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MEASUREMENT OF BENZOYLECGONINE AND COCAINE IN URINE, SEPARATION OF VARIOUS COCAINE METABOLITES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The application of reversed-phase high-performance liquid chromatography to the measurement of benzoylecgonine and cocaine in urine is described. Following a simple extraction and clean-up procedure, chromatography is performed using a column containing an octadecylsilica coated packing, elution with 17% acetonitrile in pH 2.7 phosphate buffer and ultraviolet detection at 200 or 235 nm. The detection limit is *ca.* 0.1 μg of drug per ml urine, and using the ethyl ester of benzoylecgonine as an internal standard, benzoylecgonine and cocaine are quantified with coefficients of variation of 7.0 and 2.8%, respectively. The procedure has been applied to urines from subjects receiving intranasal cocaine, and compared to the enzyme multiplied immunoassay technique. The chromatography procedure also permits the separation of norcocaine and benzoylnorecgonine.

INTRODUCTION

Benzoylecgonine (BE) has been identified as the major urinary metabolite of cocaine in man (Fish and Wilson)[1]. Peak concentrations in urine after intranasal application of 15–150 mg of cocaine vary from *ca.* 3 to 25 μg/ml (Van Dyke *et al.*)[2]. Generally no BE is detected 24–36 h after cocaine use.

Sensitive measurement of BE in urine is of interest to drug abuse screening

programs as well as to pharmacologists. The most widely used procedure for detection of cocaine use in drug abuse screening is the semiquantitative enzyme multiplied immunoassay technique (EMIT, Syva Corp., Palo Alto, Calif., U.S.A.) for BE. More recently a radioimmunoassay procedure has become available[3]. Thin-layer[4–7] and gas–liquid[8–11] chromatographic procedures have also been reported. The latter require derivatization. Fish and Wilson[1], and Wallace[9], converted BE into cocaine prior to gas chromatography. Others have prepared derivatives suitable for electron capture detection[10,11]. Misra *et al.*[12] have used radioactive tracer techniques in combination with liquid chromatography for the sensitive measurement of benzoylecgonine in biological fluids in animal studies.

High-performance liquid chromatography (HPLC) is especially attractive for the separation of polar, non-volatile compounds such as benzoylecgonine and related compounds. Using reversed-phase HPLC, we are able to measure BE and cocaine in urine. These compounds as well as their N-demethylated derivatives can all be separated by HPLC without derivatization.

MATERIALS AND METHODS

Apparatus

We used a Model 601 Liquid Chromatograph equipped with Model LC-55 variable wavelength ultraviolet detector (both Perkin-Elmer, Norwalk, Conn., U.S.A.), The chromatograph was equipped with a 25 cm × 4.6 mm reversed-phase (octadecyl-silica coated) column (Partisil-10 ODS; Reeve-Angel, Whatman, Clifton, N.J., U.S.A.).

Chromatography

Elution was performed with 0.25 *M* phosphate buffer, pH 2.7, containing 17% (v/v) acetonitrile at a flow-rate of 2 ml/min. The column (oven) was maintained at 40°. Studies were performed with UV detector settings of 200 and 235 nm.

Chemicals and reagents

Internal standard. Benzoylecgonine, *n*-ethyl ester (ETBE). This cocaine homolog was synthesized from BE. 50 mg of BE was added to 100 ml of ethanolic hydrochloric acid which had been prepared fresh by the careful addition of 5 ml of acetyl chloride to 100 ml of 100% ethanol. This mixture was refluxed overnight in a round-bottomed flask, and then evaporated to dryness. The residues was dissolved in 0.5 ml ethanol and submitted to preparative thin-layer chromatography on 20 cm × 20 cm × 0.5 mm Anasil H (silica gel) thin-layer plates (Analabs, North Haven, Conn. 06473, U.S.A.), developed with ethyl acetate–methanol–concentrated ammonium hydroxide (85–10–5). The compounds were located by spraying the plate margin with Dragendorff's reagent followed by 5% sodium nitrite. Under these conditions the ETBE has an R_R of *ca.* 0.7, and unreacted BE, a R_R of less than 0.1. The ETBE bands were scraped from the plate, eluted into diethyl ether which was dried with a sodium sulfate buffer and evaporated to dryness at room temperature under nitrogen. The isolated compound was dissolved in ethanol and submitted to gas chromatography using a 6 ft. long glass column packed with 3% OV-17, isothermally at 255°. Under these conditions the ETBE showed a single peak with a retention time of 4.0 min (1.1 relative to

cocaine). Its structure was further confirmed by mass spectrometry. The internal standard was diluted to *ca.* 1 μg/ml with ethanol and stored at *ca.* −15°.

