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DETECTION OF BENZOYLECGONINE IN HUMAN URINE

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SUMMARY

A thin-layer chromatography (TLC) method is described that can be used to detect benzoylecgonine (BE), a metabolite of cocaine, in human urine. It is a two-part procedure that can be integrated into a rapid screening program for drug abuse. The first part of the method utilizes two TLC solvent systems to identify a variety of drugs, including BE. The second part is specific for the cocaine metabolite and can be used as a confirmation method. The procedure is sensitive to 3–4 μg/ml of BE in urine.

INTRODUCTION

Owing to the recent increase in the illicit use of cocaine, a method of detecting its presence in human urine is essential in a drug-screening program. Since very little unmetabolized cocaine is found in urine, one of its major metabolites, benzoylecgonine (BE) must be detected instead[1–3]. The development of methods for the extraction and detection of BE has been difficult owing to the water solubility and amphoteric nature of this metabolite[1,3]. Various published procedures[2–8] have been investigated in this laboratory and were found to be either too expensive, time-consuming, or difficult to integrate with our existing procedures.

This paper presents a reliable thin-layer chromatographic (TLC) method for detecting and confirming BE in human urine. Amberlite® XAD-2 resin is utilized for the extraction of drugs from urine and two individual solvent systems are used for the identification of BE.

EXPERIMENTAL

Materials

All chemicals were reagent grade. Amberlite XAD-2 resin (Rohm & Haas, Philadelphia, Pa., U.S.A.) was washed with one bed volume of methanol and ten bed volumes of deionized water prior to use. The thin-layer medium was Instant Thin-

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Layer Chromatography sheets (ITLC), Type SG (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.). The thin-layer developing solvent systems were: ss I: chloroform–concentrated ammonium hydroxide (50:0.1); ss II: benzene–hexanes–diethylamine (25:10:1); ss III: ethyl acetate–methanol–concentrated ammonium hydroxide (10:5:1); and ss IV: acetone–diethylamine (30:1). Acidified iodoplatinate was prepared by mixing 5% platinic chloride, 10% potassium iodide and distilled water (1:9:9) as a stock solution and adding an equal volume of 2 *N* HCl just before use. Abuscreen® radioimmunoassay kits for benzoylecgonine, which are not yet commercially available, were donated by Roche Diagnostics (Division of Hoffmann-LaRoche, Nutley, N.J., U.S.A.). The authentic BE standard was purchased from Technam (Park Forest So., Ill., U.S.A.). For use as an authentic standard in solution, various amounts of the BE were dissolved in spectroanalyzed methanol.

All urine samples were obtained from the Drug Abuse Treatment and Rehabilitation wards and clinics, Veterans Administration Center, Wood, Wisconsin.

Methods

Part I. Drugs were extracted from 50 ml of urine using an Amberlite XAD-2 resin column and eluted with methanol which is evaporated, as previously described[9,10]. Further purification of the extracted drugs was accomplished by dissolving the dried methanol eluate in 0.5 ml of 0.1 *M* carbonate–bicarbonate buffer (approximately pH 9) and extracting with 5 ml of chloroform–2-propanol (13:7, v/v). After vortexing for 10 sec, the mixture was centrifuged for 5 min. The organic layer was transferred to another test tube and evaporated on a Buchler Evapo-Mix® (Buchler Instruments, Fort Lee, N.J., U.S.A.) at 45° under reduced pressure. This dried residue was dissolved in 100 μl of methanol. Fifteen microliters were spotted on each of two ITLC sheets, which were developed in ss I and II for 10 and 13 min, respectively. The chromatograms were air dried, sprayed with acidified iodoplatinate, and analyzed together for a variety of basic drugs as previously described[10]. Benzoylecgonine appeared immediately as a brownish-purple spot between R_F values of 0.1 and 0.35 on ss I. At high concentrations, BE was visible between the origin and R_F 0.15 on ss II.

Part II. After the iodoplatinate had faded, the area corresponding to BE was cut from the chromatogram developed in ss I, and shredded into a test tube. The chromatogram pieces were eluted twice for 15 min with consecutive volumes of 5 and 2.5 ml of methanol. The methanol eluates were combined in a conical test tube, centrifuged to settle any silica gel, transferred to another test tube, and evaporated to dryness under the above conditions. Fifty microliters of methanol were added to dissolve the dried residue, which was completely spotted in two successive aliquots. Fifteen microliters of authentic BE standards at concentrations of 5.0, 1.0, and 0.5 mg/ml were spotted on the ITLC sheet with the samples. The chromatogram was developed in ss III for 10 min and air dried. After spraying with acidified iodoplatinate, BE appeared as a purple spot between R_F values of 0.45 and 0.60. Depending on the amount of BE present, the spot appeared immediately or within 5 to 10 min.

