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High-performance liquid chromatographic confirmation of cocaine and benzoylecgonine in biological samples using photodiode-array detection after toxicological screening

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Abstract

In the later stages after intake, the important markers of cocaine abuse are its main metabolites in urine, benzoylecgonine and ecgonine methyl ester. The efficiency of the extraction of amphoteric benzoylecgonine together with cocaine from aqueous media by means of various solvents at various pH values and by means of a mixed solid phase was tested. The extraction of benzoylecgonine with diethyl ether is not efficient, whereas chloroform, dichloromethane or mixed solid-phase extraction give satisfactory results. The analytical strategy for the general chromatographic screening and identification of unknown drugs in biological samples based on diethyl ether extraction was modified to permit the sensitive detection of cocaine abuse also on the basis of benzoylecgonine. A complementary high-performance liquid chromatographic method with photodiode-array detection after solid-phase extraction was introduced for specific confirmation and determination of cocaine and benzoylecgonine.

1. Introduction

Cocaine, the main alkaloid of *Erythroxylon coca*, is nowadays one of the most important drugs of abuse. Illicit cocaine hydrochloride is administered intranasally (*i.e.*, sniffed, the most common route) or by intravenous or intramuscular injection. It can also be taken orally. Cocaine free base ("crack") can be inhaled by smoking in special devices. This route of administration is characterized by a particularly rapid pharmacological effect. The average single lethal dose is 1.2 g, but addicts can take 5–10 g daily. However, 30 mg can be fatal for susceptible persons [1,2]. In addition, the toxicity of the dose is dependent on the route of application [3].

Cocaine is converted in the body into ecgonine methyl ester and benzoylecgonine by different mechanisms [4–9]. The major biotransformation pathways are shown in Fig. 1. Ambre and coworkers [10–13] demonstrated that cocaine is eliminated in urine as benzoylecgonine (46%), as ecgonine methyl ester (41%) and in the original form (3%), the remaining 10% representing minor demethylated and hydroxylated metabolites. The range of excreted metabolites is influenced by the urine pH value. The maximum elimination of cocaine occurs at pH 5.3, whereas that of benzoylecgonine occurs at pH 7.35 [14]. Norcocaine is the only known active metabolite,

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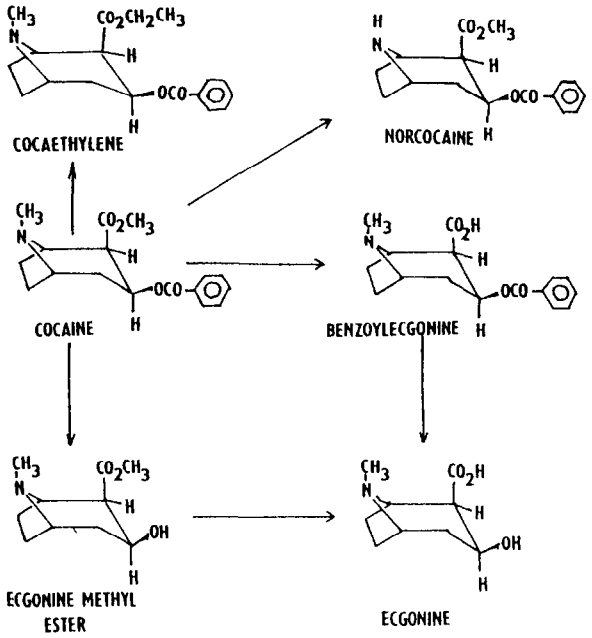

Fig. 1. The main metabolic pathways of cocaine.

but the main metabolite found in blood is benzoylecgonine [15].

