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DETECTION OF BENZOYL ECGONINE (COCAINE METABOLITE) IN URINE BY GAS CHROMATOGRAPHY*

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SUMMARY

A method is described for the detection of 1 μg/ml levels of benzoyl ecgonine, the principal metabolite of cocaine, in urine by gas chromatography. Of the several methods described, the method of choice is as follows:

(1) The benzoyl ecgonine is salted out of the urine into 95% ethanol.

(2) The ethanol extract is evaporated to dryness and purified by thin-layer chromatography.

(3) Benzoyl ecgonine is then removed from the thin-layer plate, methylated and detected by gas–liquid chromatography.

Results are given for pure benzoyl ecgonine spiked into drug-free urine and for urine samples of cocaine users.

INTRODUCTION

Although cocaine is a widely used drug in the United States, very few drug screening programs report significant numbers of cocaine-positive urines. The principal reason for this lack of detected positives is that little or no cocaine appears in the urine of cocaine users—rather, two metabolites of cocaine, benzoyl ecgonine and ecgonine, appear in the urine[1]. Since both of these compounds are amino acids, they are extracted from aqueous solution, *e.g.*, urine, by organic solvents poorly or not at all. Although benzoyl ecgonine can be extracted from a low-ionic-strength aqueous solution by XAD-2 resin, it cannot be extracted directly from urine by this resin. Since organic solvent extraction and resin extraction of the XAD-2 type are the principal methods of extraction used in drug screening, these metabolites are not extracted from urine in the usual drug screening methods. Further, the thin-layer chromatographic (TLC) method cited by Clarke[2] requires that a large amount of benzoyl ecgonine (approximately 20 μg) be spotted on the TLC plate to be visible, and gas–liquid chromatographic (GLC) methods using the usual OV-17, OV-1 or SE-30 columns require derivatization of the benzoyl ecgonine and ecgonine before they will pass through the column. Therefore, the methods currently used in routine drug screening do not effectively detect the cocaine metabolites. The purpose of the study described in this paper was to develop a sensitive and reliable method for the detection of the principal metabolite of cocaine, benzoyl ecgonine.

* Contribution No. 49.

MATERIALS AND METHODS

Gas–liquid chromatography

All samples were run on a Hewlett-Packard Model 402 gas chromatograph equipped with a flame ionization detector. A 2.5% SE-30 on 100–120 mesh Gas-Chrom Q in a 6 ft. × 1/4 in. U tube column was operated at 200° with the injection port at 250° and the detector at 230°. The gas flow-rates were: 25 ml/min at 40 p.s.i. for the nitrogen carrier gas, 30 ml/min at 10 p.s.i. for the hydrogen and 250 ml/min at 20 p.s.i. for the compressed air.

Extraction and purification procedure

The method given here is a modification of the salting out method of Bastos, *et al.*[3]. The procedure is as follows:

Wash a 10-ml sample of urine twice with 10 ml of chloroform. Each time centrifuge the two phases at 3000 rpm for 5 min and discard the chloroform and any denatured proteins appearing at the liquid–liquid interface. Transfer the washed sample to a 17 × 150 mm glass test tube and add 10 ml of 95% ethanol, 5 g of K_2HPO_4 and 5 g of KH_2PO_4. Shake vigorously for 30 sec and allow phases to separate. Transfer the upper ethanol phase to a clean 15 × 150 mm glass test tube. Repeat the previous extraction with an additional 5 ml of ethanol (without adding more phosphate salt). Combine the second 5-ml ethanol fraction with the first 10-ml ethanol fraction and centrifuge at 3000 rpm for 5 min. Remove sediment with a disposable capillary pipette and transfer the clear ethanol solution to a 15-ml conical test tube. Dry under a stream of nitrogen as completely as possible.

Alternately, the following primary extraction method may be used:

Place 10 ml of urine in a 30-ml serum bottle. Freeze in a dry-ice–acetone mixture, and then lyophilize. Reconstitute the lyophilized urine sample with 10 ml of methanol, shake well, then centrifuge. Decant the methanol solution into a 15-ml conical-bottom test tube and dry it down to a syrupy residue in a water-bath at 80° using a nitrogen stream.

Redissolve the viscous material from either of the above procedures by adding a minimal amount (200–400 μl) of methanol. Draw the solution up into a 500-μl pipetting syringe with repeating dispenser. Leave any solid material behind.