Carbonate buffer. This was a mixture of 17.5 parts of Na_2CO_3 and 20 parts of $NaHCO_3$, 100 mg added to 5 ml urine yielded a pH of 9.4–9.6.

Sulphate buffer. A solution of 0.5 *M* H_2SO_4 was used.

Reference standards. Stock standards of cocaine, benzoylecgonine, benzoylnorecgonine, and norcocaine were prepared and stored in methanol at concentrations of 1 mg/ml.

Acetonitrile. UV grade was used.

Phosphate buffer (0.1 M, pH 2.7). 0.1 *M* KH_2PO_4 was adjusted to pH 2.7 with 0.1 *M* H_3PO_4.

Solvent (elution). Phosphate buffer–acetonitrile (83–17).

PROCEDURE

5.0 ml urine was placed in a 50 ml round-bottomed centrifuge tube, and 10 μg of internal standard and 0.5 ml of 0.5 *M* H_2SO_4 were added. The acidified urine was extracted for 3–5 min with 10 ml diethyl ether. After centrifugation the organic solvent was removed and discarded, and the aqueous phase alkalinized by addition of about 400 mg (*ca.* 1/8 teaspoon) of the dry carbonate buffer mixture. The aqueous phase was then extracted for 5 min with 10 ml of chloroform–ethanol (80:20 v/v). After centrifugation the organic (lower) phase was removed, and evaporated to dryness under a stream of air. The residue was dissolved in 50 μl of methanol, and 10 μl chromatographed. Drug-free urines supplemented with exact quantities of BE and cocaine, and carried through the entire extraction procedure served as standards.

Quantitation

Identification was based upon retention times relative to that of the internal standard. Relative peak height ratios (BE or cocaine/internal standard) of unknowns were compared with those of the extracted urine standards for quantitation.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of cocaine and its various metabolites under the chromatographic conditions described. With a new (unused) column it was necessary to raise the temperature to 50° and increase the acetonitrile concentration slightly to achieve the same separation.

Peaks height ratios of BE and cocaine showed a linear relationship to concentration over a range of 0.5–10 μg/ml of urine. Although satisfactory results were obtained at both 200 and 235 nm, sensitivity was *ca.* 2.5 times greater at 200 nm. Comparison of the peak height absorbance at the two wavelengths can be useful for verification. Figs. 2 and 3 show chromatograms obtained from a negative urine, of a volunteer who received intranasal cocaine, and from a street abuser. The latter (Fig. 3) showed a relatively large quantity of BE, probably reflecting repeated use over a short period. In subjects who were followed over a period of time following a single application of cocaine, BE persisted long after cocaine was no longer detectable. Using gas chromatography with a nitrogen detector (detection limit 5 ng/ml)[13], we

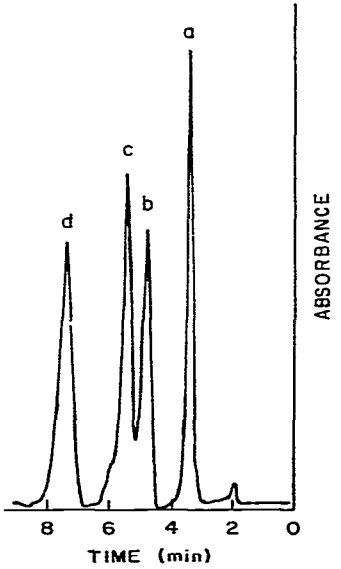

Fig. 1. Chromatogram of a mixture of norbenzoylecgonine (a), benzoylecgonine (b), norcocaine (c), and cocaine (d).

have found that cocaine can be detected in plasma for *ca.* 6 h after a single application[14]; BE can be detected in urine at concentrations greater than 0.5 μg/ml for *ca.* 24 h.

The detection limit of the procedure was *ca.* 0.1 μg/ml of urine. This limit was dependent on biologic background rather than detector sensitivity. Possibly by decreasing the acetonitrile concentration, better separation from early eluting contaminants could be achieved at the cost of broader peaks and later retention times.

The coefficients of variation at 2.0 μg/ml urine were 7 and 2.8% for BE and cocaine, respectively.

Drug-free urines showed no peaks with the same retention time as cocaine, BE or the internal standard. The initial clean-up extraction of acidified urine reduces endogenous chromogens without significantly affecting the extraction efficiency (non-corrected recovery) of BE or cocaine which were 80 and 84%, respectively. Wallace *et al.*[7,9] are to be credited with showing that BE can be efficiently extracted with chloroform containing 20% ethanol (v/v).

