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Simultaneous detection in urine of cocaine and its main metabolites*

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Abstract: The simultaneous detection in urine of cocaine (CO), and the main biotransformation products, benzoylecgonine (BE) and ecgonine methyl ester (EME), is difficult due to their different physicochemical properties.

The method presented involves a bonded silica solid-phase extraction procedure that allows mixed ionic and apolar interactions with the analyte. After extraction the compounds are derivatized sequentially with ethyliodide to obtain the BE ethyl ester derivative, and with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) to obtain the *O*-TMS derivatives. The derivatized compounds are then analysed by a capillary (methylphenylsilicone) gas chromatographic system equipped with a specific nitrogen–phosphorus detector.

The method is suitable for the confirmation and quantitation of CO and its main metabolites in urine. BE levels in urine samples (n = 20) measured by the described method and by an immunological technique were in close agreement (r = 0.999).

Keywords: *Cocaine metabolites; capillary gas chromatography; derivatization; urine.*

Introduction

The simultaneous detection of benzoylecgonine (BE) and ecgonine methyl ester (EME), the main biotransformation products of cocaine (CO) [1] is a reliable way of confirmation of CO use. Most assays for the identification of CO use are designed for the detection of BE only [2, 3, 5, 7–9, 11] in urine. Reports on the simultaneous detection of EME and BE are very few [4, 6].

The simultaneous detection of BE and EME is difficult due to their different physicochemical properties. Some liquid–liquid extraction procedures with organic solvents are devoted specifically to the detection of each compound separately [12]. The co-extraction of both compounds is possible with solid–liquid extraction procedures [4, 6, 10]. The different derivatization techniques used for the detection of BE and EME lead in most cases to the duplication of chromatographic systems for their detection and quantitation. Alternatively, if EME is analysed underivatized, specific extraction procedures and chromatographic systems are needed.

In the present work, we have developed an analytical method that overcomes some of the problems cited above.

Materials and Methods

Chemicals and reagents

Cocaine hydrochloride, BE and EME were given by Research Triangle Institute (RTI, Durham, NC, USA). Internal standard levallorphan bitartrate was given by Roche (Basel, Switzerland). Positive urine samples to CO were obtained from patients at the Hospital del Mar (Barcelona, Spain). Methanol, chloroform, acetone, potassium dihydrogen phosphate, potassium carbonate and hydrochloric acid were reagent grade (Merck, Darmstadt, FRG). Isopropyl alcohol and ammonium hydroxide 25% reagent grade, were supplied by Scharlau (Barcelona, Spain). Ethyliodide synthesis grade (Merck, Darmstadt, FRG), and *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, FRG) were used as derivatization reagents.

Bond Elut CERTIFY™ columns were provided by Analytichem International (Harbor

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City, USA). Deionized water was obtained in our laboratory with a MILLI-Q System (Waters, Mulheim, France).

Fluorescence polarization immunoassay (FPIA, ADX™ system) was provided by Abbott Laboratories (Irving, TX, USA).

Experimental standard solutions

Stock standard solutions of CO, BE, EME and levallorphan were prepared in methanol at a concentration of 1 mg ml^{-1} (S1). Solutions of 10 μg ml^{-1} were prepared by diluting 100 μl of S1 to 10 ml with methanol (S2). Solutions were checked by UV spectrophotometry and were stored at −20°C.

Instrumentation

Analyses were performed using a Hewlett Packard 5890A model gas chromatograph equipped with a nitrogen–phosphorus detector and interfaced with a 7673A Hewlett Packard autosampler. The separation was carried out using a cross-linked capillary column (Hewlett Packard, Palo Alto, USA) 25 m × 0.2 mm 5% phenyl-methyl silicone gum (0.33 μm film thickness). The injector (splitless mode) and detector were operated at temperatures of 280 and 290°C, respectively. Oven temperature was programmed from 150 to 280°C at a rate of 10°C min^{-1}. Helium was used as carrier gas at a flow rate of 0.75 ml min^{-1}. The detector was operated using helium as a make up gas at a flow rate of 37 ml min^{-1}. Air and hydrogen flow rates were 79 and 4 ml min^{-1}, respectively. Chromatograms were recorded on a 3392A model Hewlett Packard integrator.

A vacuum manifold to operate with solid-phase extraction columns in a semiautomatic mode was supplied by Analytichem International.

Extraction procedure

The samples were prepared by adding to 2.5 ml of urine, previously centrifuged at 3000 rpm for 10 min, 250 μl of a methanolic solution of levallorphan (S2) and 1 ml of 0.1 M phosphate buffer (pH 7). The mixture was vortexed and the pH was adjusted when necessary to 6–7.

