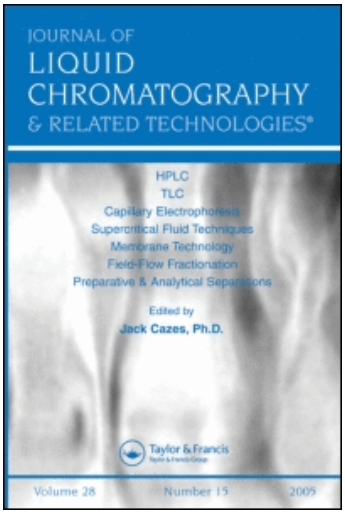

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Use of High Performance Liquid Chromatography for the Determination of Cocaine and Benzoylecgonine in Human Hair

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ABSTRACT

A high performance liquid chromatographic method involving diode array detection (HPLC–DAD) is proposed for the determination of cocaine and benzoylecgonine in human hair. Samples were washed, dried, and subjected to enzyme hydrolysis and liquid–liquid extraction in Toxitubes A® prior to analysis on a Nova-Pak C18 (150 × 3.9 mm) column with a 70 : 30 (v/v) methanol–0.02 M phosphate buffer pH 7, at a constant flow-rate of 0.4 mL/min as the mobile phase, and articaine as reference compound. The detector response was linear over the concentration range 2–200 ng/mg hair for both drugs. The detection and quantitation

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limits were 1 ng/mg and 2 ng/mg, respectively. The precision was quite acceptable, with coefficients of variation less than 5%. An overall 52 specimens from cocaine users were analysed with the proposed method and found to contain cocaine and benzoylecgonine concentrations over the ranges 4.2–554.7 ng/mg (average 57.3 ng/mg) and 0–1167.6 ng/mg (average 159.7 ng/mg), respectively.

Key Words: HPLC–DAD; Cocaine; Benzoylecgonine; Hair.

INTRODUCTION

Many drugs can be entrapped and retained in a stable manner by the keratin matrix of hair, even after low doses.[1] The mechanisms via which drugs can be incorporated into hair include passive diffusion from blood to developing hair cells at the base of the follicle; transfer from sweat and sebaceous and apocrine glands after the final hair has formed,[2] external contamination from ambients where drugs are smoked or analysed in large batches; and diffusion from blood and sweat to the skin.

Technically, hair is not any more difficult to analyse than are other biological matrices. Unlike urine, samples cannot be diluted by drinking large amounts of liquids. These advantages have fostered the use of drug tests for hair as routine procedures for detecting the presence of drugs even months after use; the results have been used in clinical, forensic, epidemiological, and historical research studies, as well as presented as evidence before civil, criminal, and military courts. Cocaine and other drugs in hair were initially detected by radioimmunoassay (RIA);[3,4] this technique, however, cannot discriminate the drugs from their metabolites. In subsequent years, the gas chromatography–mass spectrometry (GC–MS) gained ground for this purpose.[5–9] By contrast, high performance liquid chromatography (HPLC) has scarcely been used in this context.[10–12]

In this work, we developed an HPLC method using a diode array detector (DAD) for the determination of cocaine and its main metabolite, benzoylecgonine (BEG), in hair, and applied it to the analysis of samples from cocaine users.

EXPERIMENTAL

Chemicals

Cocaine hydrochloride and benzoylecgonine were purchased from Sigma–Aldrich (Madrid, Spain). Articaine hydrochloride was obtained from

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Inibsa, S.A. (Barcelona, Spain). Pronase E, 1,4-dithioerythritol (DTT), methanol Lichrosolv, trihydroxymethylaminomethane (Tris), hydrochloric acid, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were supplied by Merck (Darmstadt, Germany). Finally, Toxitubes A® were obtained from Varian (Lake Forest, USA).

A 0.02 M phosphate buffer of pH 7 was made by adding 320 mL of 0.02 M dipotassium hydrogen phosphate to 680 mL of 0.02 M potassium dihydrogen phosphate. A 0.1 M Tris buffer of pH 7.2 was made by mixing 50 mL of a 12.1 mg/mL solution of Tris with 44.7 mL of 0.1 N HCl and making to 100 mL with Milli-Q water.

Pronase and DTT solutions containing a 2 and 12 mg/mL concentration, respectively, in Tris buffer of pH 7.2 were prepared.