A large number of basic drugs were evaluated and did not interfere. These included tricyclic antidepressants, morphine and related alkaloids, and benzodiazepine drugs.

To further verify the procedure the peak corresponding to BE obtained from a patient who had used cocaine was scanned under stop-flow conditions, and its UV spectrum compared with that of authentic BE. The two spectra were identical (Fig. 4).

Urine from human subjects receiving topical cocaine as part of a study of the acute effects of cocaine in man were analyzed for BE by the HPLC procedure described here and by the EMIT technique. The latter is generally considered a highly specific but semiquantitative procedure for the detection of BE in urine. Its lower limit of detection is *ca.* 0.5 μg/BE/ml of urine. The results of this comparison are shown in

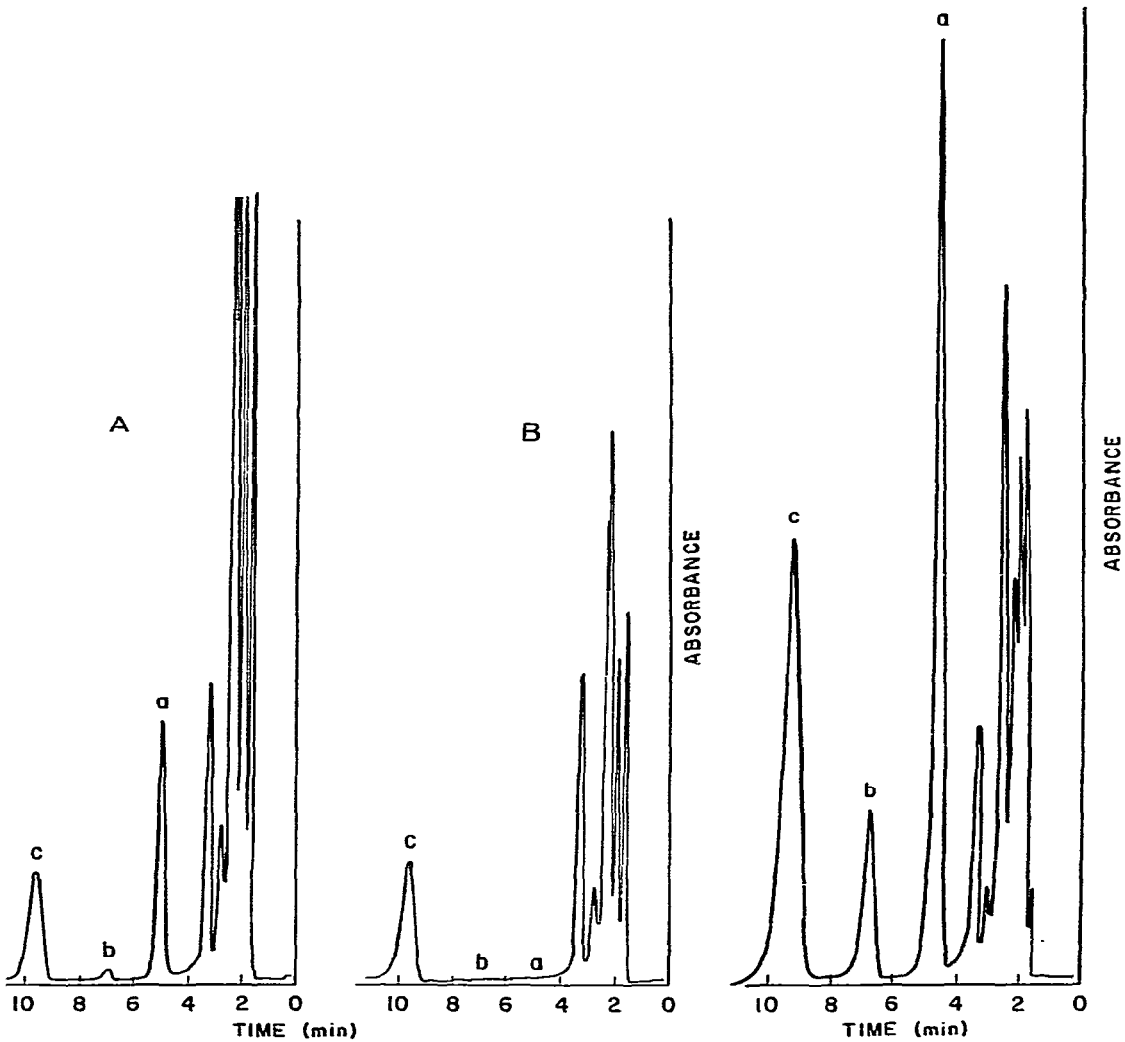

Fig. 2. (A) Chromatogram of urine extract from subject after administration of cocaine showing benzoylecgonine (a), cocaine (b), and internal standard (c). (B) Chromatogram of urine extract from subject before receiving cocaine. a and b are where benzoylecgonine and cocaine peaks would be, if present; internal standard (c).

