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Rapid Confirmation of Enzyme Multiplied Immunoassay Technique (EMIT®) Cocaine Positive Urine Samples by Capillary Gas-Liquid Chromatography/Nitrogen Phosphorus Detection (GLC/NPD)

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ABSTRACT: A rapid gas-liquid chromatographic (GLC) method was developed for the confirmation of benzoylecgonine (BE) positive urine samples screened by the enzyme multiplied immunoassay technique (EMIT®) assay. The procedure is performed by solvent extraction of BE from 0.1 or 0.2 mL of urine, followed by an aqueous wash of the solvent and evaporation. The dried residue was derivatized with 50 μL of pentafluoropropionic anhydride and 25 μL of pentafluoropropropanol at 90°C for 15 min. The derivatizing reagents were evaporated to dryness, and the derivatized BE, and cocaine if present, were reconstituted and injected into the gas chromatograph. The column was a 15-m by 0.2-mm fused silica capillary column, coated with 0.25 μm of DB-1, terminating in a nitrogen phosphorus detector (NPD). Cocaine and the pentafluoro BE derivatives retention times were 3.2 and 2.6 min, respectively. Nalorphine was used as reference or internal standard with a retention time of 4.78 min. The complete procedure can be performed in approximately 1.5 h. The EMIT cutoff between positive and negative urine samples is 300 ng/mL of BE. The lower limit of sensitivity of this method is 25 ng of BE extracted from urine. Validation studies resulted in confirmation of 101 out of 121 EMIT cocaine positive urine samples that could not be confirmed by thin-layer chromatography (TLC). This represents 84% confirmation efficiency.

KEYWORDS: toxicology, urine, benzoylecgonine, cocaine, immunoassay, chromatographic analysis

Cocaine abuse is very common in all sections of the population [*1*]. Public safety may be endangered when cocaine is used at the workplace. For this reason, drug abuse testing is becoming widespread in private industry and government. Identification of cocaine abusers is performed mostly by urinalysis [*2*]. Enzyme immunoassay is a practical and objective method for benzoylecgonine (BE) screening. The remote possibility that cross-reacting sub-

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stances cause false positive results by the immunoassays requires confirmation by a chromatographic method. The enzyme multiplied immunoassay technique (EMIT®)-cocaine assay cutoff for BE is 300 ng/mL between positive and negative results [3]. Thus, methods of confirmation should detect less than 300 ng/mL of BE. Chromatographic methods such as gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), and gas chromatography/mass spectrometry (GC/MS) are useful for BE confirmation because of their low nanogram sensitivity. The method of choice is GC/MS because specific identification is possible of BE or cocaine or both by fragmentation analysis. Some laboratories, however, do not have GC/MS capability or GC/MS testing is too expensive for the client. This method was developed for rapid, reliable, and inexpensive confirmation of BE positive urine samples.

Experimental Procedure

Materials

Chloroform and ethyl acetate were purchased from J. T. Baker Chemical Co. Isopropanol was glass distilled, acquired from Burdick and Jackson Co. Reagent grade potassium carbonate was purchased from Mallincrodt and sodium bicarbonate from J. T. Baker Chemical Co. Pentafluoro-propionic anhydride (PFPA) and 1H, 1H pentafluoropropanol (PFPOL) were purchased from Pierce Chemicals and PCR/SCM Specialty Chemicals, Gainesville Florida, respectively. Only deionized water was used (Millipore Filtration System).

Extraction of Benzoylecgonine from Urine

To 15 mL of centrifuge tubes 0.8 mL of deionized water containing 300 ng of nalorphine hydrochloric acid (HCl) and 0.2 mL of urine were added. The diluted urine samples were saturated with a mixture of solid crystals of sodium bicarbonate/potassium carbonate ($NaHCO_3/K_2CO_3$) (2:1) pH 9.5 and extracted into 5 mL of chloroform/isopropanol (95:5). The samples were shaken for 10 min in a mechanical shaker and centrifuged at 2000 rpm for 5 min, and the aqueous phase (top layer) was aspirated. One millilitre of deionized water was added; shaking, centrifugation, and aspiration of the aqueous wash was repeated. The organic phases, containing BE, were transferred into clean centrifuge tubes and evaporated to dryness under the flow of N_2 at 48°C.