Bond Elut CERTIFY™ columns were inserted into the vacuum manifold and conditioned by washing once with 2 ml of methanol and 2 ml of 0.1 M phosphate buffer pH 7. The columns were prevented from running dry before applying the sample. Samples were poured into each column and gently sucked through. The columns were washed with 3 ml of deionized water, 3 ml of 0.1 M hydrochloric acid and 9 ml of methanol. Elution of the analytes was performed with 2 ml of a mixture of chloroform–isopropyl alcohol (80:20%, v:v) with 2% of ammonium hydroxide. The eluates were evaporated to dryness under a gentle stream of nitrogen at room temperature.

Sequential derivatization

Ethyl ester derivative of carboxylic acid groups. 180 μl of acetone, 20 μl of ethyliodide and 10 mg of potassium carbonate were added to the dried residue and vortexed for 10 s. The tubes were incubated for 3 h at 55°C.

TMS derivatives of hydroxyl and phenolic groups. After derivatization with ethyliodide, the tubes were cooled to room temperature and 20 μl of MSTFA was added. The mixture was vortexed and incubated for 10 min at 60°C. An aliquot of 2 μl of this mixture was injected into the gas chromatograph.

Calibration procedure

Standard curves were prepared with blank human urine over the concentration range 100–2000 ng ml^{-1} for CO and BE, and over the range 250–2500 ng ml^{-1} for EME.

Known amounts of CO and BE equivalent to 100, 250, 500, 1000 and 2000 ng ml^{-1}, and ecgonine methyl ester equivalent to 250, 500, 1000, 2000 and 2500 ng ml^{-1}, were taken to dryness before adding urine (drug free).

Peak height ratios between CO, BE, EME and the internal standard were subjected to least-squares regression analysis.

Results

A higher chromatographic response factor for the BE ethyl ester derivative, prepared as described, had previously been obtained as compared with the BE trimethylsilyl derivative using MSTFA–trimethylsilyliodide–dithioerythritol (100:2:2%, v/v/v) as a silylating agent. Therefore our approach has been focused towards the ethylation of the carboxyl group. The two successive derivatization procedures described here have been shown to be compatible. Both derivatization procedures (carboxyl ethylation and *O*-silylation) can be used

sequentially to derivatize EME, BE and other CO metabolites (Fig. 1A). Additionally, CO was found to be stable during all the derivatization process. When the extraction and derivatization procedures were applied to real urine samples, good chromatograms free of interferences were obtained (Figs 1B and 1C). Relatively high recoveries for CO (87.5%) and BE (83.5%) were obtained with this extraction procedure. Best results for EME (41.5%) were obtained by reducing the volume of sample (from 5 to 2.5 ml). Some other minor metabolites of CO can also be easily detected, as shown in Figs 1A and 1C.

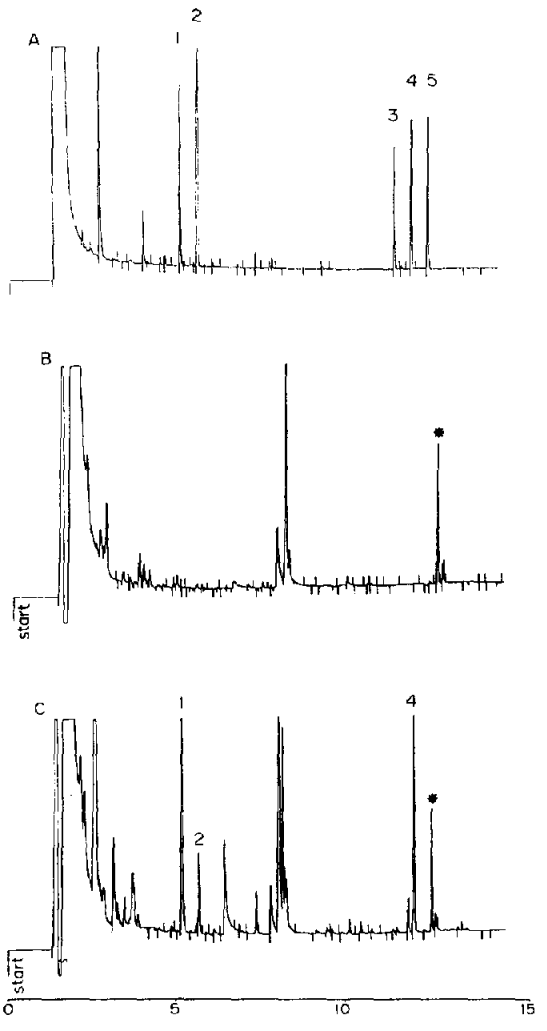

Figure 1
(A) Chromatogram of a mixture of different metabolites of cocaine (1 μg ml^{-1} of each) after selective derivatization. (B) Chromatogram of a blank urine. (C) Chromatogram of a cocaine positive urine (immunoassay technique). 1, ecgonine methyl ester (*O*-TMS); 2, ecgonine (*O*-TMS, ethyl ester); 3, cocaine; 4, benzoylecgonine (ethyl ester); 5, benzoylnorecgonine (*N*-TMS, ethyl ester); *, ISTD, levallorphan (*O*-TMS).