Fig. 3. Chromatogram of urine extract from street user of cocaine showing benzoylecgonine (a), cocaine (b), internal standard (c). Urine was diluted 1:50 prior to extraction because of very high concentrations.

Table I. Note that concentrations below 0.5 μg/ml by HPLC were negative by EMIT.

Although the described procedure permits the simultaneous measurement of BE and cocaine in urine, it was designed primarily for BE which has been difficult to analyze. It is probable that cocaine alone can be more sensitively measured in urine by gas chromatography. Certainly it can in plasma using gas chromatography with nitrogen sensitive[13], and electron capture detectors[10,11]. On the other hand no

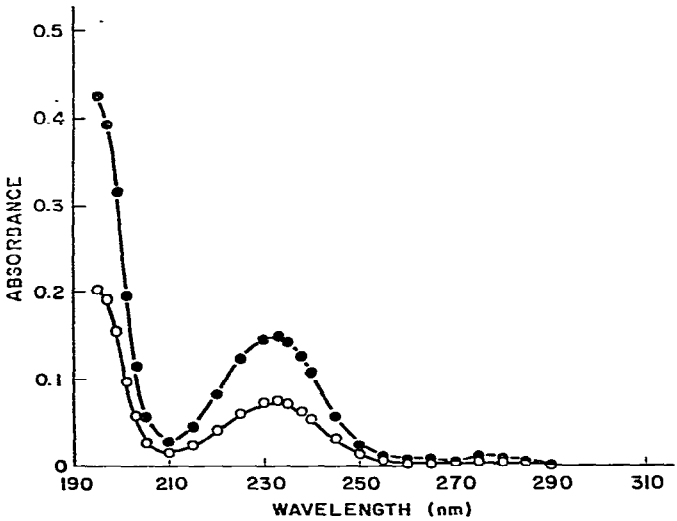

Fig. 4. Stopped-flow ultraviolet absorption spectrum (scan) of benzoylecgonine peak obtained from subject's urine (●) compared with that of authentic benzoylecgonine (○).

other procedure permits the easy and sensitive quantitation of underivatized BE in urine. This procedure should be useful for pharmacokinetic studies as well as for confirmation in drug abuse screening programs. Graffeo *et al.*[15] used HPLC as a preliminary step for purification of BE prior to gas chromatography–mass spectrometry of the trimethylsilyl derivative.

Cocaine, BE, norbenzoylecgonine, and norcocaine are all easily separated by

TABLE I

COMPARISON OF HPLC WITH EMIT OF BENZOYLECGONINE IN URINE

Less than 0.5 μg/ml is considered negative with the EMIT procedure. EMIT values are rounded off to two significant figures.

Benzoylecgonine (μg/ml)			
HPLC	*EMIT*	*HPLC*	*EMIT*
34.4	37	1.2	1.5
0.6	1.0	2.8	3.0
0.1	negative	1.9	1.9
0.16	negative	0.5	0.5
34.8	29	21.8	8.8
2.0	1.6	16.5	10
25.8	20	22.4	15
20.0	17	26.5	20
0.1	0.6	16.1	5.5
1.3	1.2	11.0	6.2
8.2	5.8	0.8	negative
2.2	1.8	6.3	5.0
0.3	negative	2.7	2.7
1.7	3.3	1.3	0.5
0.5	0.6	13.0	5.8

reversed-phase chromatography, but only the first two have been identified in humans. The others have been found in animals given cocaine[16–18]. The procedure as described here for the extraction and measurement of cocaine and BE is not necessarily optimized for the analysis of norcocaine and norbenzoylecgonine in biological materials.

Notably absent from this discussion are ecgonine and ecgonine methyl ester. These debenzoylated compounds did not show sufficient absorption in the ultraviolet to permit their sensitive detection, even at 200 nm. A number of hydroxylated metabolites have been postulated, and/or identified in animals[17]. These polar compounds should also be very suitable for analysis by HPLC.

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