Derivatization

To the residue containing BE, 50 μL of PFPA and 25 μL of PFPOL were added and heated at 90°C for 15 min. The BE derivatives formed were stable both in the reagent (for months) and after the evaporation of derivatizing reagents for at least 24 h. The derivatizing solvents and reagents were evaporated under the flow of N_2 at 48°C. The samples were reconstituted with 25 μL of ethylacetate and 1- to 2-μL aliquots were injected into the gas chromatograph. Figure 1 shows chromatograms of three derivatizing conditions: Fig. 1*a* represents a tracing from the reaction of 200 ng of BE + 50 μL of PFPA; Fig. 1*b* shows a tracing of 200 ng of BE + 50 μL of PFPA + 50 μL of ethylacetate; and Fig. 1*c* is 200 ng of BE + 50 μL of PFPA + 25 μL of PFPOL. The results on the figure indicate that pure PFPA, or PFPA in solvent, did not react with BE to form halogenated BE. Derivatization occurred only in the presence of the pentafluoropropanol (Fig. 1*c*).

Recovery

To each of four test tubes 25 ng of BE were added, and the samples were derivatized and chromatographed as described. A comparative set of four test tubes with 25 ng of BE and 0.2

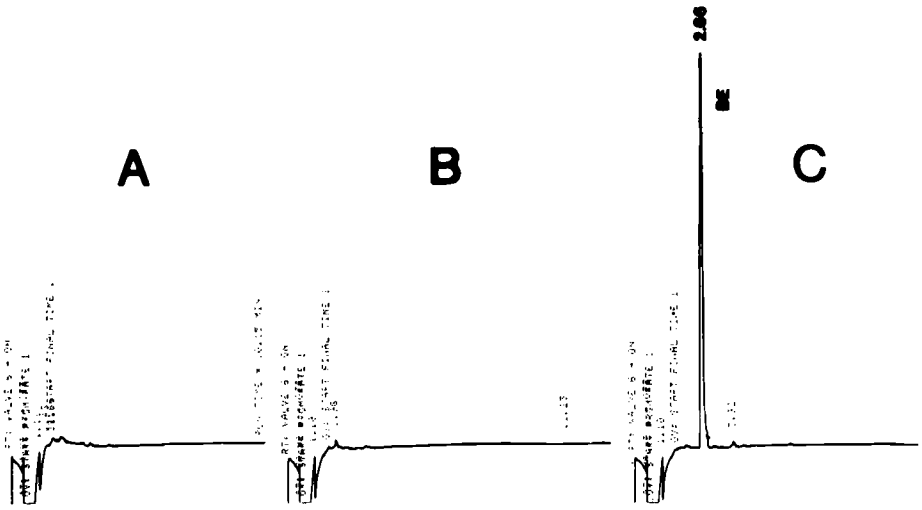

FIG. 1—*Chromatographic tracings of the substances formed under various conditions for the derivatization of benzoylecgonine* (BE): (a) *BE 200 ng + PFPA 50 μL at 90°C for 15 min,* (b) *BE 200 ng + PFPA: ethylacetate (50 μL: 50 μL) at 90°C for 15 min, and* (c) *BE 200 ng + PFPA + PFPOL (50 μL: 25 μL) 90°C for 15 min.*

mL of blank urine were also extracted, derivatized, and chromatographed. The comparison of the absolute and the extracted BE concentration resulted in the calculated recovery of 56.4 ± 3.1% BE.

Instrumentation and Chromatography

Analysis of BE derivatives was performed on a Hewlett Packard GLC 5880A, equipped with a 15-m by 0.2-mm inside diameter (id) wall-coated open tubular (WCOT) fused silica capillary column with 0.25-μm DB-1 coating and a NPD detector. The injection port and detector temperatures were 220 and 300°C, respectively. The instrument was operated in the splitless mode. The column temperature was programed from 190°C for 0.7 min and 30°C/min to the final temperature of 220°C. Helium was the carrier gas, flowing at 1.5 mL per min, H_2 at 3 mL/min, and air at 90 mL/min. The retention times for the pentafluoro derivative of benzoylecgonine and cocaine were 2.6 and 3.2 min, respectively (Fig. 2). Figure 3 shows a nalorphine standard in the first panel and BE and nalorphine in BE positive urine samples in the second and third panels.

