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ROUTINE IDENTIFICATION OF COCAINE METABOLITES IN HUMAN URINE

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SUMMARY

A method was developed whereby the major metabolites of cocaine (ecgonine and benzoylecgonine) were extracted from human urine, butylated and subsequently isolated and identified on Polygram silica gel sheets. A comparison of the butylated ecgonine thin-layer chromatographic technique (BETT) with the enzyme multiplied immunoassay technique (EMIT) for benzoylecgonine indicated complete agreement on the presence or absence of cocaine metabolites in 88% of the human urine samples analyzed. Disagreement on the positive urine samples was ascribed to the lower level of sensitivity of BETT (3–5 μg/ml of ecgonine or benzoylecgonine) in comparison to EMIT (1 μg/ml benzoylecgonine). Of the urine samples positive for the cocaine metabolites 18% were negative for other drugs of abuse and 71% were positive for methadone. Cross-reactivity studies with EMIT indicated a high degree of specificity for benzoylecgonine. The BETT and EMIT assays were found to be highly reliable, valid and sensitive tests for the presence of cocaine metabolites in human urine.

INTRODUCTION

Cocaine is extensively metabolized[1–3] and, therefore, little free cocaine is available for detection in urine. Apparently, the major metabolites[1] of this drug are benzoylecgonine and ecgonine. Unfortunately, these biotransformation products are water-soluble and thus not easily extracted by organic solvents, nor are they extracted readily by ion-exchange resins or absorbed on styrene-divinylbenzene copolymers (*i.e.*, XAD-2). For these reasons, cocaine, an apparently widely abused drug, is seldom detected in urinalysis screening programs for drugs subject to abuse.

This communication describes a method for extracting cocaine and its metabolites from human urine, the butylation of these metabolites, their thin-layer chromatographic detection, and subsequent comparison with the enzyme multiplied immunoassay technique for benzoylecgonine.

EXPERIMENTAL

Materials and methods

All chemicals were of reagent grade. Cocaine was obtained commercially from Merck and Co. (Rahway, N.J., U.S.A.). Ecgonine and benzoylecgonine were prepared in our laboratory from cocaine[4]. Benzoylecgonine and benzoylnorecgonine were also obtained as gifts from Dr. E. L. May, National Institute of Health, and Dr. R. J. Bastiani of the Syva Corporation. The urine samples were obtained from NACC treatment and rehabilitation centers as well as from the Syva Corporation. The assay for benzoylecgonine by the enzyme multiplied immunoassay technique (EMIT) was performed as suggested by the manufacturer[5].

Procedure

To 10 ml of urine in a 16 × 150 mm test tube about 250 mg of solid sodium bicarbonate is added to adjust the pH between 8–9. Five milliliters of a chloroform–ethanol (3:2) mixture are added, followed by mixing with a Genie Vortex for about 20 sec. After centrifuging the sample at about 1100 *g* for about 10 min, the upper aqueous phase is transferred to a clean test tube. The remaining organic layer contains unmetabolized cocaine and/or other extractable organic bases.

To the ethanolic aqueous layer containing the cocaine metabolites sufficient solid anhydrous potassium carbonate for saturation is added. The sample is mixed thoroughly using the Genie Vortex and allowed to stand for a few minutes. A dark brown suspension floats to the surface of the tube. It is then centrifuged at 1100 *g* for 10 min to separate the alcohol phase and subsequently transferred to a 40-ml centrifuge tube with a Pasteur pipette. One drop of 6 *N* HCl is added to the ethanolic extract and the pH is determined. If the pH is not below 7, additional 6 *N* HCl should be added.

