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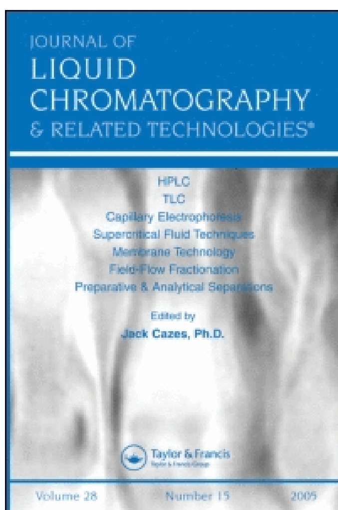

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Bile Analysis for Cocaine and Benzoylecgonine in Overdose Cases

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Abstract: A method for determining cocaine and benzoylecgonine (BEG) in human bile using high performance liquid chromatography (HPLC) with ultraviolet detection at 235 nm is proposed. The method uses a Lichrospher RP18 column and methanol-phosphate buffer as mobile phase. Following solid phase extraction with Bond Elut Certify cartridges, the linearity of the method was examined over the analyte concentration range 0.125–5 μg/mL in bile. The precision of the method was acceptable, with coefficients of variation less than 5%. The average extraction yield was 82% for cocaine and 76% for BEG. The proposed method was applied to 30 bile samples from individuals fatally poisoned with cocaine.

Keywords: Benzoylecgonine, Bile, Cocaine, HPLC, Solid-phase extraction

INTRODUCTION

Although the number of deaths caused by cocaine alone is small, abuse of this drug is the origin of many cases of acute and chronic poisoning, which have grown substantially in number in recent years, especially among the young.

Cocaine overdose is diagnosed largely from laboratory tests. In fact, a number of procedures exist for identifying and quantifying cocaine and

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its metabolites in biological samples;[1–4] few such procedures, however, are carried out in bile.

Bile is the principal vehicle for the release of many substances including cocaine. Its aqueous nature facilitates the release of water soluble substances and the bile acids it contains increase the solubility of lipophilic substances.[5] This makes bile useful for detecting a wide variety of substances, or even as a screening fluid in the absence of urine and/or blood samples.[6] Experiments with bile have shown that the concentrations of cocaine and its metabolites in it are significantly higher than those in blood. Some authors even claim that negative tests for the drug in blood should be confirmed with a bile test.[7–9]

In this work, we developed a method for determining cocaine and its metabolite benzoylecgonine (BEG) in bile, using solid phase extraction with Bond Elut Certify cartridges in combination with high performance liquid chromatography (HPLC) for quantitation.

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EXPERIMENTAL

Reagents

Standards containing 1 mg/mL cocaine, BEG or methaqualone in the mobile phase were used to prepare working strength solutions containing 1, 10, and 100 μg/mL cocaine and BEG, and 1 μg/mL methaqualone. All solutions were stored refrigerated at 4°C prior to use.

Collection and Treatment of Samples

Real bile samples were obtained by direct puncture of the biliary vesicle during the course of autopsies performed at the Institute of Legal Medicine of Santiago de Compostela (NW Spain) and stored frozen at −20°C until analysis.

In each run, a volume of 1 mL of bile was supplied with 1 mL of Milli-Q water, sonicated for 10 min, and centrifuged at 2500 rpm for 10 min. The supernatant thus obtained was used to prepare the calibration solutions.

Chromatographic Conditions

The chromatographic system consisted of a Model 717 Plus autosampler, a Model 616 pump, a Lichrospher RP18 stainless steel column (125 × 4 mm id, 5 μm particle size), a Model 490 UV detector operated at

235 nm, and a 70:30 v/v mixture of methanol and 0.02 M phosphate buffer at pH 7 as mobile phase. The flow gradient used was as follows (time, flow rate): 0 min, 0.4 mL/min; 3 min, 0.4 mL/min; 6 min, 0.7 mL/min; 8 min, 1.0 mL/min; 10 min, 0.7 mL/min; 11 min, 0.6 mL/min; and 12 min, 0.4 mL/min.

Procedure

The detector response was studied by injecting 25 μL volumes of solutions containing cocaine and BEG at concentrations of 0.5, 1, 2, 5, 10, and 20 μg/mL in the mobile phase plus a 1 μg/mL concentration of methaqualone (compound of reference).