Discussion

A quick and sensitive confirmation method for BE positive urines has been described. Although other GLC methods for BE in urine have been published, the sensitivity was not sufficient in five out of six methods and most were not practical for routine toxicological confirmation [*4-10*]. In Table 1 a summary is presented comparing six published methods with this one. The following categories were compared: substance detected (BE/cocaine or both), sensitivity of the method (ng/mL), internal standard (if used), volume of sample needed for analysis, number of manipulative steps for extraction and derivatization procedures, type of derivatization, GLC columns and detectors, and the relative GLC retention times. This method requires the least number of preparation steps among the methods listed.

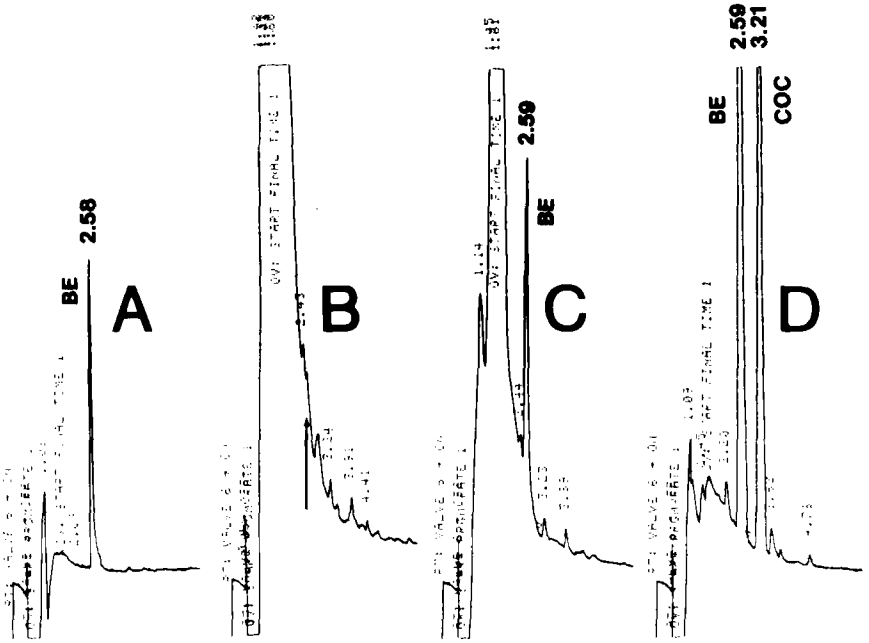

FIG. 2—*Benzoylecgonine* (BE) *and cocaine* (Coc) *PFPA derivatives:* (a) *benzoylecgonine standard,* (b) *blank urine, arrow at benzoylecgonine retention time,* (c) *sample with benzoylecgonine, and* (d) *sample containing benzoylecgonine and a large cocaine peak.*