To butylate the extracted cocaine metabolites 2 ml of 1-butanol and 0.1 ml of concentrated H_2SO_4 are added to the ethanolic extract and heated for 30 min in a glycerin bath maintained at 120–130°. The solution is allowed to cool to room temperature, 5 ml of toluene are added and the entire mixture is transferred to a clean test tube. The centrifuge tube is washed with 5 ml of water and the wash added to the toluene–butanol mixture. The mixture is shaken for 10 sec, centrifuged at 275 *g* for 5 min and the upper organic layer aspirated. The remaining aqueous layer is saturated with solid sodium bicarbonate and extracted with 10 ml of cyclohexane. The cyclohexane is separated from the aqueous layer by centrifugation at 275 *g* for 5 min. The organic phase is transferred to a clean tube and evaporated to dryness in a water-bath. The residue is dissolved in about 150 μl of chloroform and applied to a 0.25-mm Polygram silica gel sheet made by Macherey, Nagel & Co., Düren, G.F.R. (supplied by Brinkmann Instruments, Westbury, N.Y., U.S.A.). The sheets are developed in various solvent systems (see Table I), however, the 1 + 7 system is most commonly used. This solvent system combination (1 + 7) is also used for two-dimensional chromatography. Following development the chromatographic sheet is removed from the tank, air dried for 30 min, or placed in an oven at 90° for 5 min. The thin-layer sheet is sprayed with the iodoplatinate reagent[5], which reacts with butylated ecgonine to provide a bluish purple spot on a light background and with butylated benzoylecgonine or benzoyl-

norecgonine to provide a purple-brown spot. For optimum color development one should spray once and then respray 5 min later.

RESULTS

In Table I R_F values and sensitivity data are listed for the butylated cocaine derivatives and R_F values for morphine. Using a single solvent system it was difficult to separate morphine from butylated ecgonine, or the butylated compound was located close to the solvent front, associated with urinary extractable impurities, thus making detection difficult. A single solvent system was effective provided morphine was not present in the sample. Solvent systems No. 5 and No. 6 were acceptable, however, some interference from extractable substances was observed. All other

TABLE I

R_F VALUES AND SENSITIVITY LIMITS FOR DETECTION OF BUTYLATED COCAINE DERIVATIVES

Solvent systems: No. 1 = ethyl acetate–methanol–water (7:2:1), No. 2 = ethyl acetate–methanol–ammonia (15:4:1), No. 3 = chloroform–acetone–diethylamine (5:4:1), No. 4 = chloroform–acetone–ammonia (5:4, saturated), No. 5 = methanol–ammonia (100:1.5), No. 6 = benzene–ethyl acetate–methanol–ammonia (80:20:1.2:0.1), and No. 7 = chloroform–acetone–ammonia (5:94:1).

Solvent system No.	*$R_F \times 100$**				*Sensitivity (μg/ml of urine)***	
	Morphine	*Butylated ecgonine*	*Butylated benzoyl-ecgonine*	*Butylated benzoyl-norecgonine*	*Butylated ecgonine*	*Butylated benzoylecgonine*
1	7	8	45	49	3	3–5
2	31	82	92	78	10	15
3	7	84	91	79	20	40
4	12	87	85	87	10	30
5	34	52	64	58	5	15
6	0	14	32	7	5	3
7	9	78	75	64	10	40
1+2	36	87	—	—	3	3–5
1+3	20	92	—	—	3	5
1+4	19	88	—	—	3	3–5
1+5	44	55	—	—	3	3–5
1+7	23	83	—	—	3	3

* Morphine was applied directly to the thin-layer chromatographic sheets (10–15 μg). The cocaine derivatives (ecgonine, benzoylecgonine and benzoylnorecgonine) were dissolved in either water or ethanol–water (95:5) in concentrations of 1 mg/ml and then butylated and extracted as described under Methods. The compounds were detected by spraying the sheets with the iodoplatinate reagent, which provided blue to purple color reactions. When two solvent systems were used the R_F was relative to the second solvent front and no R_F value was obtained when the derivatives were located above the second solvent front. Thus, the R_F value for these compounds would be the same as for the single solvent system No. 1, since the second solvent system was only allowed to travel a distance of 4–5 cm from the origin.