The standard solutions of cocaine and BEG at concentrations of 0.125, 0.25, 0.5, 1.25, 2.5, and 5 μg/mL in bile were prepared and extracted using Bond Elut Certify cartridges that were preconditioned with 2 mL of methanol and 2 mL of phosphate buffer at pH 6. A volume of 0.2 mL of bile plus 1.8 mL of Milli-Q water was added and then, 3 mL of water, 3 mL of 0.1 N HCl, 9 mL of methanol, and 3 mL of 0.3 M ammonia was passed. After applying vacuum for 5 min, the cartridge was eluted with 2 mL of 4:1 v/v chloroform-isopropanol and evaporated to dryness with a nitrogen stream, the extract being reconstituted in 50 μL of a 1 μg/mL methaqualone solution in the mobile phase. Because the initial bile volume was 200 μL and the dry extract reconstituted in 50 μL, the postextraction solutions were 4 times more concentrated than the initial solutions. Therefore, the final concentrations were 0.5, 1, 2, 5, 10, and 20 μg/mL. An aliquot of 25 μL was injected into the chromatograph in each of the six runs performed at each concentration level and analyte to metaqualone area ratios were plotted against postextraction concentrations.

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Validation of the Method

We examined the linearity of the proposed method for cocaine and BEG in the mobile phase and bile, using the least squares method to construct regression lines of the type $y = ax + b$. Linearity was assumed when the slope of the calibration curve, a, was statistically non-zero, the independent term, b, was not statistically different from zero and the correlation coefficient was not significantly different from unity.[10]

The limit of detection (LOD) is the lowest analyte concentration in a sample that can be distinguished with a probability of 95% from the blank signal under the experimental conditions used. The limit of quantitation (LOQ) is the lowest analyte concentration, which

can be determined with acceptable accuracy and precision under the experimental conditions established; such a concentration has been identified with the lowest level in the calibration curve.

The between-day precision of the proposed method was evaluated by conducting reproducibility tests at two concentration levels (1 and 10 μg/mL, $n = 6$) and expressed as relative standard deviations or coefficients of variation. The accuracy of the analytical method, a measure of its ability to provide results as close as possible to the true value, was also calculated at two concentration levels (1 and 10 μg/mL, $n = 6$).

RESULTS AND DISCUSSION

High performance liquid chromatography in combination with UV detection is especially useful and usually simpler than gas chromatography to determine cocaine and its metabolites.[11] Injected volumes are larger but the samples require less pretreatment, which allows compounds of low volatility to be more expeditiously detected. The principal shortcoming of HPLC-UV is the absence of a useful absorption band for determining other metabolites such as ecgonine methyl ester.

The chromatographic conditions were optimized for the identification and quantitation of cocaine and BEG in bile. The compound of

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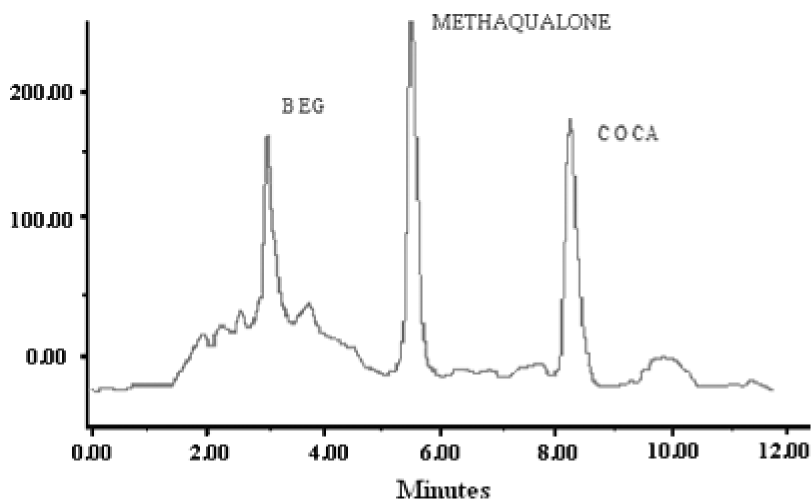

Figure 1. Chromatogram for a bile sample containing 5 μg/mL cocaine (COCA) and benzoylecgonine (BEG). Compound of reference: methaqualone.

Table 1. Figures of merit of the regression analysis

	a	*b*	*r*
Cocaine in mobile phase	0.1726	−0.0179	0.9998
BEG in mobile phase	0.3469	−0.0187	0.9998
Cocaine in bile	0.1464	−0.0247	0.9901
BEG in bile	0.2446	0.0156	0.9977

reference used, methaqualone, is not present in biological fluids and is chromatographically similar to the target analytes but differs from both in the retention time. The sequence of appearance in the chromatogram was BEG (3.81 min), methaqualone (5.61 min), and cocaine (8.38 min); the overall analysis time was 12 min (Figure 1).