The kinetic parameters of cocaine distribution, biotransformation and elimination cause variable analytical findings depending on the sampled material and the time elapsed. It has been reported that the original form can be found in human urine only 3–6 h after application, whereas ecgonine methyl ester and benzoylecgonine still appear after 24–60 h [13]. From the forensic toxicological point of view, these two metabolites are the important markers of cocaine abuse. Cocaine and metabolites in biological samples can be identified by various toxicological methods, *e.g.*, thin-layer chromatography (TLC) with colour reactions (CR) [16], gas chromatography with mass-selective or nitrogen–phosphorus detection (GC–MS or GC–NPD) [4,12], high-performance liquid chromatography (HPLC) with various detectors [17], depending on the aims and the laboratory equipment available.

In this work, we evaluated the efficiencies of various sample preparation procedures including solid-phase extraction (SPE) followed by HPLC with photodiode-array detection of cocaine and benzoylecgonine.

2. Experimental

2.1. Chemicals

Analytical-reagent grade chemicals were used, unless indicated otherwise. Organic-free water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). Methanol, 2-propanol, diethyl ether, chloroform, ethyl acetate, ammonia solution (25%), anhydrous ethanol (spectral grade), sodium dihydrogenphosphate dihydrate and phosphoric acid (85%) were supplied by Lachema (Brno, Czech Republic) and triethylamine, dichloromethane and *n*-butyl chloride by Fluka (Buchs, Switzerland).

SPE NARC-2 cartridges (125 mg/3 ml) were obtained from J.T. Baker (Phillipsburg, NJ, USA).

The drug standards were kindly donated by the United Nations Drug Control Programme, Scientific Support/Laboratory Section (Vienna, Austria).

2.2. Conditions for TLC screening

TLC plates were made in the laboratory from Kieselgel G (Merck, Darmstadt, Germany). The mobile phase was ethyl acetate–ethanol–25% ammonia solution (36:2:2, v/v/v) and detection was effected with Dragendorff reagent followed by dilute sulphuric acid (1:1) [16] (for details of the composition of Dragendorff reagent, see also ref. [1]).

2.3. Conditions for HPLC confirmation

The modular isocratic chromatograph used consisted of a Waters Model 510 pump, a Waters U6K injector, a Waters Model 990 photodiode-array detector and an APC IV NEC Power Mate 2 computer (Waters Division of Millipore, Milford, MA, USA). UV spectra were obtained in the range 210–400 nm with 2-nm resolution. Guard (30 × 3 mm I.D.) and analytical Separon SGX CN cartridges (150 × 3 mm I.D.), packed with cyanobonded silica, particle size 7 μm, were

obtained from Tessek (Prague, Czech Republic). The mobile phase, methanol–buffer (15:85, v/v), was filtered through a 22-μm Durapore filter (Millipore) and briefly degassed under vacuum. The buffer consisted of 1 l of 0.1 *M* phosphate and 1 ml of triethylamine, the pH being adjusted to 3.5 with phosphoric acid. The flow-rate of the mobile phase was set at 0.7 ml/min, which resulted in an analysis time of 25 min.

2.4. Recovery

Extraction recovery tests from different buffers (12 ml) with different solvents (6 ml) were performed. The time of extraction was 5 min. For comparison, SPE on NARC-2 columns was carried out [18]. The efficiencies of the different procedures were tested at concentration levels of 2 μg/ml for cocaine and benzoylecgonine and 5 μg/ml for the intended internal standard, benzoctamine. The recoveries were evaluated by HPLC (detection at 233 nm), by the external calibration method and comparisons of areas theoretically expected with those observed in reality.

2.5. Calibration and detection limits in serum and urine by HPLC

The linearity of calibration for 2-ml serum samples was tested with blank bovine serum spiked with benzoylecgonine and cocaine in the range 20–6000 ng/ml using benzoctamine (20 μg/ml) as an internal standard.

Detection limits for both analytes were also tested in 2-ml samples of negative human urine spiked with standards (20, 50, 200 and 500 ng/ml) using a signal-to-noise ratio of 2.