Standards and Controls

Standards containing 1 mg/mL cocaine, benzoylecgonine, or articaine in methanol were used to obtain working-strength solutions containing 0.1, 0.5, 1, 2, 5, and 10 μg/mL cocaine or BEG, as well as 10 μg/mL articaine, which was used as a reference compound. One-milliliter aliquots of these solutions were spiked into blank hair samples (50 mg) to obtain controls of 2, 10, 20, 40, 100, and 200 ng/mg hair, according to the equation:

$$\text{Hair conc. (ng/mg)} = \frac{\text{methanol conc. (μg/mL)} \times 10^3 \times \text{methanol solution vol. (1 mL)}}{\text{hair weight (50 mg)}}$$

Chromatographic Conditions

A Waters HPLC System (Milford, MA) consisting of a Model 717 Plus autosampler, a Model 616 pump, and a Model 996 DAD was used for chromatographic measurements. Data were analysed by using Millennium 32 system software, vs. 3.05.01 for Windows 98. Analyses were conducted at room temperature on a 150 × 3.9 mm ID Nova-Pak C18 column (particle size 5 μm), using 70 : 30 (v/v) methanol–0.02 M phosphate buffer of pH 7, at a flow-rate of 0.4 mL/min, as the mobile phase. The injected sample volume was 25 μL and detection was performed at 231 nm by scanning the wavelength range from 200 to 400 nm.

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Hair Specimens

Hair specimens were obtained from individuals brought before the judge and were submitted by the courts of first instance to the Institute of Legal Medicine of Santiago de Compostela, to check for the presence of cocaine or other abuse drugs. Based on the recommendations of the Society for Hair Testing, hair (100–200 mg) was cut as close to the scalp (occipital region) as possible in order to span as long an exposure period as possible—hair grows approximately 1 cm per month. Samples, thus obtained, can be stored in tubes or bags at room temperature.

Sample Processing

Hair specimens were placed in glass tubes, washed with neutral liquid soap, and rinsed with distilled water several times to remove any drug potentially introduced through passive contamination, as well as dirt and grease on the outer layer of hair. Samples were then dried in a stove at 40°C for 24 h and hairs cut into segments 2–3 mm long to expose as much cortex and marrow (where drugs are retained) to the action of the extracting solvent. An amount of 50 mg of each sample was mixed with 500 μL of DTT and placed in a stove at 37°C for 2 h. Then, 500 μL of pronase solution was added and the tube was kept closed in the stove at 37°C for a further 12 h. The liquid obtained upon enzymatic digestion was subjected to liquid–liquid extraction in Toxitubes A and the organic layer was transferred into a conical-bottom flask, evaporated to dryness in a bath at 60°C under a nitrogen stream, and reconstituted in 1 mL of a 10 μg/mL articaine solution used as reference compound. The mixture components were thoroughly mixed by shaking and a 25 μL aliquot of the solution was injected into the chromatograph.

RESULTS

We initially identified the analytes from their UV spectra (λ_{max}: 231 nm for cocaine and 232 nm for BEG) and retention times (ca. 3.4 min for BEG, 5.5 min for articaine, and 8.6 min for cocaine). Figure 1 shows a 3-D representation (time × wavelength × absorbance units) obtained at a 10 μg/mL concentration of each analyte in methanol (200 ng/mg hair).

The above-described procedure was applied to 10 specimens of drug-free hair from individuals who had neither used nor been in contact with cocaine in order to confirm the absence of compounds that might interfere with the method; in fact, only the reference compound (articaine) was detected.

