30 s and then centrifuged at 1700 *g* for 20 min. The aqueous phase was discarded and 4 ml of the organic phase were dried at 45 °C under nitrogen. The sample was reconstituted in 300 μl of mobile phase for injection into the HPLC system.

Solid-phase extraction. A 80-μl sample was mixed with 720 μl of 0.5 *M* ammonium sulfate, pH 8.7, containing 450 ng LID as the internal standard, and injected through a Chromprep PRP-1 80-μl cartridge (Hamilton, Reno, NV, U.S.A.) at an average rate of 1.5 ml/min. The cartridge was then washed with 3 ml of 0.01 *M* ammonium sulfate, pH 8.7, and 1 ml of 0.01 *M* phosphate, pH 2.0, followed by 80 μl of water and 250 μl of diethyl ether. The cartridge dead-space was cleared of diethyl ether by injecting air. The drugs were eluted with 300 μl of methanol and the eluate was dried at 45 °C under nitrogen. Samples were reconstituted in 300 μl of mobile phase. Siliconized glassware was used throughout the extraction and reconstitution procedure.

Gas chromatography–mass spectrometry

Samples were analyzed by the Toxicology Department of The Oregon Health Sciences University with a method based on the work of Griesemer et al. [14] and Clark and Hajar [15]. The method has been modified to use the deuterated compounds [$^2H_3$]COC and [$^2H_3$]BE as internal standards instead of SKF 525-A. BE was derivatized to ethylbenzoylecgonine. EME can also be determined by this method. Sample preparation and GC–MS analysis was performed as described previously [15]. Samples were triply extracted progressing from basic to acidic to basic pH in a solvent extraction method. The following ions (*m/z*) were monitored in the GC–MS selected-ion monitoring mode: *m/z* 303 for COC; *m/z* 306 for [$^2H_3$]COC; *m/z* 361 for BE; *m/z* 364 for [$^2H_3$]BE; *m/z* 96 for EME. Quantitation was based on ion peak-area ratios for the undeuterated and deuterated fragments.

Animals

Experiments were carried out using pregnant Dunkin-Hartley guinea pigs (Charles River, Wilmington, MA, U.S.A.; Simonsen Labs., Gilroy, CA, U.S.A.) in the last half of gestation (term is 65 days). Animals were housed indoors with controlled light cycles, continuous ad libitum food and water and daily health checks. The Animal Care Department is fully accredited by the American Association for Accreditation of Laboratory Animal Care. The Department operates in compliance with the Animal Care Act and has an assurance letter on file at the National Institutes of Health. Maternal samples were obtained by the method of Olsen et al. [16] with the following changes: isoflurane replaced halothane as the anesthetic and an external jugular vein catheter was introduced as well as a carotid artery catheter. Dams were allowed to recover from surgery at least 24 h before experiments were performed. Samples of 1 ml were drawn into heparinized disposable syringes and placed in polypropylene microfuge tubes containing 2.5 mg/ml sodium fluoride. After centrifugation at 5°C, the plasma was removed and stored at −15°C until assayed.

RESULTS AND DISCUSSION

Several published methods have been adapted to develop a microassay for the simultaneous determination of COC and metabolites. The mobile phase was a modification of that of Svensson [17]. Several mobile phase modifiers have been tested and the one used by Garret and Seyda [18], TBA-OH, was selected for use because it resulted in the best separation of drug peaks. Three compounds, LID, *m*-toluic acid, and the ethyl ester of benzoylecgonine (ETBE) were evaluated as internal standards. ETBE was synthesized as described by Jatlow et al. [11] and purity was verified by GC–MS. Of the three, LID was chosen as the internal standard because of its short retention time.

Fig. 1 shows the separation of COC, BE, BN, NOR and LID under the chromatographic conditions described. Extractions were originally performed by the solvent method but this proved unsatisfactory for the small sample size due to relatively poor recoveries (average 50%), high coefficients of variation (average 15%) and decreased sensitivities (varying from 35 ng for BE to 300 ng for NOR). A solid-phase extraction method similar to one used in this laboratory for morphine and its glucuronides [13] was modified to purify COC and the three metabolites from biological fluids. Because the solid-phase method gave much higher recoveries and increased sensitivity compared to the solvent method (Table I), all subsequent extractions were done using the solid-phase method. Considering the 80 μl sample size and dilutions inherent within the assay, the sensitivity of the assay was 18 ng for BE and BN and 35 ng for COC and NOR, while the lowest quantifiable levels for BE and BN were 35 ng/ml and 75 ng/ml for COC and NOR. The coefficient of variation within an assay was less than 7% for COC and the metabolites tested (Table I). The assay is