3. Results and discussion

3.1. Recovery

It was confirmed experimentally that benzoylecgonine can be extracted simultaneously with cocaine from neutral or basic media with chloroform or dichloromethane. The extraction efficiency is enhanced by additions of alcohols (ethanol, 2-propanol), whereas extraction with diethyl ether, diethyl ether–benzene or *n*-butyl chloride is not effective (Table 1). However, the addition of alcohols to chloroform or dichloromethane increases the recovery at the expense of the purity of the extracts of biological samples and thus deteriorates the detection limits.

The pH of the buffer has little influence on the recovery of benzoylecgonine, in contrast to cocaine (Table 2). Basic ecgonine methyl ester can be expected to behave similarly to cocaine.

Optimum extraction results for both cocaine and benzoylecgonine were achieved by solid-phase extraction on NARC-2 columns or by dichloromethane–2-propanol extraction at alkaline pH (Table 3). The capacity of a NARC-2 column was sufficient for the examination of 2-ml serum or 2–5-ml urine samples.

Table 1
Extraction recovery (%) of benzoylecgonine, cocaine and internal standard benzoctamine from aqueous media with various solvents at pH 7.0 ($n = 3$)

Solvent	Recovery (%)		
	Benzoylecgonine	Cocaine	Benzoctamine
Diethyl ether	0	101	96
Diethyl ether–benzene (1:3)	2	70	71
n-Butyl chloride	1	101	105
Dichloromethane	38	93	78
Dichloromethane–2-propanol (4:1)	66	99	78
Chloroform–ethanol (4:1)	62	102	81

Table 2
Influence of pH on extraction recovery of benzoylecgonine, cocaine and internal standard benzoctamine from aqueous media into dichloromethane–2-propanol (4:1) ($n = 3$)

pH	Recovery (%)		
	Benzoylecgonine	Cocaine	Benzoctamine
3.0	64	42	54
7.0	66	99	78
10.4	78	101	82

3.2. Detection of cocaine and ecgonine methyl ester by TLC screening in urine

The usefulness of the screening based on TLC with the system of colour reactions [16] has already been verified in toxicological cases investigated for cocaine abuse in our laboratory. It has been used for rapid estimations and its results influence the further strategy. The general urine extraction with diethyl ether from acid and then from alkaline media permits the detection of cocaine and ecgonine methyl ester (the efficiency of extraction is about 85%) with a limit of 50 ng per spot of cocaine and 2000 ng per spot of ecgonine methyl ester. The detection by this method of both bases in 50-ml urine extracts from some drug addicts is illustrated in Fig. 2.

The detection and identification of benzoylecgonine are outside the scope of this method. Extraction with diethyl ether was not effective and the mobile phase used for TLC–CR screening was not suited to the amphoteric character of benzoylecgonine, resulting in a lack of migration.

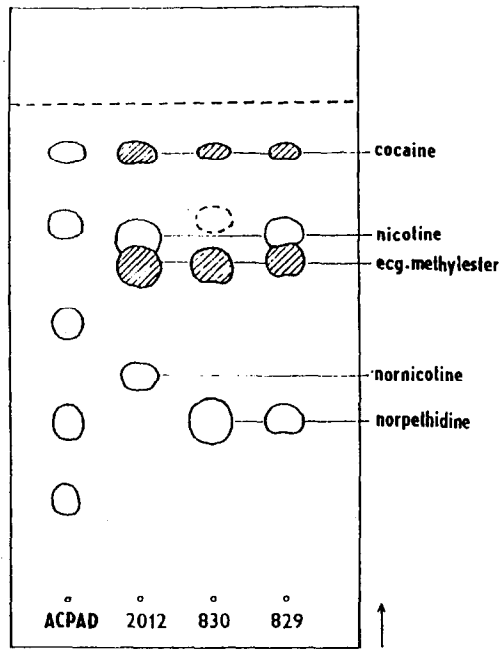

Fig. 2. Example of general TLC screening of urine samples from drug addicts. ACPAD is a mixture of reference standards (atropine, codeine, phenmetrazine, aminophenazone, diazepam). 2012, 830, 829 are numbered urine extracts extracted by diethyl ether from alkaline media. The spots detected were identified in other analytical steps as given.