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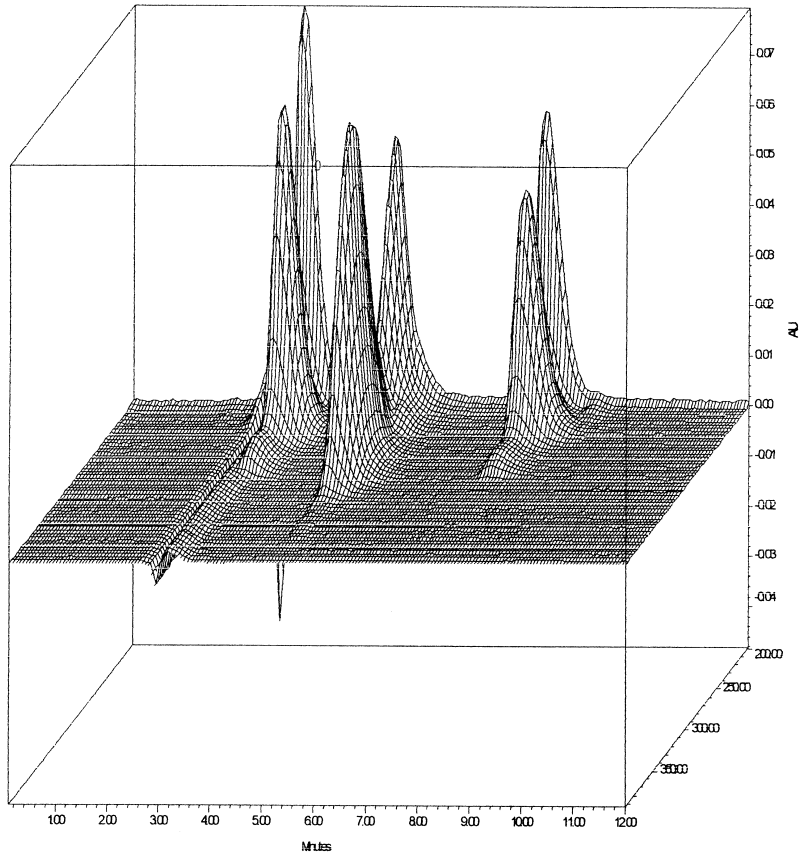

Figure 1. 3-D representation (time × wavelength × absorbance units) of BEG (3.4 min), articaine (5.5 min), and cocaine (8.6 min). Drugs concentration: 200 ng/mg hair.

Calibration curves were obtained by plotting the drug-to-articaine area ratio against the drug concentration over the range 0.1–10 μg/mL methanol (2–200 ng/mg hair):

$$\text{Cocaine: } y = 0.3305x + 0.0093 \quad R^2 = 0.9999$$

$$\text{BEG: } y = 0.8651x + 0.0277 \quad R^2 = 0.9997$$

According to the criteria of Bressolle et al.,[13] linearity of the method was demonstrated by showing that the slopes were statistically different from 0, the

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intercepts were not statistically different from 0, and the regression coefficients were not statistically different from 1.

The sensitivity of the method was assessed via the limit of detection (LOD) and limit of quantitation (LOQ). They were determined experimentally,[14] obtaining LOD values of 1 ng/mg and LOQ values of 2 ng/mg for both drugs.

The precision refers to reproducibility of the results; it was expressed as the coefficient of variation at each concentration level, which was obtained in sextuplicate and ranged from 1.79% to 4.53% for cocaine, and from 1.69% to 4.87% for BEG.

In order to avoid false positives due external contamination, several hair specimens from non-users of cocaine working at a laboratory that analysed large batches of seized cocaine daily were also studied. The specimens were washed as described above and the last washings were analysed for cocaine. Tests were all negative, which confirms the effectiveness of the washing procedure used.

Finally, 52 real samples from cocaine users (86.5% males and 13.5% females) were analysed and the results shown in Table 1.

DISCUSSION

The use of a DAD in the proposed HPLC method makes it highly specific. In fact, it allows the wavelength range from 200 to 400 nm to be scanned, and hence, three-dimensional chromatograms showing absorbances and wavelengths against time to be obtained. In addition, a spectral library for the studied substances was constructed and subsequently used for their unequivocal identification. Consequently, the proposed method allows cocaine and BEG to be detected not only from their retention times, but also from their UV spectral data; this facilitates the detection of possible impurities and/or coextracted drugs with retention times similar to those of cocaine and BEG, but with spectra clearly departing from theirs.

The reference compound used should not be one of the substances typically used to adulterate the drug; also, it should be chromatographically similar to the analytes but possess a different retention time in order to avoid interferences with their analyses. The most effective choice for this purpose was articaine.

The precision results were good. In fact, the coefficients of variation never exceeded 5%; the highest value (4.87%) corresponded to a 10 ng/mg concentration of BEG.