Good linearity (ratio to ISTD versus concentration) was obtained over the ranges studied (r = 0.998, intercept = −0.034, slope = 0.00125 for CO; r = 0.999, intercept = −0.002, slope = 0.00106 for BE; and r = 0.998, intercept = 0.029, slope = 0.001 for EME). Intraday relative standard deviations were not greater than ±3.1% for CO and BE and not greater than ±9.5% for EME at a concentration of 1000 ng ml^{-1} of each compound. Detection limit was established about 100 ng ml^{-1} for CO and BE, and 250 ng ml^{-1} for EME, based on signal to noise ratios higher than 3.

The analytical method was evaluated for BE by comparison with qualitative and quantitative results obtained by fluorescence polarization immunoassay (FPIA) in a set of urine samples routinely tested for drugs of abuse. Fifty prescreened samples for CO by FPIA were analysed in a blind mode by the present analytical procedure. There was a full agreement in a qualitative point of view in terms of presence (n = 20) or absence (n = 30) of CO consumption. From a quantitative point of view the concentrations for BE obtained by both methods were evaluated in the 20 positive samples. The results obtained (see Fig. 2) showed a good correlation (r = 0.999, intercept = 0.28, slope = 0.93).

Discussion

The modifications introduced in the extraction procedure of Analytichem (Bond Elut Cocaine Extraction Procedure, Analytichem International, 1989) increased the recovery for EME to 41.5% from an initial 10%. In order to improve the recovery of EME, different approaches including variations in pH and ionic strength of buffer used to condition the columns, and pH adjustment of samples, had been attempted. The fact that best results have been obtained just by reducing the volume of sample, indicates that most probably at higher volumes, there is a saturation of the column by interfering substances present in urine. This phenomenon is of relevance when the interaction with the solid phase is weak, as is the case of EME, because of its low hydrophobicity.

The derivatization techniques proposed have the advantage that the reagents are compatible

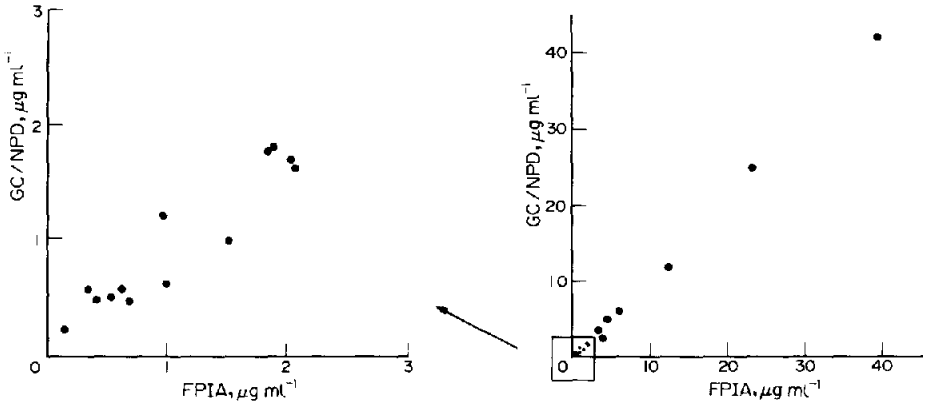

Figure 2
Correlation of quantitation for benzoylecgonine between the GC–NPD method and an immunoassay technique (FPIA).

and the main metabolites of CO can be easily derivatized and then analysed without degradation of CO. The main disadvantage is the time needed to carry out the whole derivatization procedure. In the future, particularly for the ethylation reaction, faster procedures will be examined for example using trimethylanilinum hydroxide as a reagent (TMAH derivatization of THC metabolite, Analytichem International, 1990) but maintaining the compatibility with the trimethylsylilation reaction.

The method shows good linearity and reproducibility in the range of concentrations studied for the quantitation of CO, BE and EME. The good correlation in the quantitation of BE by the present analytical technique, as compared to that obtained using the FPIA technique, and allows the present analytical procedure to be used as a confirmatory technique for presumptive immunological tests. The sensitivity achieved (100 ng ml^{-1}) for BE is good enough for the confirmation of CO abuse in drug testing when compared with the standards demanded by some regulatory agencies like NIDA (300 ng ml^{-1}) [13]. In addition, there is the advantage that the ingestion of CO can be confirmed with the simultaneous detection and quantitation of EME and the detection of some other minor metabolites. From a pharmacological point of view, more comprehensive studies on the excretion profile of CO can be undertaken applying this analytical technique.

Conclusions

The method developed is suitable for the confirmation of CO use. The method allows the detection in a unique procedure of main biotransformation products of CO: BE and EME, and some other minor metabolites. Quantitation of BE by the present method has been validated by comparison with an immunoassay highly specific for this compound. The sensitivity achieved for BE (100 ng ml^{-1}) is well above the confirmatory cut-off values of the NIDA guidelines [13].

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