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Confirmation of cocaine exposure by gas chromatography–mass spectrometry of urine extracts after methylation of benzoylecgonine

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Abstract

Volatility and thermal stability are necessary physical–chemical properties for analysing a substance by gas chromatography. A derivatization step is required before gas chromatography of benzoylecgonine (the main metabolite of cocaine). In the literature, reactions such as silylation, perfluoroalkylation or alkylation are the most frequently used to derivatize benzoylecgonine. However, they allow the formation of products sensitive to moisture or require a purification step. So, a procedure to derivatize benzoylecgonine with diazomethane before gas chromatographic analysis was evaluated. A study was performed to evaluate the efficiency of conversion of benzoylecgonine in cocaine, the necessary time for reaction and the stability of ethereal solution of diazomethane. The reaction was shown to be very fast in mild conditions and there was no need for a further purification step. When benzoylecgonine was extracted from urine by solid-phase extraction and derivatized with diazomethane, concentrations as low as 25 ng/ml could be detected using GC–MS in the full scan mode.
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1. Introduction

Methods for the analyses of benzoylecgonine, the main cocaine metabolite found in urine have been proposed, employing different techniques such as immunoassay [1], thin-layer chromatography [2], gas chromatography (GC) [3], liquid chromatography [4,5] and gas chromatography–mass spectrometry (MS) [6,7].

Automated immunoassays (enzyme immunoassay and fluorescence polarization immunoassay) are the most common techniques in screening benzoylecogonine due to their simplicity, rapidity and no sample pre-treatment needed. Confirmation of positive results from screening analyses must be performed by GC–MS [8]. However, the hydrophilic and amphoteric properties of benzoylecgonine make the gas chromatographic analysis of this substance difficult. Conventional liquid–liquid extraction does not provide good recovery. In the last years, solid-phase extraction (SPE) has been the technique of choice for benzoylecgonine analysis. Clean extracts, good recovery and rapidity in the detection procedure were reported after SPE use [5,9]. For derivatization of benzoylecgonine, silylation, perfluoroalkylation and alkylation are the most commonly used techniques. Both silylation and perfluoroalkylation procedures are simple and rapid and there is no need for further purification of the product obtained. Nevertheless, silyl derivatives of benzoylecgonine are moisture sensitive [10,11] and

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the stability of perfluoroalkyl derivatives do not exceed 48 h [6]. The decomposition of derivatives can be a disadvantage considering the increasing use of autoinjectors and the large number of samples in a GC–MS batch analyses [11]. Other alkyl derivatives of benzoylecgonine are more stable and have good chromatographic characteristics as well. Derivatization with dimethylformamide-dialkylacetal, alkyl iodides or alcohol and acids have been proposed in the literature to form alkyl derivatives. For applying these techniques an additional purification step of the formed product is required and very low yields of alkylation with alkyl iodides ranging from 32 to 64% for propylbenzoylecgonine and from 13 to 22% for isopropylbenzoylecgonine are obtained [11]. Furthermore, approximately 14–42% of the products are decomposed during purification. The same authors proposed a one-step esterification of benzoylecgonine with dimethylformamide-dialkylacetal in the presence of pyridine at 100°C for 30 min. The developed procedure is very simple because no extra step of re-extraction is necessary.

Another alternative technique to avoid the purification step is the use of extractive alkylation solvent. By this method, acidic substances such as benzoylecgonine can be extracted and derivatized simultaneously due to the presence of an alkyl halide in the organic phase [12]. Its limitation is related to the possibility of competition between the analyte and other anions for the phase transfer reagent [13].

Diazomethane is another useful reagent for the methylation of a wide variety of substances containing an active hydrogen [14]. The reaction is very fast, takes place in mild conditions and produces minimal by-products. In the study presented the advantages and disadvantages of the use of diazomethane to convert urinary benzoylecgonine in cocaine for further GC–MS analysis were evaluated. Benzoylecgonine isopropyl ester was used as internal standard (I.S.).

2. Experimental

2.1. Chemicals, reagents and solid-phase columns

N-Nitroso-*n*-methyl-4-toluenesulfonamide, used to prepare ethereal diazomethane solution was purchased from Fluka (Buchs, Switzerland). Diazomethane solution was prepared according to Vogel [15]. Benzoylecgonine, benzoylecgonine isopropyl ester and cocaine solutions (1 mg/ml) were purchased from Radian International (Austin, TX, USA). Other reagents of common use in the laboratory were of analytical grade purchased from Merck (Darmstadt, Germany).

Solid-phase columns (Bond Elut Certify) were obtained from Varian (Harbor City, CA, USA).

2.2. Preparation of standard solutions

Working solutions of benzoylecgonine, benzoylecgonine isopropyl ester and cocaine at concentrations of 10 μg/ml were prepared in acetonitrile with volumetric glassware and stored at −20°C when not in use.