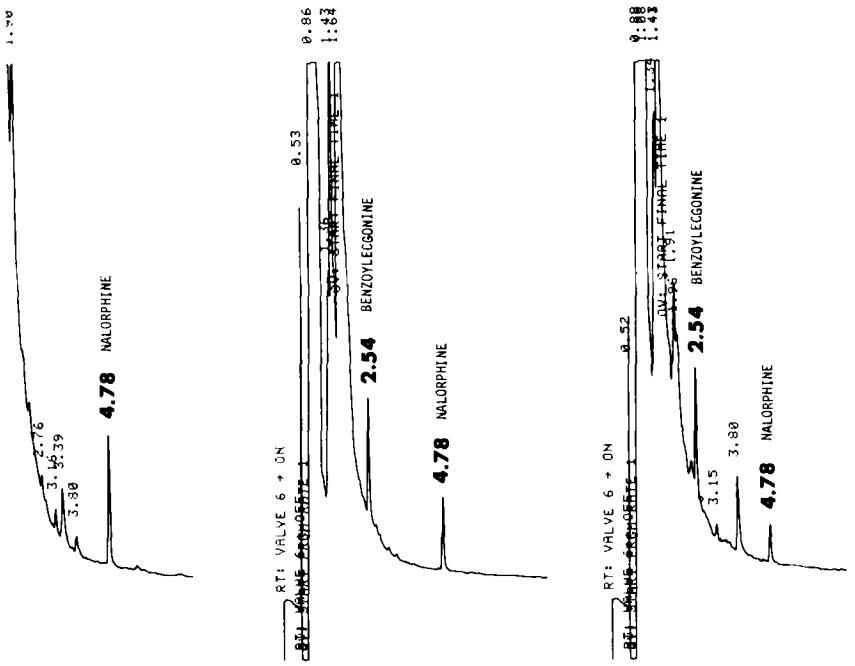

FIG. 3—*Nalorphine standard (4.78 min) and two BE positive urine samples (BE 2.54 min and nalorphine 4.78 min).*

TABLE 1—*Summary of various GLC methods for benzoylecgonine or cocaine or both determination in urine.*[a]

References	Substance Detected	Sensitivity, ng/mL B/C	Internal Stnd./Urine Volume	No. of Procedures Extraction/and Type of Derivatization	Detector Type	Retention Time, min	Columns Capillary/ Packed
4	B/C	B = 1000 C = 200	ND 5.0 mL	N.D. heptafluoro	ECD	C = 4.0 B = 5.8	5% OV-1 packed
5	B/C	B = 500 C = 500	isopropyl BE 1.0 mL	4/1 = 5 on column alkylated	FID	B = 4.7 C = 2.7	3% OV-17 packed
6	B/C B/C	B = 1000 C = 500	scopolamine 1.0 mL	6/1 = 7 sylilated	FID	B = 0.93 C = 0.78	3% SE-30 packed
8	B/C	B = 200 C = 200	... 5.0 mL	2/9 = 11 propylated	FID	B = 7.0 C = 5.5	3% SE 30 packed
7	B	B = 300	codeine 5.0 mL	2/9 = 11 butylated	FID	B = 3.39	DB-1 capillary
9	B/C	B = 200 C = 100	Butylanthraqui- none 5.0 mL	2/6 = 8 methylated	FID	C = 3.5	3% OV-17
this paper	B/C	B = 125 C = 75	nalorphine 0.2 mL	3/2 = 5 pentafluoro	NPD	B = 2.6 C = 3.2	DB-1 capillary

[a]B = benzoylecgonine, C = cocaine, ND = not determined for biological materials, and NPD = nitrogen/phosphorous detector.

BE analysis presents two main obstacles: the recovery of BE from urine and the formation of stable BE derivative. Organic extraction of polar metabolites, such as BE, from urine often is a problem. As the polarity of the extracting solvent increases, the recovery of BE also increases along with the recovery of unwanted contaminants. Chloroform:isopropanol 95:5 was chosen for extraction of BE [5]. At higher alcohol content BE recovery increased but the background became too dirty for proper chromatographic presentation and interpretation. At the 95:5 polarity BE recovery was only 56.4% but the blank urine chromatograms were clean in the area of BE elution, see arrow (Fig. 2*b*).

Cocaine when present is also detected by this method. Only about 4 to 10% of unchanged cocaine is excreted into the urine, mainly during the first few hours after the dose [9]. Thus the presence of cocaine in urine is indicative of recent use. An example when both BE and cocaine was present in a urine sample is shown in Fig. 2*d*.

Underivatized BE has poor chromatographic properties and very low detector response. For this reason derivatization is necessary. Sylilation was unreliable, confirming the observation of Jain et al. [5]. Methylation of BE to convert it back to cocaine was better and stable, but it confirms the presence of cocaine not BE, and the EMIT assay is for BE detection. The combined use of an anhydride and an alcohol for BE derivatization was described by Javaid et al. [4]. We have adopted a similar derivatization system and found it extremely reliable, forming stable derivatives. When the anhydride, PFPA alone, was mixed with BE and heated, no halogenated derivatives formed, indicating that an acidic condition is a prerequisite for derivatization of BE. This was achieved by the combined use of PFPA and PFPOL. Figure 1*c* shows the pentafluoro-BE peak at 2.66 min. Greater detection sensitivity of halogenated BE can be attained by the use of electron capture (EC) detectors. However, the EC detectors are very sensitive to contaminants and routine injections of urine extracts would require frequent cleaning of the EC detectors.