Although this procedure has been standardized to the use of 50-ml urine samples, in routine clinical application 5–70 ml of urine are used.

Benzoylecgonine radioimmunoassay (BE RIA) kits were used according to the manufacturer's instructions.

RESULTS AND DISCUSSION

An experiment was conducted to determine whether Amberlite XAD-2 resin extracted BE from urine. Fifty milliliters of drug-free urine spiked with BE to a final concentration of 4 μg/ml of urine and a drug-free control urine were extracted as described in Part I of *Methods* and spotted on chromatograms for ss I and II. As a reference, 15 μl of a 1 mg/ml authentic BE standard solution were directly spotted with the samples on chromatograms to be developed in ss I and II. The 1 mg/ml BE standard remained at the origin in both solvent systems but did react with acidified iodoplatinate, appearing as a brownish-purple spot. The 4 μg/ml standard extracted from urine revealed a similarly colored spot at R_F 0.1 in ss I, although no migration was observed in ss II. The control urine did not show any brownish-purple spots. Therefore, it was concluded that BE was extracted by the Amberlite XAD-2 procedure and that the R_F of BE in an extracted urine sample was higher than that of BE dissolved in methanol.

To determine the effect of various concentrations of BE on the R_F value, drug-free urine samples were spiked with BE in final concentrations from 2 to 20 μg/ml of urine. These samples were extracted, spotted, and chromatographed in ss I and II, re-chromatographed in ss III as described in Parts I and II of *Methods*. Brownish-purple spots were observed in all samples on the chromatogram developed in ss I. Larger spot size and an increased R_F value were seen with each increase in concentration of BE, as shown in Fig. 1a. Fig. 1b illustrates the appearance of brownish-purple spots above the origin on the ss II chromatogram as the concen-

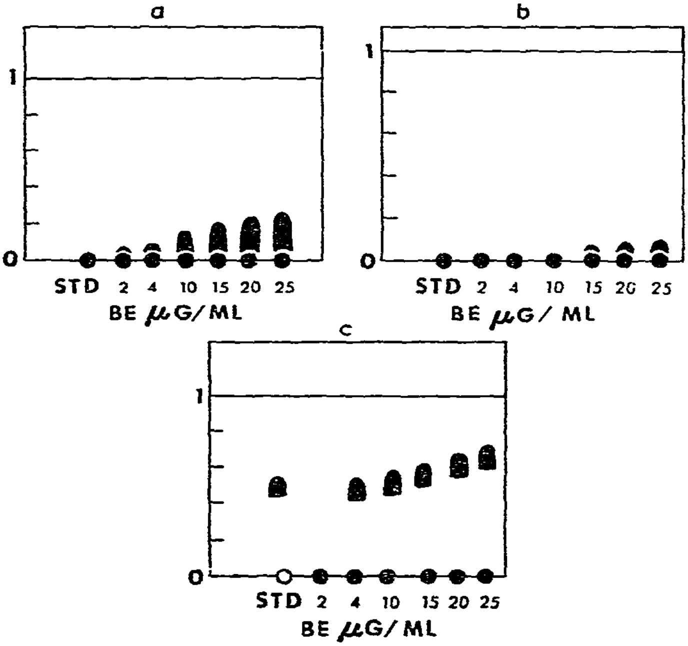

Fig. 1. The average R_F values of extracted urine samples with increasing concentrations of BE chromatographed in ss I (a), II (b), and III (c). In solvent systems I and II, the R_F change with concentration is much more pronounced than it is with ss III.

tration of BE increased from 15 μg/ml. In ss III, as seen in Fig. 1c, BE at a concentration of 4 μg/ml of urine appeared at an R_F of 0.45 and migrated higher at greater concentrations. The R_F of BE therefore, visualized as a brownish-purple spot, consistently shifted upward with increases in concentration on all three solvent systems.

The sensitivity of ss I and ss III was determined using 50 ml drug-free urine samples spiked with BE to final concentrations ranging from 0.5 to 5 μg/ml of urine in a single blind study. The samples and a control urine were extracted as in Part I of *Methods* and spotted in random order on duplicate chromatograms which were developed in ss I. The minimum concentration reliably detected on ss I was between 1 and 2 μg/ml. Owing to loss of material in re-extraction from ss I, the sensitivity of ss III was 3–4 μg/ml.