3.3. Confirmation of cocaine and benzoylecgonine by HPLC with photodiode-array detection

GC–MS is a commonly accepted method for forensic identification of substances, but requires derivatization of benzoylecgonine [4]. The alternative HPLC method with photodiode-array detection proved suitable for confirming the

Table 3
Comparison of extraction recoveries of benzoylecgonine, cocaine and internal standard benzoctamine with various solvents from alkaline buffer (pH 10.4) and on solid-phase NARC-2 ($n = 3$)

Solvent/SPE	Recovery (%)		
	Benzoylecgonine	Cocaine	Benzoctamine
Diethyl ether	0	104	96
Dichloromethane	52	103	76
Dichloromethane–2-propanol (4:1)	78	101	82
NARC-2	85	95	76

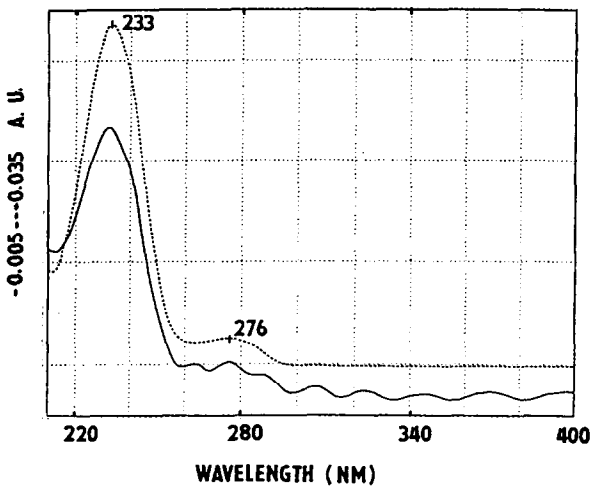

Fig. 3. Typical UV spectrum of the benzoylecgonine peak in bovine serum spiked with 50 ng/ml (full line) or 1000 ng/ml (dotted line).

presence of benzoylecgonine in its original form in biological samples after SPE on NARC-2 columns. Identification of cocaine and benzoylecgonine detected in extracts was based on two criteria: characteristic retention times and UV spectra of selected peaks. The retention data for standards had to be updated daily in order to eliminate the influence of ageing of the column and the variability of batches of mobile phases. The average retention times were 7.0 min for benzoylecgonine, 10.0 min for cocaine and 15.0 min for internal standard benzoctamine. The UV spectra of cocaine and benzoylecgonine did not display significant differences and both had maxima at 233 and 276 nm (the spectrum of benzoylecgonine is shown in Fig. 3). It appears that the identification of both substances at trace concentrations in biological material can be hampered by the deterioration of the quality of the spectrum.

The lowest concentration of both compounds that could be confirmed in 2-ml serum samples was estimated to be about 50 ng/ml (Fig. 4), and in 2-ml urine samples about 200 ng/ml (Fig. 5). The sensitivity in urine is sufficient to confirm the results of the most widespread immunoassays and TLC screenings.