The method reported here has been used for the confirmation of 121 EMIT-BE positive urine samples. The samples received for confirmation could not be confirmed by TLC, indicating BE concentrations below 1000 ng/mL. Out of 121 tests, 101 were confirmed positive by the capillary GLC system, representing 84% confirmation efficiency. Of the samples 16% were not confirmed. In many of these samples (which were ultimately judged negative) a small peak appeared on the GLC tracing and the recorder printed out the correct retention time for the BE derivative, but the background was too "noisy" to call them positive.

In summary, this capillary GLC assay is quick and practical to confirm the presence of BE in EMIT-BE positive urine samples. The simplicity of the assay allows confirmation within 1.5 h after sample receipt.

Acknowledgments

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References

[1] Washton, A. M. and Gold, M. S., "Recent Trends in Cocaine Abuse as Seen from the "800-Cocaine" Hotline," in *Cocaine: A Clinician's Handbook*, A. M. Washton and M. S. Gold, Eds., Guilford Press, New York, 1987, pp. 10-22.

[2] Verebey, K., "Cocaine Abuse Detection by Laboratory Methods," in *Cocaine: A Clinician's Handbook*, A. M. Washton and M. S. Gold, Eds., Guilford Press, New York, 1987, pp. 214-218.

[3] SYVA-EMIT Cocaine Metabolite Assay, EMIT®-dau package insert, P.O. Box 10058, Palo Alto, CA 94303.

[4] Javaid, J. I., Dekirmenjian, H., Brunngraber, E. G., and Davis, J. M., "Quantitative Determination of Cocaine and Its Metabolites Benzoylecgonine and Ecgonine by Gas-Liquid Chromatography," *Journal of Chromatography*, Vol. 110, 1975, pp. 141-149.

[5] Jain, N. C., Chinn, D. M., Budd, R. D., Sweath, T. S., and Leuing, W. J., "Simultaneous Determination of Cocaine and Benzoylecgonine in Urine by Gas-Chromatography with On-Column Alkylation," *Journal of Forensic Sciences*, Vol. 22, No. 1, Jan. 1977, pp. 7-16.

[6] Kogan, M. J., Verebey, K. G., DePace, A. C., Resnick, R. B., and Mule, S. J., "Quantitative Determination of Benzoylecgonine and Cocaine in Human Biofluids by Gas-Liquid Chromatography," *Analytical Chemistry*, Vol. 43, No. 13, 1977, pp. 1965-1969.

[7] Falk, P. M. and Harrison, B. C., "Use of DB-1 Capillary Columns in the GC/FID Analysis of Benzoylecgonine," *Journal of Analytical Toxicology*, Vol. 9, Nov.-Dec. 1985, pp. 273-274.

[8] Von Minden, D. L. and D'Amato, N. A., "Simultaneous Determination of Cocaine and Benzoylecgonine in Urine by Gas-Liquid Chromatography," *Analytical Chemistry*, Vol. 49, No. 13, Nov. 1977, pp. 1974-1977.

[9] Wallace, J. E., Hamilton, H. E., King, D. E., Bason, D. J., Schwertner, H. A., and Harris, S. C., "Gas-Liquid Chromatographic Determination of Cocaine and Benzoylecgonine in Urine," *Analytical Chemistry*, Vol. 48, No. 1, Jan. 1976, pp. 34-38.

[10] Wallace, J. E., Hamilton, H. E., Christenson, J. G., Schimek, E. L., Land, P., and Harris, S. C., "An Evaluation of Selected Methods for Determining Cocaine and Benzoylecgonine in Urine," *Journal of Analytical Toxicology*, Vol. 1, Jan.-Feb. 1977, pp. 20-26.

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