** Ecgonine and benzoylecgonine were added to urine in concentrations of 1–100 μg/ml and carried through the entire extraction and butylation procedure as described under Methods. Each value represents the minimum concentration detectable. A low level of detection (10–40 μg/ml) was due to extractable substances that interfered with the color reactions for the drugs in the various solvent systems.

single solvent systems were not considered effective for the detection of the cocaine derivatives. A double solvent system was used to separate morphine from butylated ecgonine. This separation is important since morphine glucuronide is extracted by the method described above and subsequently hydrolyzed to free morphine during the butylation procedure. However, little free morphine is finally extracted into cyclohexane. The solvent system combination 1 + 7 was found to be the most useful for the isolation and subsequent detection of butylated ecgonine. The R_F values for butylated benzoylecgonine and butylated benzoylnorecgonine with the double solvent system technique were the same as for the single solvent system No. 1, since these compounds were located above the second solvent system front. The sensitivity for detection of butylated ecgonine and benzoylecgonine varied from 3 to 40 μg/ml of urine. The greatest sensitivity was observed with solvent system No. 1 and the least with solvent systems No. 3 and No. 4. Using the double solvent system, of course, did not change sensitivity (3–5 μg/ml) since these values were related to the extraction and solvent system No. 1.

The butylated derivatives of ecgonine and benzoylecgonine may be effectively

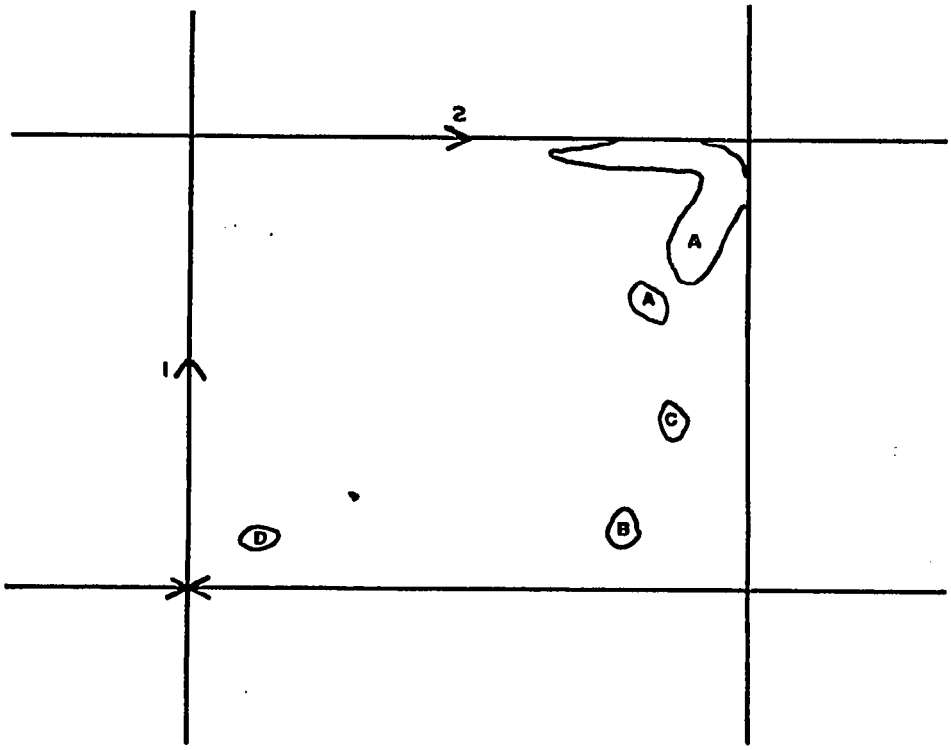

Fig. 1. Urine samples containing ecgonine, benzoylecgonine (10 μg/ml each) and control urines (no drug) were carried through the entire procedure as described under Methods. The butylated extracts as well as free morphine (10 μg) were applied at the origin and the chromatogram was developed in two different solvent systems (two-dimensional chromatography). The solvent system used for the first dimension was ethyl acetate–methanol–water (7:2:1) (Solvent system No. 1) and that used for the second dimension was chloroform–acetone–ammonia (5:94:1) (Solvent system No. 7). A = Urinary impurities; B = butylated ecgonine; C = butylated benzoylecgonine; D = morphine; X = origin.

separated from morphine and urinary impurities through the use of two-dimensional chromatography (Fig. 1). This technique, however, is limited and time consuming for the routine urinalysis of thousands of samples.