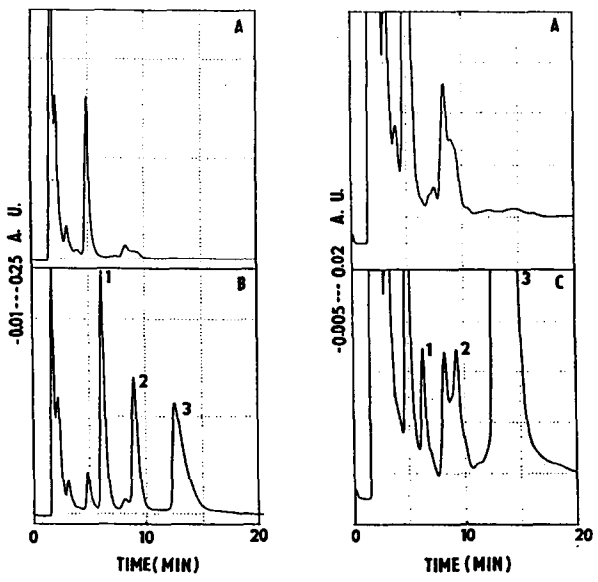

Fig. 4. HPLC at 233 nm of extracts (NARC-2) of 2 ml of bovine serum. (A) Blank; (B) spiked with 1000 ng/ml; (C) spiked with 50 ng/ml. Peaks: 1 = benzoylecgonine; 2 = cocaine; 3 = benzoctamine (internal standard).

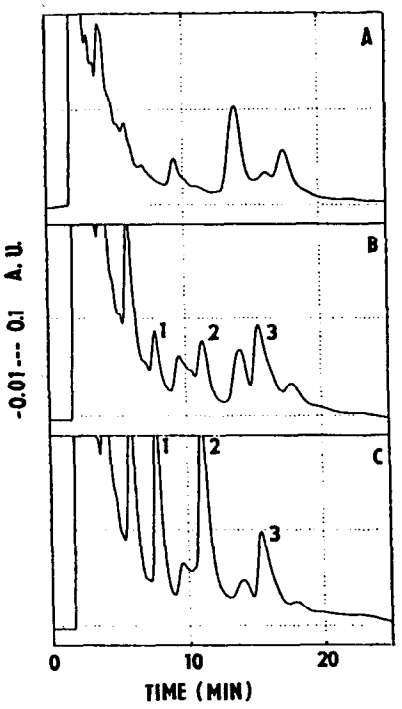

Fig. 5. HPLC at 233 nm of extracts (NARC-2) of 2 ml of human urine. (A) Blank; (B) spiked with 200 ng/ml; (C) spiked with 1000 ng/ml. Peaks: 1 = benzoylecgonine; 2 = cocaine; 3 = benzoctamine (internal standard).

3.4. Calibration for serum

The linearity of calibration was checked for a broad range of therapeutic, toxic and fatal concentrations (50–6000 ng/ml) in 2-ml aliquots of bovine serum spiked with standards and extracted on NARC-2 columns. The calibration dependences were linear over the whole range tested. The calibration graph obtained from the peak-area ratios of cocaine to internal standard at 233 nm (y) *versus* concentration in ng/ml (x) yielded the linear regression equation $y = -0.0007 + 0.0005436x$ and the correlation coefficient was $r = 0.9997$. The analogous equation obtained for benzoylecgonine was $y = 0.0155 + 0.0007239\ x$ ($r = 0.9987$). The concentration range involves the expected values in serum from addicts (hundreds of ng/ml) after a single intranasal street dose (0.4–2.0 mg/kg) and from subjects after a fatal overdose (thousands of ng/ml) [1,3,19]).

3.5. Case reports

One of the first cases in which we confirmed cocaine abuse by HPLC was a nurse who was suspected of intravenous application of 300 mg of cocaine hydrochloride. After 2 h a blood sample was taken for toxicological investigation and the cocaine abuse was confirmed by analysis: cocaine (1394 ng/ml) and benzoylecgonine (3777 ng/ml) were detected in serum.

The second case was a patient admitted to a clinic in an unclear collapse state. A sample of urine was sent for toxicological examination. By the general TLC–CR screening and further TLC analyses, nicotine, caffeine and its metabolites and products of the hydrolysis of metabolites of diazepam were identified. No clear evidence of the presence of cocaine or ecgonine methyl ester in the urine was found. Another 5-ml aliquot of this urine sample was subjected to SPE on NARC-2 and subsequent HPLC. The presence of benzoylecgonine was confirmed. In addition, this extract was simultaneously analysed by GC with thermionic detection and a peak with a retention time corresponding to the other metabolite, ecgonine methyl ester, was detected.