Before cocaine and BEG in hair can be determined, both drugs must be isolated from the keratin matrix that retains them. The conditions used to

Table 1. Concentrations in hair specimens.

Cases	Cocaine (ng/mg)	BEG (ng/mg)	Cases	Cocaine (ng/mg)	BEG (ng/mg)
1	7.5	43.4	27	554.7	629.4
2	244.2	28.4	28	7.4	—
3	9.9	16.1	29	81.4	—
4	113.8	87.7	30	6.9	137.6
5	18.4	76.1	31	9.7	86.5
6	7.7	—	32	10.6	—
7	64.1	129.6	33	175.0	—
8	7.1	148.5	34	10.3	215.3
9	42.6	53.8	35	204.6	—
10	9.3	—	36	5.6	19.4
11	151.6	—	37	40.2	308.7
12	23.9	—	38	10.1	—
13	26.0	—	39	71.6	947.7
14	146.9	—	40	14.2	276.1
15	70.1	—	41	108.5	—
16	11.9	181.7	42	25.6	170.3
17	115.9	236.2	43	4.5	—
18	9.7	—	44	4.7	522.4
19	13.7	68.6	45	14.6	176.1
20	77.1	—	46	13.6	464.0
21	80.9	594.5	47	17.3	—
22	18.2	434.4	48	22.1	712.9
23	71.9	—	49	4.4	—
24	130.8	1167.6	50	10.6	317.3
25	52.0	54.6	51	5.1	—
26	4.2	—	52	16.7	—

release them should facilitate their extraction from the sample without causing their alteration; this is rather difficult as cocaine is a very labile, readily hydrolysed drug. Usually, the analytes are isolated by direct extraction with an organic solvent, after degradation with a strong acid or base or enzymatic digestion of the keratin using a proteolytic enzyme such as pronase,[15] proteinase K,[16] or β-glucuronidase arylsulphatase.[17] In this work, we used DTT and pronase,[18] which avoid degradation of cocaine and allow hair to be thoroughly dissolved and the drug to be fully released as a result. The liquid–liquid extraction procedure used provided adequately clean extracts, as confirmed by analyses of several drug-free specimens.

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Finally, the proposed method was applied to 52 hair specimens from cocaine users. Preliminary RIA tests were all positive for cocaine and revealed the concomitance of opiates in 43 cases. Additional abuse substances were rarely encountered: there were only four positive tests for benzodiazepines, four for methadone, and one for cannabis. The cocaine doses and alterations of its kinetics, because of presence of these drugs, accounts for the high dispersion in the results. In fact, the concentrations in hair ranged from 4.2 to 554.7 ng/mg (average 57.3 ng/mg) for cocaine and from 0 to 1167.6 ng/mg (average 159.7 ng/mg) for BEG; these values are comparable to those previously found by Tagliaro et al.[19] All samples tested positive for cocaine, whereas only 29 tested positive for BEG. This prevalence of cocaine over its main metabolite is consistent with previous findings of Kintz and Manguin,[20] but not with those of Moeller et al.,[21] who detected both cocaine and BEG in all their positive cases. However, in 27 of the 29 cases where both analytes were present in our specimens, BEG was found at higher levels than cocaine. The BEG/cocaine ratio ranged from 1.05 to 111.15 (average 15.56).

In interpreting the results, one must take into account that the extent to which the drug incorporates into hair is a function of its lipophilic character, based on which cocaine should be present in greater amounts than BEG. However, the opposite may hold if the individuals concerned use alkaline soaps for their personal hygiene as these hydrolyse cocaine,[20] or if a long enough time for the cocaine to be metabolized to BEG has elapsed.

The proposed HPLC–DAD method has some advantages over existing RIA and GC–MS alternatives. Thus, RIA does not allow cocaine to be discriminated from its metabolites, which is indispensable with a view to distinguishing drug use from external contamination; also, cross-reactions between the analytes and antiserum can lead to false positives. On the other hand, GC–MS is a more expensive technique that usually requires a prior derivatization of the analytes and only allows the determination of volatile substances. The use of the DAD allows one to determine cocaine and BEG without previous derivatization, improving the method sensitivity as it facilitates selection of the optimum wavelength for maximizing the chromatographic response of the two analytes (viz. 231 nm); also, it gives a high specificity to the proposed method as it provides information about the purity of the corresponding peaks.

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