A total of 259 human urine samples analyzed for benzoylecgonine by EMIT were analyzed by the butylated ecgonine thin-layer chromatographic technique (BETT). The data (Table II) indicate that 88.0% of the samples that were either positive or negative by EMIT were confirmed by BETT and 11.9% were not. The largest

TABLE II

COMPARISON OF THE EMIT ASSAY FOR BENZOYLECGONINE WITH THE BETT ANALYSIS IN 259 HUMAN URINE SAMPLES

BETT is performed as described under Methods and EMIT as described by Syva Co., Palo Alto, Calif.[4]. About 3000 urine samples were screened by EMIT and those positive for benzoylecgonine as well as a random selection of negative samples were subsequently analyzed by BETT.

*Results**	*Urine samples*	*%*
True positive	170	65.6
True negative	58	22.4
Total	228	88.0
False positive	26	10.0
False negative	5	1.9
Total	31	11.9

* All values are in reference to BETT.

percentage of false determinations were false positives (10.0%) *vs.* 1.9% for false negatives. Of the false positives 80.8% (21 samples) were at concentration levels in urine of 1–2 μg/ml of benzoylecgonine and 19.2% (5 samples) were between 2–3.5 μg/ml. The maximum sensitivity level for BETT was found to be 3–5 μg/ml (Table I). It is quite possible, therefore, that almost all the false positive samples were labeled so because of the limiting sensitivity of the confirmation technique (BETT). The false negatives may be ascribed to possible technician error in the application of either or both the EMIT and/or BETT analysis.

It was of interest to ascertain whether other drugs subject to abuse were present in the urine samples analyzed for cocaine. These data appear in Table III. Methadone was present in 71.0% of the urine samples truly positive for cocaine usage (confirmed by BETT) and in 76.0% of those samples unconfirmed by BETT (false positives). Morphine was present in 4.7% of the cocaine-positive urines, and quinine in 14.2%. Of the total samples (257) analyzed for cocaine metabolites, 21.4% were negative for other drugs subject to abuse, 69.6% contained methadone, 12.5% quinine, and 3.1% each were positive for morphine, barbiturates, and a miscellaneous group of drugs.

Many drugs chemically similar to derivatives of cocaine (Table IV) as well as those drugs commonly abused were tested for cross-reactivity in the EMIT assay for benzoylecgonine. Ecgonine was the only compound that provided a reasonable cross-reactivity, equivalent to 9.5 μg/ml of benzoylecgonine (relative reactivity of 0.105).

TABLE III

OTHER DRUGS OF ABUSE DETECTED IN 257 URINE SAMPLES ANALYZED FOR COCAINE METABOLITES BY EMIT AND BETT

The analysis for drugs other than cocaine was performed as described previously[6,7]. The data are presented both as whole numbers and percentages of the cocaine results.

Cocaine results	*Other drugs*					
	Negative	*Methadone*	*Quinine*	*Morphine*	*Barbiturates*	*Miscellaneous**
True positive (169)	31 18.3%	120 71.0%	24 14.2%	8 4.7%	8 4.7%	5 3.0%
True negative (58)	16 27.6%	38 65.5%	6 10.3%	—	—	1 1.7%
False positive (25)	5 20.0%	19 76.0%	2 8.0%	—	—	2 8.0%
False negative (5)	3 60.0%	2 40.0%	—	—	—	—
Total (257)	55 21.4%	179 69.6%	32 12.5%	8 3.1%	8 3.1%	8 3.1%

* This includes the following: *d*-methorphan, chlorpromazine (Thorazine), phenylpropanolamine, *d*-amphetamine, and thioridazine (Mellaril).

Cocaine cross-reactivity in this assay was 460 μg/ml, a relative reactivity ratio of 0.002. The methyl ester of ecgonine and benzoylnorecgonine cross-reacted at concentrations of 380 and 335 μg/ml, respectively. All other compounds did not cross-react at concentrations ranging from 500 to 1000 μg/ml.