The last case described was a fatal cocaine overdose by a dealer. A damaged 4-g packet of cocaine smuggled in the body caused the man's death. After the autopsy the tissues and urine were analysed by TLC and were positive for cocaine and metabolites. An aliquot of 1 g of liver tissue was taken for parallel HPLC analysis. The SPE procedure used for liquids had to be modified in this instance. The liver sample was homogenized and extracted at neutral pH with dichloromethane and the extract was purified by

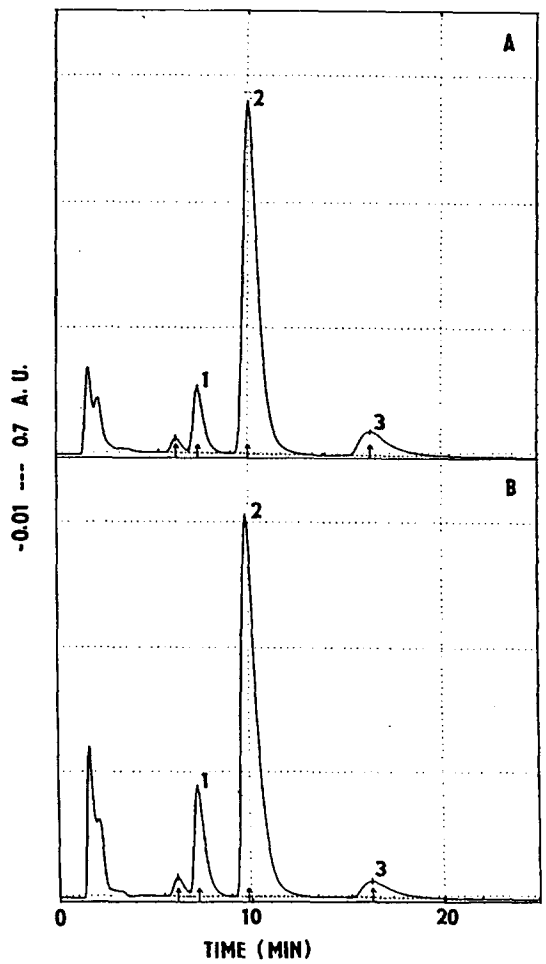

Fig. 6. Example of fatal cocaine overdose. HPLC at 233 nm of (A) the extract of 1 g of liver sample from a dealer (A) and (B) the extract of the same material after additions of 2000 ng/g of (1) benzoylecgonine and (2) cocaine. Internal standard, benzoctamine (3), 40 μg/g. Combined liquid–liquid and solid-phase extraction.

SPE on a NARC-2 column. We attempted to determine the cocaine and benzoylecgonine contents in the liver tissue. The calibration method based on standard additions to the sample was expected to be the most suitable for eliminating matrix effects. However, no reproducible and linear correlations in tissues were achieved. Therefore, only qualitative findings for cocaine and benzoylecgonine were possible and are shown in Fig. 6.

4. Conclusions

Cocaine and one of its main metabolites, ecgonine methyl ester, are efficiently extracted with diethyl ether and can be detected by the general TLC–CR screening system [16] used for unknown drugs in biological materials. The second main metabolite, benzoylecgonine, is not extracted with diethyl ether and its detection and confirmation require another special toxicological method.

The HPLC method with photodiode-array detection after independent SPE on NARC-2 columns is suitable for confirming or complementing the results of toxicological screening or as a targeted method to detect and identify cocaine and benzoylecgonine in small volumes of biological samples or in samples taken in later stages after the application of cocaine when the TLC results need not be positive.

Combination of independent toxicological methods is always beneficial for increased reliability of analytical results.

5. Acknowledgements

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