The calibration curves for cocaine and BEG in mobile phase were linear over the range 0.5–20 μg/mL (Table 1). The analyses in bile were done by using a solid phase extraction to remove potential interferences; the calibration curves for cocaine and BEG were linear over the range 0.125–5 μg/mL, which corresponds to postextraction concentrations from 0.5 to 20 μg/mL (Table 1).

The precision was acceptable because the coefficients of variation never exceeded 5%.[10] This was also the case with the accuracy, which was less than 3% (Table 2). The limits of detection and quantitation for cocaine and BEG (Table 2) were slightly higher than those previously obtained by Soriano et al.,[12] but still acceptable based on the concentrations obtained in the real samples from our series and others.[13,14]

Drug recovery was examined at two different concentration levels (1 and 10 μg/mL) and amounted to $81.97 \pm 4.20\%$ for cocaine and $75.75 \pm 3.55\%$ for BEG; both values are comparable to those reported by other authors.[12]

The proposed method was applied to 30 bile samples from individuals fatally poisoned with cocaine, where males prevailed (83%) and the average age was 30 years. Figure 2 shows the chromatogram for a selected real sample of bile. Most of the individuals were addict to parenteral drugs and used one or more drugs in addition

Table 2. Validation of the proposed method

	CV (%)	Accuracy (%)	LOD (μg/mL)	LOQ (μg/mL)
Cocaine	4.54	+2.75	0.037	0.125
BEG	4.89	+0.70	0.033	0.125

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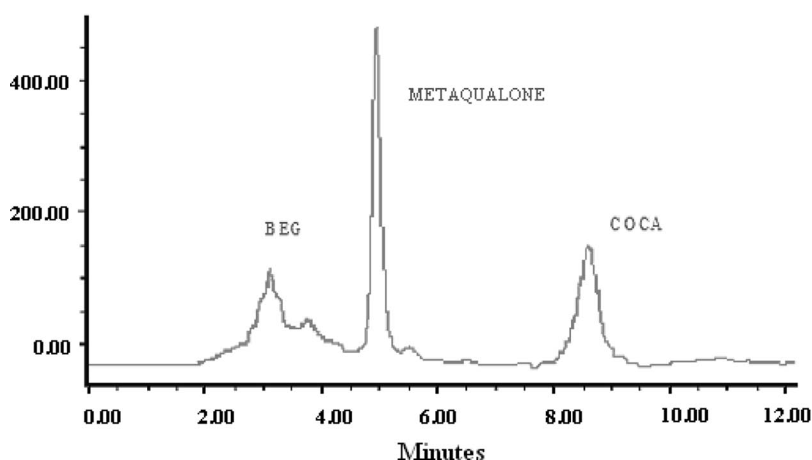

Figure 2. Chromatogram for a real bile sample.

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to cocaine. Thus, 67% were found to have consumed morphine, 60% benzodiazepines, 20% alcohol and methadone, and 17% cannabis. The high variability in the concentrations found may have resulted from the presence of other drugs, which can alter the metabolism of cocaine.

Table 3 shows the results for our series. The BEG concentration exceeded the cocaine concentration in 83% of cases, and the BEG/COCA ratio as calculated from the average levels of the two substances was 3.4, which is consistent with the fast metabolism of cocaine in the liver. The cocaine concentrations for our series are similar to other previously reported values,[9,15,16] but different from those obtained by other authors.[8,17,18]

Blood samples from 15 individuals fatally poisoned with cocaine in our series were analyzed by radioimmunoassay in parallel to the bile samples. The drug levels in bile invariably exceeded those in blood; the ratio between the two ranged from 2.2 to 470.6 (average 64.8). These results are consistent with those obtained by other authors, who assigned bile a greater diagnostic value than blood in order to identify cocaine fatal poisoning.[9]

Table 3. Drug concentrations found in 30 real bile samples

	Positive cases	Mean ± SD (μg/mL)	Maximum (μg/mL)	Minimum (μg/mL)
Cocaine	27	0.50 ± 0.38	1.81	0.13
BEG	30	1.71 ± 1.44	5.43	0.15

Extrapolating the drug concentrations in postmortem samples to those present at the time of death, and correlating them with the final effects of the drug at such a time is rather complicated for both toxicologists and pathologists.[19] This is a result of the frequent unavailability of data such as the dose and time elapsed between intake and death, and also between death and sample collection. Also, the response of individuals varies widely depending on the degree of tolerance developed during chronic use of the drug.

CONCLUSION

Based on the results for 30 poisoned individuals, the proposed chromatographic method is sensitive and specific enough for the joint determination of cocaine and its main metabolite, benzoylecgonine, in bile. Therefore, bile constitutes an effective supplementary sample for collection during autopsies with a view to accurately diagnosing overdose in cases of fatal poisoning where drugs might go undetected if sought in blood alone.

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