TABLE IV

CROSS-REACTIVITY OF VARIOUS DRUGS IN THE EMIT ASSAY FOR BENZOYLECGONINE

Drug	*Equivalent to 1.0 μg/ml of benzoylecgonine (μg/ml)*	*Relative reactivity**
Benzoylecgonine	1.0	1.000
Ecgonine	9.5	0.105
Methyl ester of ecgonine	380	0.003
Benzoylnorecgonine	335	0.003
Norecgonine	NR_{1000}**	0.001
Cocaine	460	0.002
Atropine	NR_{500}	—
Nicotine	NR_{500}	—
Homatropine	NR_{500}	—
Scopolamine	NR_{500}	—
d-Amphetamine	NR_{500}	—
Morphine	NR_{500}	—
Methadone	NR_{500}	—
Secobarbital	NR_{500}	—

* All values relative to benzoylecgonine.

** NR = No cross-reactivity at the concentration indicated.

DISCUSSION

Cocaine apparently is a widely abused psychoactive drug that has been extremely difficult to detect on routine urine screening because of the extensive biotransformation of this drug. We therefore developed a method whereby ecgonine as well as benzoylecgonine could be extracted from biological material (urine) and butylated with subsequent isolation and detection on a thin-layer chromatogram. Because of extractable urinary substances and the possible presence of morphine, a double solvent system (1 + 7, Table I) proved to be most effective for the identification of butylated ecgonine. Two-dimensional chromatography, of course, may also be effectively used in detecting the butylated cocaine derivatives. Morphine present in the final extract was derived from morphine glucuronide, extractable with ecgonine and congeners and subsequently acid hydrolyzed to free morphine in the butylation procedure. Even though relatively little free morphine is extracted into cyclohexane, it is necessary to separate morphine (heroin metabolite) from the cocaine metabolites since heroin addicts quite commonly use cocaine.

A comparison of the EMIT assay for benzoylecgonine and the BETT assay on 259 urine samples indicated the reliability and validity of these tests. Although BETT confirmed only 88.0% of the EMIT tests, further inspection of the data revealed that all the false positive samples were in the concentration range of 3.5 μg/ml or less of benzoylecgonine. The sensitivity limits for the BETT assay were determined at 3–5 μg/ml, thus it appears that the false positives were simply due to the sensitivity limits of the confirmation assay (BETT). With the EMIT assay false positives may also be due to native lysozyme activity in the urine. This may be easily determined by analyzing the urine in the absence of antibody and the enzyme–drug complex (*i.e.* no reagent). The false negatives (1.9%) appear to be due primarily to technical error.

It is interesting to note that a high percentage of the samples positive for cocaine were also positive for methadone (71%) and that only 18% of the cocaine-positive samples were negative for other drugs. Quinine was present in 14% of the cocaine-positive samples and morphine was confirmed in 5%. Barbiturates and miscellaneous groups of drugs were present in 3 to 5% of the true positive cocaine samples. The data are indicative of the fact that cocaine is generally not used alone, but in conjunction with other drugs, especially heroin and methadone.

The EMIT assay for benzoylecgonine appears to be quite specific. All the drugs tested, with the exception of ecgonine, cross-reacted in the EMIT assay, at concentrations greater than 300 μg/ml benzoylecgonine equivalents.

It appears that the EMIT and BETT assays are highly reliable, valid and relatively sensitive tests for the determination of cocaine metabolites in human urine. These tests may be used independently to detect cocaine abuse, but preferably in combination as complimentary confirming tests.

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REFERENCES

1 F. Fish and W. D. C. Wilson, *J. Pharm. Pharmacol.*, 21 (1969) 135S.
2 L. A. Woods, F. G. McMahon and M. H. Seevers, *J. Pharmacol. Exp. Ther.*, 101 (1951) 200.
3 A. R. McIntyre, *J. Pharmacol. Exp. Ther.*, 57 (1936) 133.
4 M. R. Bell and S. Archer, *J. Amer. Chem. Soc.*, 82 (1960) 4642.
5 *Emit (TM) Operators Manual*, Syva Corporation, Palo Alto, Calif., October, 1972.
6 S. J. Mulé, M. L. Bastos, D. Jukofsky and E. Saffer, *J. Chromatogr.*, 63 (1971) 289.
7 M. L. Bastos, D. Jukofsky, E. Saffer, M. Chedekel and S. J. Mulé, *J. Chromatogr.*, 71 (1972) 549.