Preparative TLC is now performed on a 20 × 20 cm glass TLC plate precoated with 0.25 mm of SIL G-25 silica gel without gypsum. Draw a light pencil line about 3 cm from the bottom of the plate. This line provides a guide for streaking the extract. On the center of the guide line spot 5 μl of a 10 μg/μl benzoyl ecgonine in methanol solution. This will provide a "tracking spot" which will locate the band on the plate*. Now streak the methanol solution in the syringe evenly over the guide line using an entire 20 × 20 cm plate for each sample. The streak must be kept as narrow as possible without overloading the plate. Develop the dried plate in methanol–ammonia hydroxide (100:1.5). Dry the plate with a hot-air stream. Mask a 1.5-cm vertical section which contains the tracking spot and spray with acidified iodoplatinate solution. The benzoyl ecgonine will have an R_F value of about 0.4 and will be bluish-purple in color. Scrape a 3-cm band corresponding to the tracking spot from the unsprayed portion

* Tracking spot must be applied prior to urine streak for meaningful determination of the benzoyl ecgonine band.

of the plate. To the silica gel in a clean test tube add 4 ml of distilled water and mix vigorously. Place the test tube in a water-bath at 80° for 5 min, then centrifuge and decant the solution. Repeat this wash once more. Combine the washings in a conical test tube and evaporate to dryness. Add 30 μl of pure DMF dimethyl acetal (commercially available from Pierce Chem Co., Rockford, Ill., U.S.A.), cover with parafilm and place in a water-bath at 70–80° for 1 h. Evaporate off the methylating agent using a nitrogen stream, reconstitute the residue with 25 μl of chloroform and inject 2 μl into the gas chromatograph.

An ion-exchange approach to the extraction and purification procedure was also investigated. The weakly basic anion-exchange (Bio-Rad AG 3-X4, 100–200 mesh) resin was used. First the resin form was changed from chloride to bicarbonate with a carbonate solution. Then, the resin was washed with a pH 7.0 bicarbonate solution until there was no chloride ion detectable by $AgNO_3$–HNO_3 reaction.

A 20-ml sample of urine was made alkaline with NaOH to pH 9. Then 20 ml of chloroform was added, shaken and the chloroform phase was discarded. The pH of the aqueous phase was either changed to 6.5 at this point or further washing was performed. The extra washing involves adjusting the pH to 2.0 with HCl and treating with chloroform. Then, the aqueous phase is brought back to pH 6.5 with NaOH.

5 g of resin were packed in a column and washed twice with a pH 7.0 bicarbonate solution. The aqueous phase was passed through the column, eluted with 20 ml of methanol three times. The first wash and the aqueous phase were discarded. The second wash and the third wash are dried, methylated and GLC analyzed.

Spin immunoassay

Recently, a spin immunoassay for detection of opiates in urine has been developed[4,5]. Yield values for extraction steps and verification of benzoyl ecgonine positive samples were determined by a similar spin immunoassay (Syva, Co., Palo Alto, Calif. 94304, U.S.A.).

RESULTS AND DISCUSSION

The yields of the various extraction procedures were determined prior to methylation and GC detection by spin immunoassay. It was found that the yields of benzoyl ecgonine in the methanolic extraction of lyophilized urine and that of salting out into ethanol are both 90–100%. The yield of the preparative TLC procedure is 30% to 40%, while that for the second wash of the ion-exchange column is 30% to 50%.

Using the ethanolic extraction followed by preparative TLC, methylation and GLC detection, the following samples were assayed: (1) A negative urine; (2) the same urine spiked at levels of 1 μg/ml, 5 μg/ml, and 10 μg/ml with benzoyl ecgonine; (3) urines from known addicts positive on spin immunoassay at levels of 5.0, 2.8, 1.7, and 1.3 μg/ml benzoyl ecgonine.

All positive samples were easily detected. Gas chromatographs for the 1 μg/ml and 5 μg/ml levels of benzoyl ecgonine in urine are given in Fig. 1. Using lyophilization followed by preparative TLC, methylation and GLC detection, a negative urine, a 3-μg/ml, a 5-μg/ml and a 40-μg/ml spike were run. In addition, benzoyl ecgonine determinations were performed on two urines from known cocaine users and urine control containing 5 μg/ml of the following drugs: benzoyl ecgonine, morphine,

methadone, methadone metabolite (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), amphetamine, methamphetamine, secobarbital, phenobarbital, amobarbital, pentobarbital, butabarbital and ecgonine.

The gas chromatogram for the 5 μg/ml benzoyl ecgonine in urine control and the two urines from known cocaine users are given in Fig. 2. Both methods appear to provide reliable detection of levels as low as 1 μg/ml of benzoyl ecgonine in urine.

With the ion-exchange method, in one set of experiments, three spikes were run to determine the detection level. The levels 10, 5 and 3 μg/ml of benzoyl ecgonine in urine were assayed along with positive urine samples obtained from addicts known to have taken cocaine and determined positive by the spin immunoassay technique. The results show detection of the spikes and all of the positive samples. However, quantitation by this method was not adequate. Since other extraction methods were working well, this particular approach was not pursued further. However, it seems very likely that extraction by ion-exchange resin could be developed into a reliable method with some further work.