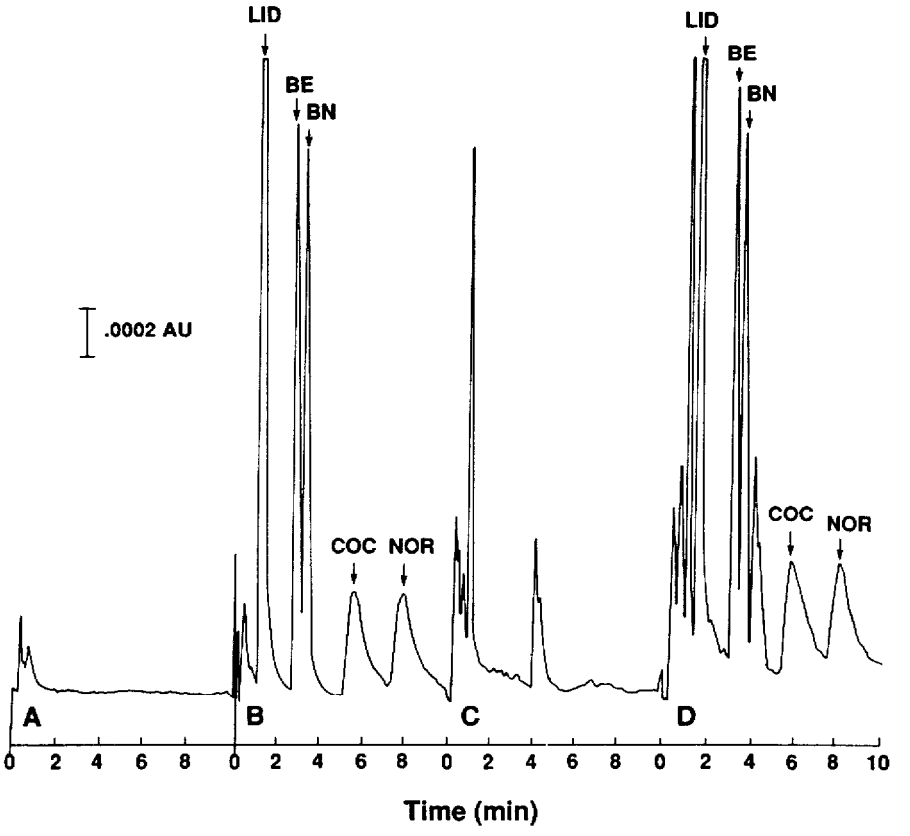

Fig. 1. Chromatograms of (A) mobile phase, (B) mobile phase with 15 ng each of BE, BN, COC and NOR and 150 ng of LID, (C) extracted undiluted guinea pig plasma and (D) guinea pig plasma with 15 ng each of BE, BN, COC and NOR and 150 ng of LID. All extractions were done by the solid-phase method. Extraction volume was 80 μl and injection volume was 100 μl.

also specific for COC, NOR, BN and BE. Relative retention times for other drugs compared to COC are listed in Table II.

Fig. 1 shows chromatograms of extracted guinea pig control plasma and plasma with 15 ng of COC, BE, BN and NOR and 150 ng of LID. There are no interfering peaks eluting with COC, BN, NOR or LID. Occasionally, a small plasma peak coelutes with BE but because it is relatively small, its area can be subtracted from that of the samples. Various solvents were tested for their ability to remove interfering peaks from the solid-phase extraction. These included hexane, heptane, chloroform–isopropanol (95:5), chloroform–ethanol (80:20) and diethyl ether. The method presented proved to be the most effective at cleaning the samples. The peak eluting at 4.5 min is a constituent from the Chromprep cartridge which elutes with the sample. However, it does not interfere with the quantitation of BN or BE.

TABLE I

COMPARISON OF LIDOCAINE, COCAINE, BENZOYLECGONINE, BENZOYLNORECGONINE AND NORCOCAINE RECOVERY, ACCURACY AND COEFFICIENT OF VARIATION FOR SOLVENT AND SOLID-PHASE EXTRACTION METHODS

For the solvent extraction method, 80-μl samples in a basic phosphate buffer were extracted with 95:5 chloroform–isopropanol. Benzoylnorecgonine was not studied with this method. In the solid-phase method, 80-μl samples in a basic ammonium sulfate buffer were injected onto a Chromprep cartridge, washed with acidic phosphate buffer, water and diethyl ether and eluted with methanol. For all solvent extraction values $n=6$ and for solid-phase values $n=15$, except for cocaine $n=12$. Accuracy is expressed as the percentage error of the difference between expected and observed values.

Drug	Recovery (%)		Accuracy (%)		Coefficient of variation (%)	
	Solvent	Solid	Solvent	Solid	Solvent	Solid
Lidocaine	59	92	–	–	9.7	4.4
Cocaine	57	86	33	3.8	16	6.0
Benzoylecgonine	66	91	46	2.1	14	3.5
Benzoylnorecgonine	–	92	–	0.6	–	5.6
Norcocaine	21	85	14	4.0	15	7.0

TABLE II

RELATIVE RETENTION TIMES OF VARIOUS SUBSTANCES COMPARED TO COCAINE

Non-extracted samples in mobile phase were injected and the chromatogram recorded for 30 min. A 30-ng amount each of NOR, COC, BN and BE was injected. Because of decreased sensitivity, 150 ng of LID and 250 ng of the remaining drugs were injected. No peaks were seen during the 30 min for morphine, morphine-3-glucuronide, ecgonine methyl ester and ecgonine. Retention time relative to cocaine is listed.