As shown in Fig. 2a, certain drugs such as quinine, the phenothiazines, some tricyclic antidepressants and their metabolites were known to chromatograph to the region where BE appears in ss I[10]. To accomplish positive identification and confirm BE in the presence of these potentially interfering drugs, an additional solvent system was required. Various problems hampered the development of this system. Polar solvent systems that suitably chromatographed authentic BE standards were not compatible with urine extracts which consistently overloaded in them. Attempts to reduce the amount of urinary constituents causing the overloading by using less polar extraction solvents resulted in the loss of BE as well. The problem was solved by cutting the BE spot off the ss I chromatogram, eluting it, and re-chromatographing it in another solvent system. This separated the BE from most interfering substances, produced a relatively pure extract, and avoided the problem of overloading. The partial purification also resulted in better correlation of sample and standard R_F values on subsequent solvent systems. Studies of various solvent systems using an authentic BE standard solution indicated that ss III produced the best R_F for re-chromatographing the BE spot. Fig. 2b shows the good separation of the same drugs as shown in Fig. 2a after the BE area was re-chromatographed in ss III.

To determine the concentration of BE visible on ss I in the presence of interfering drugs with similar R_F, 50-ml urine samples from drug abuse patients receiving clinical doses of quinine, phenothiazines, tricyclic antidepressants or propoxyphene were spiked with BE at varying concentrations up to 10 μg/ml of urine. The extraction

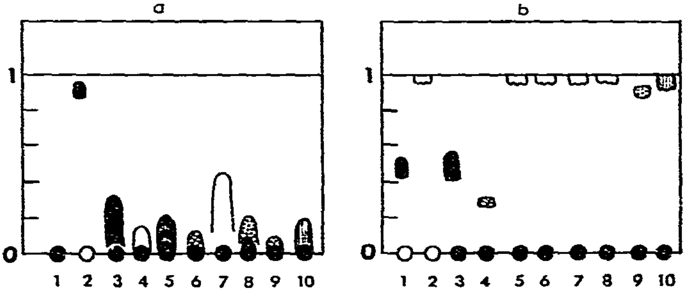

Fig. 2. Spots of pertinent drugs on ss I (a) and III (b). Sample numbers 1 and 2 are chemical standards of BE and cocaine respectively, number 3 is an extracted cocaine-positive urine, and numbers 4–10 are extracts of urine samples from drug abuse patients administered the following drugs: 4 = promazine; 5 = thioridazine; 6 = desipramine; 7 = methadone; 8 = propoxyphene; 9 = morphine; and 10 = quinine.

procedure was performed as described in Part I of *Methods* and 15 μl of each sample were spotted on an ITLC sheet that was developed in ss I. The characteristic brownish-purple color of BE was visible when the concentration of BE was 10 μg/ml of urine. When BE was present at a concentration below 10 μg/ml, it was difficult to distinguish from the above drugs. However, by cutting the spot in question from the ss I chromatogram and re-chromatographing it on ss III, BE was visible at all concentrations to the sensitivity limit of 3–4 μg/ml.

The only drug found to interfere with the detection of BE in both ss I and ss III was trimethobenzamide (Tigan®). The excreted metabolites of trimethobenzamide completely masked the R_F of BE in both solvent systems. The metabolites of trimethobenzamide can be identified using the brown spots at R_F values of 0.25–0.35 and 0.45–0.55 on ss II, along with the spot on ss I. If trimethobenzamide is detected in the initial screening by ss I and II, the chromatogram should be developed in ss III, dried, and not sprayed until it is re-developed in ss IV for 16 min. The metabolites of trimethobenzamide appear at R_F 0.5 and at the solvent front in this solvent system; BE migrates to R_F 0.75.

In the surveys of urine samples from drug abuse patients and staff conducted for this publication, the drugs listed in Table I showed no interference with BE detection on ss I and III. These drugs were tabulated from prescribed medications and the results of urine surveillance by TLC, gas–liquid chromatography, and RIA[10]. The drugs listed in Table II migrated to the same area as BE on ss I, but did not interfere with BE detection on ss III.

Since cocaine could not be given investigationally to humans, a controlled study of metabolized cocaine in human urine could not be set up to examine the effect of the concentration of BE on R_F value. Therefore, urine samples from drug abuse patients were screened for BE spots. The observed brownish-purple BE spots had an average R_F of 0.1–0.35 in ss I. Certain samples were observed in which the concentration of BE must have been very high since the BE spot extended from the origin to an R_F of 0.6 in ss I and 0.15 in ss II. In these samples, cocaine was frequently present with the metabolite, appearing as a purple-brown spot at R_F 0.9 in ss I and at the solvent front in ss II.