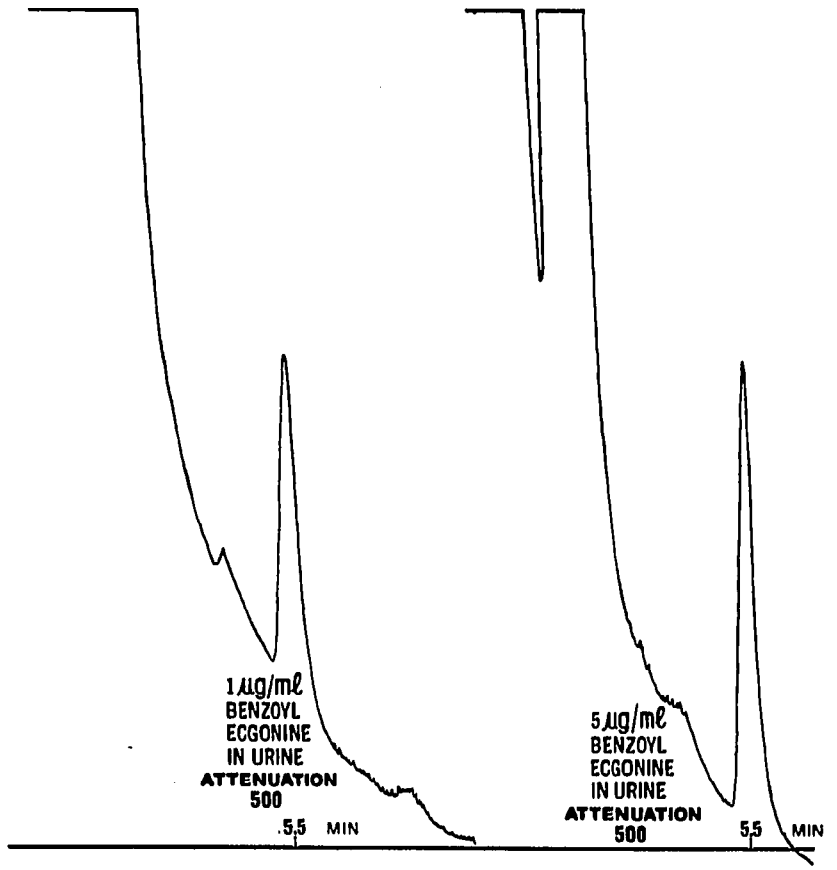

Fig. 1. Gas chromatograms of benzoyl ecgonine extracted from urine by salting out into ethanol. GC conditions are given in the Materials and Methods section of the text. Peaks shown at 5.5 min are for the benzoyl ecgonine after it has been methylated.

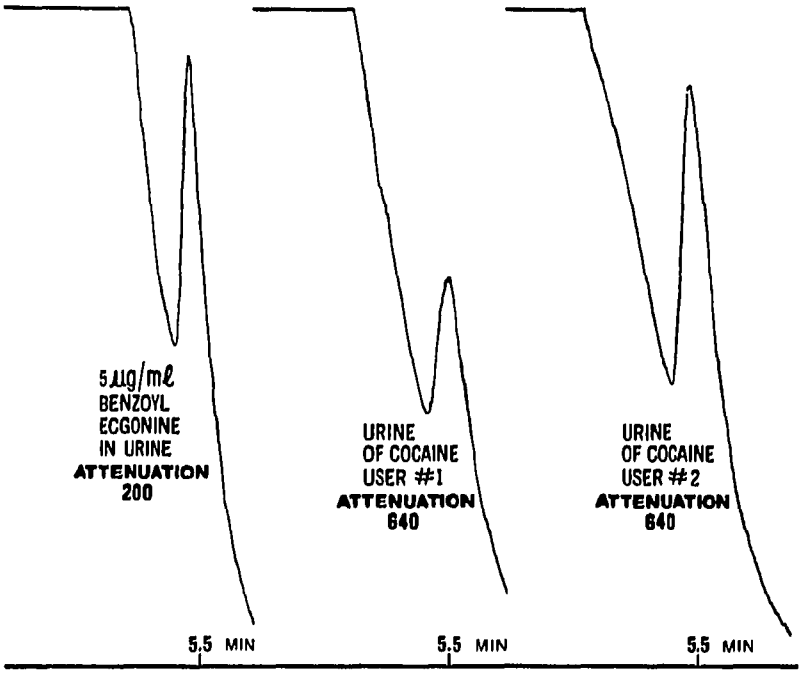

Fig. 2. Gas chromatograms of benzoyl ecgonine extracted from lyophilized urine into methanol. GC conditions are given in the Materials and Methods section of the text. Peaks shown at 5.5 min are for benzoyl ecgonine after it has been methylated. Note: Higher attenuation settings give proportionately lower peak sizes.

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