2.3. Instrumentation

GC–MS analyses were performed on a Carlo Erba 8000 series gas chromatograph coupled with a Fisons TRIO 1000 mass spectrometer. Chromatographic separation was achieved on a CP-Sil 8 CB Chrompack fused-silica capillary column (15 m×0.25 mm, 0.10 μm film thickness) using helium as carrier gas. The injector port and interface temperatures were 250°C. The oven temperature was maintained at 100°C for 1 min and was increased to 250°C at a rate of 20°C/min. The final temperature was held for 1 min.

The mass spectrometer was operated in the scan mode, from 50 to 340 u and autotuned daily with perfluorotributylamine. Identification of cocaine was based on comparison of relative retention time to benzoylecgonine isopropyl ester and the similarity (match index) with the spectrum of cocaine standard.

2.4. Reaction time for derivatization of benzoylecgonine with diazomethane

Diazomethane (100 μl) was added to dried benzoylecgonine (7.5 μg) and benzoylecgonine isopropyl ester (7.5 μg) in 12 vials. The reaction mixture was kept at room temperature in closed vials for 1, 5, 10 and 15 min. The reaction was conducted in triplicate for each reaction time. Immediately after

the time proposed, the excess reagents were evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1 ml of methanol and 1 μl was injected into the GC–MS system. The area ratio of ions 182 (cocaine) and 210 (benzoylecgonine isopropyl ester) was calculated.

2.5. Efficiency of benzoylecgonine conversion in cocaine

Diazomethane (100 μl) was added to dried benzoylecgonine (7.5 μg) and benzoylecgonine isopropyl ester (7.5 μg) in six vials. The reaction mixture was kept at room temperature in closed vials for 1 min. After the time of reaction, the excess of reagents was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 1 ml of methanol and 1 μl was injected into the GC–MS system. The area ratios of ions 182 (cocaine) and 210 (benzoylecgonine isopropyl ester) were calculated for each replicate.

At the same time, 7.5 μg of dried cocaine and benzoylecgonine isopropyl ester were reconstituted with 1 ml of methanol in six vials. A 1-μl volume of this solution was injected into the GC–MS system. The area ratios of ions 182 and 210 were calculated for each replicate. The percentage of efficiency of benzoylecgonine conversion in cocaine was evaluated by comparison of the average of area ratio obtained from methylation of benzoylecgonine and the average of area ratio obtained from the cocaine solution at the same concentration.

2.6. Study of stability of ethereal solution of diazomethane

The ethereal solution of diazomethane was stored at −20°C in amber flask. Weekly, 100 μl of diazomethane solution was added to dried benzoylecgonine (7.5 μg) and benzoylecgonine isopropyl ester (7.5 μg) in triplicate. After 1 min at room temperature, the excess of reagents were evaporated to dryness and reconstituted with 1 ml of methanol. A 1-μl volume of this solution was injected into the GC–MS system. The average of area ratios of ions 182 and 210 was calculated for each week.

2.7. Sample preparation

Solid-phase extraction (SPE) was performed according to manufacturer's recommendations. The column was preconditioned with 2 ml of methanol and 2 ml of phosphate buffer (pH 6.0). To 2.5 ml of urine, distilled water (2.5 ml), 0.1 *M* phosphate buffer, pH 6.0 (2 ml) and 75 μl of I.S. solution (containing 10 μg/ml of benzoylecgonine isopropyl ester) were added. The mixture was transferred to the column that was further washed with 6 ml of deionized water, 3 ml of 0.1 *M* HCl and 9 ml of methanol. The elution was performed with 2 ml of methylene chloride–2-propanol–ammonium hydroxide (80:20:2, v/v). The eluate was evaporated at 40°C under a gentle stream of nitrogen. To the dried extract, 100 μl of diazomethane solution was added and the mixture was kept at room temperature for 1 min. The excess reagents were evaporated. The residue was reconstituted in 100 μl of methanol and 1 μl was injected into the GC–MS system.

2.8. Intra- and inter-assay precision

Precision, defined as the relative standard deviation, was determined by intra- and inter-assays [16]. Intra-assay precision was performed by analysing samples of urine spiked with benzoylecgonine (150 ng/ml) in six replicates at a same day. Inter-assay precision was performed by analysing samples of urine spiked with benzoylecgonine (150 ng/ml) in six replicates during 6 days.

2.9. Limit of detection

The limit of detection (LOD) was determined by an empirical method that consists of analysing a series of urine samples containing decreasing amounts of benzoylecgonine [17]. The LOD was the lowest concentration value at which the results complied with the predetermined acceptance criteria (variation of relative retention time less than 1% and accordance of spectrum better than 70% in six replicates when compared with the cocaine standard spectrum in a database).

Table 1
Reaction time for derivatization of benzoylecgonine with diazomethane

Time (min)	Average of area ratios 182/210
1	0.510
5	0.503
10	0.505
15	0.509

3. Results

No differences were observed among the evaluated times for conversion of benzoylecgonine to cocaine. Therefore, the minimum time (1 min) was considered to be sufficient to complete the reaction. With this reaction time approximately 95% of benzoylecgonine is converted to cocaine with diazomethane. The averages of area ratios obtained from each reaction time are shown in Table 1.