BE RIA was used to confirm the presence of BE in drug abuse patient urine regardless of the R_F at which the BE spot appeared. Ten extracted patient samples containing brownish-purple spots with R_F varying between 0.1 and 0.6 on ss I and 0.5 and 0.8 on ss III were selected. The BE spot from each sample was cut from both chromatograms and processed as described in Part II of *Methods*, except that the final dried product from each spot was dissolved in 5 ml of distilled water and assayed with BE RIA. All samples were qualitatively positive using the radioactivity (cpm) of a 100 ng/ml BE standard as the cut-off. As a result of the above work, we concluded that a brownish-purple spot between R_F values of 0.1 and 0.6 on ss I and 0.5 and 0.8 on ss III was indicative of BE. The variations in R_F were due to the concentration of the particular patient sample, as had already been demonstrated with drug-free urine spiked with BE.

During the application of ss III to routine urine surveillance, approximately 2000 urine samples from drug abuse patients were screened for BE on ss I. Since potential interferences were still being explored, 122 of these 2000 patient urines were chosen for processing on ss III by a double criterion: either the presence of BE, or

TABLE I

DRUGS WHICH DO NOT INTERFERE WITH BE DETECTION USING SS I AND III OR BE RIA

Acetaminophen
Aluminum hydroxide
d-Amphetamine
Aspirin
Atropine sulfate
Benzathine penicillin G
Benztropine
Brompheniramine maleate
Chloral hydrate
Chlordiazepoxide
Citrocarbonate
Chlorpropamide
Codeine
Diacetylmorphine
Diazepam
Digoxin
Dioctyl calcium sulfosuccinate
Diphenhydramine
Diphenylhydantoin
Disulfiram
Doxepin
Ephedrine sulfate
Erythromycin
Ethambutol
Flurazepam
Glycerol guiacolate
Guanethidine
Haloperidol
Hydrochlorothiazide
Hyoscine hydrobromide
Hyoscyamine sulfate
Isoniazid
Lidocaine
Magnesium hydroxide
Meperidine
Methadone
Methamphetamine
Methenamine mandelate
Methocarbamol
Alpha-methyldopa
Methylphenidate
Methyprylon
Morphine
Multivitamins
Naloxone
Oxazepam
Pentobarbital
Phenobarbital
Phenylephrine hydrochloride
Phenylpropanolamine hydrochloride
Promethazine
Propantheline
propoxyphene napsylate
Propranolol
Reserpine
Simethicone
Sulfamethoxazole
Tetracycline
Theophylline ethylenediamine
Trimethoprim
Tybamate

drugs covering the same R_F on ss I. Sixty-four of these urines were positive for BE on ss III, including six urines which contained a spot indicating the presence of parent compound cocaine.

The interference of trimethobenzamide became apparent when the above 122 urine samples were evaluated using BE RIA. Six of the samples which were positive

TABLE II

DRUGS WHICH INTERFERE WITH BE DETECTION USING SS I IF BE IS PRESENT AT A CONCENTRATION BELOW 10 μg/ml OF URINE, BUT DO NOT INTERFERE WITH BE DETECTION USING SS III OR BE RIA

Amitriptyline
Chlorpromazine
Desipramine
Hydroxyzine hydrochloride
Promazine
Quinine
Thioridazine
Trifluoperazine

on ss I and III were negative when assayed with BE RIA. By correlating the patient medications and the results of ss II and IV, it was determined that these six false positives on ss I and III were due to trimethobenzamide. Seven samples, which were positive on ss I were subsequently found negative on ss III owing to the loss of material in re-extraction. However, these seven samples were positive by BE RIA using a standard solution of 100 ng/ml BE in urine as a cut-off. The results from ss III agreed with the results of BE RIA for all of the remaining 109 samples. On the basis of this study, we concluded that the drugs listed in Tables I and II, plus trimethobenzamide, do not interfere with BE RIA.

More recently, an additional 2000 patient urine samples were screened for BE. Sixty-nine samples were considered positive or questionable for BE on ss I. These 69 samples were re-chromatographed on ss III; 46 were positive and confirmed by BE RIA. The remaining 23 were negative by both methods.

CONCLUSION

The need for the detection of cocaine in urine surveillance for drug abuse has received much emphasis recently; however, research toward developing a practical procedure has been difficult owing to the chemical characteristics of the major cocaine metabolites. The procedure presented in this paper is reliable and sufficiently sensitive for clinical application. The use of two chromatography systems significantly reduces the probability of interference. Since TLC is the primary method of screening in many laboratories conducting urine surveillance, this procedure is adaptable to existing laboratory facilities, providing a practical method for the detection of BE in urine.

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