Substance	Relative retention time
Norcocaine	1.41
Cocaine	1.00
Benzoylnorecgonine	0.65
Benzoylecgonine	0.62
Lidocaine	0.29
Methamphetamine	0.21
Amphetamine	0.17
Morphine	–
Morphine-3-glucuronide	–
Ecgonine methyl ester	–
Ecgonine	–

Standard curves were obtained by analysis of plasma combined with a range of drug concentrations (35–2260 ng/ml) (Fig. 2). The ratios of the area under the curve (AUC) of COC, BE, BN and NOR to LID, multiplied by 100, versus

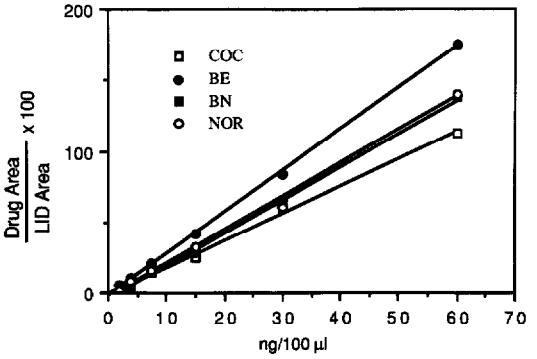

Fig. 2. Standard curves for COC, BE, BN and NOR. Guinea pig plasma (80 µl) was extracted by the solid-phase method. The extract was dried under nitrogen and reconstituted in 300 µl of mobile phase. The ratios of the AUC for each drug (COC, BE, BN and NOR) to the AUC of the internal standard (LID) multiplied by 100 are plotted as functions of the amount of COC, BE, BN or NOR in the injection volume of 100 µl.

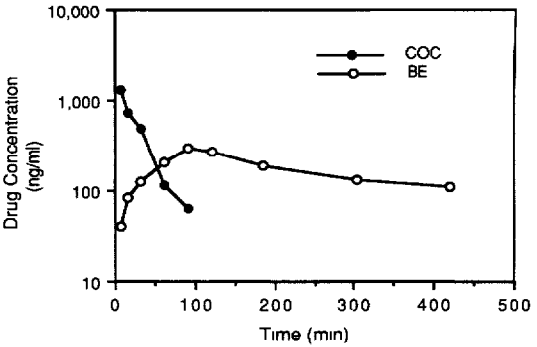

Fig. 3. Plasma concentrations of COC and BE in a material guinea pig after a 2 mg/kg intravenous dose of COC. Arterial samples were taken at 7, 16, 31, 61, 92, 121, 184, 304 and 419 min after the COC injection. COC was no longer detectable after 92 min while BE was still quantifiable at 419 min when sampling ended. The elimination half-life of COC and BE were 20 and 239 min, respectively.

the drug concentrations gave standard curves, determined by linear regression analysis, with an average r value of 0.998. Samples too concentrated to fit on the standard curve were diluted before extraction.

The HPLC method was validated for COC and BE by GC–MS. Aliquots of guinea pig plasma with 280–1130 ng/ml COC and BE were analyzed by GC–MS. By this method, the average error from the known concentrations was 13 and 8% for COC and BE, respectively. By HPLC analysis, the error for plasma samples with 560 ng/ml COC, NOR, BN and BE ranged from 1 to 4% (Table I). The larger error with GC–MS analysis found for COC and BE is probably due to the fact that the analysis was done on 0.5-ml samples in an assay that was designed to use 5 ml. The two methods have been compared using un-

known guinea pig samples and the agreement is good with an average difference of less than 10%. It should be noted that no EME was detected in guinea pig plasma by GC–MS. From Table I, Figs. 1 and 2 and the GC–MS work it is evident that the solid-phase extraction method followed by HPLC analysis can be used to accurately quantitate COC and metabolite concentrations in very small (80 μl) samples.

The solid -phase extraction and HPLC methods described here have been successfully used to measure COC and metabolite concentrations in maternal and fetal guinea pig plasma, urine and amniotic fluid samples as well as in human urine samples. Fig. 3 shows plasma concentrations of COC and BE in a maternal guinea pig after a 2 mg/kg intravenous injection. COC is no longer detectable in plasma approximately 1.5 h after COC administration and BE is still quantifiable at 7 h after injection when sampling ended. Because the elimination half-life of BE is twelve times longer than COC, BE would accumulate in plasma with repeated COC injections.

In conclusion, the HPLC microassay described here provides an accurate, sensitive way to simultaneously measure COC and metabolites BE, BN and NOR from plasma, urine and amniotic fluid. The assay is an improvement over standard solvent extractions and has been validated by GC–MS for COC and BE.

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