The ethereal solution of diazomethane showed to be stable at the storage conditions. During 4 months in which the stability tests were performed, variations of less than 10% were observed in the efficiency of methylation of benzoylecgonine.

Fig. 1 shows a chromatogram obtained with the analysis of an urine sample spiked with benzoylecgonine (150 ng/ml) and the spectrum of the cocaine formed by methylation (A) and the spectrum of benzoylecgonine isopropyl ester (B).

The RSDs obtained in the study of intra- and inter-assay precision were 4.3 and 7.0%, respectively. The LOD of benzoylecgonine in urine derivatized to cocaine was 25 ng/ml.

4. Discussion

Derivatization reaction is a very important step for the analyses of polar compounds by GC–MS. The choice of the derivatization reagent can determine the practicability and the efficiency of the method. Reactions that need purification of the product are sometimes laborious, prolong analysis time and show low recovery. Other derivatization reactions do not need removal of reagents before injection, what means time savings. It is the reason why silyl derivatives, that do not require re-extraction steps neither removal of reagents, are frequently used to analyse benzoylecgonine.

The use of diazomethane as reagent to derivatize benzoylecgonine is not frequently mentioned in the literature [18,19]. However, some special characteristics can be observed in this type of derivatiza-

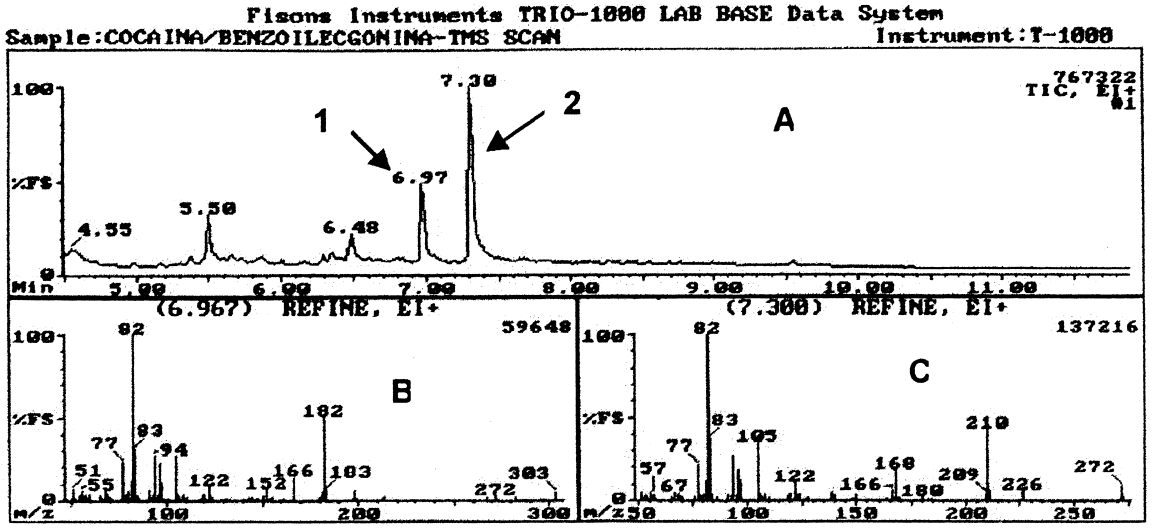

Fig. 1. (A) Chromatographic profile obtained by the analysis of a urine sample spiked with benzoylecgonine (150 ng/ml). (1) Peak corresponding to cocaine, (2) peak corresponding to benzoylecgonine isopropyl ester (I.S.). Mass spectrum of cocaine formed by methylation of benzoylecgonine with diazomethane (B) and spectrum of benzoylecgonine isopropyl ester (C).

tion: the product of the reaction is cocaine, which presents well known analytical and physical–chemical properties; there is no need for further purification of the product; the by-product of the reaction is nitrogen and the excess of ethereal diazomethane solution can be easily removed by evaporation. Furthermore, the reaction is very fast and occurs under mild conditions with an excellent yield. In our experiments, 1 min was enough to convert approximately 95% of benzoylecgonine to cocaine at room temperature.

Nevertheless, some care must be taken at manipulating with this reagent. Diazomethane is a toxic yellow gas which can explode in contact with ground glasses or when heated above 90°C [14]. It can be decomposed if water or alcohol is present. The decomposition of diazomethane can occur by the action of light and heat as well and therefore its ethereal solution must be stored in well-closed amber flask at low temperatures. Under these conditions, stability for more than 4 months was verified.

The LOD of benzoylecgonine in urine (25 ng/ml) is below of the reference value (cut off) recommended by the European Union Toxicology Experts Working Group [8] for the confirmation of urinary benzoylecgonine (150 ng/ml). The use of SPE followed by derivatization of benzoylecgonine with diazomethane proved to be very fast and highly precise (RSD<8%) and can be useful to confirm the cocaine exposure by GC–MS.

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