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Direct injection determination of benzoylecgonine, heroin, 6-monoacetylmorphine and morphine in serum by MLC

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Abstract

A simple and sensitive direct injection chromatographic procedure is developed for the determination of heroin, two of its metabolites (morphine and 6-monoacetylmorphine (6-MAM)), and benzoylecgonine (a metabolite of cocaine) in serum samples. The proper resolution of the four substances is obtained with a chemometrics approach, where the retention is modelled as a first step using the retention factors obtained in a limited number of mobile phases. Afterwards, an optimisation criterion that takes into account the position and shape of the chromatographic peaks is applied. The mobile phase selected to carry out the analysis was 0.1 mol L^{-1} SDS–4% (v/v) butanol buffered at pH 7, in which the separation is performed in less than 18 min. The limits of quantification were in the 17–36 ng mL^{-1} range. Intra- and inter-day assay accuracy and precision (below 3%) were obtained following ICH guidelines. The method developed was applied to the determination of the drugs studied in serum samples with good recoveries (90–104%). Serum samples from subjects that have been ingested cocaine and heroin were also analysed. The samples were injected directly in the chromatographic system without any pretreatment.

Keywords: Benzoylecgonine; Heroin; Morphine; 6-Monoacetylmorphine; Micellar liquid chromatography; Direct injection; Serum

1. Introduction

Cocaine, a naturally occurring stimulant found in the leaves of coca plants (*Erythroxilon coca*), is one of the most widespread illicit drugs of abuse. In blood as well as in plasma, cocaine is rapidly and extensively broken down in vivo and in vitro [1–3]. Benzoylecgonine is one of the major hydrolysis products of cocaine in human plasma formed by the chemical hydrolysis of the methyl ester group [2,4]. Although benzoylecgonine is not a pharmacologically active metabolite, it is of great interest in pharmacological/toxicological and forensic studies because it has a longer half-life than cocaine itself [5–7].

Cocaine is often ingested with other drugs like heroin (speed ball) to enhance its effects. Heroin (3,6-diacetyl morphine) is a potent narcotic analgesic and is metabolised in serum or liver esterases, or spontaneously hydrolysed to 6-monoacetylmorphine (6-MAM), which is further hydrolysed to morphine and this in turn is conjugated to morphine glucuronides [8–10]. Morphine may be further metabolised in both the liver and the intestine to normorphine and codeine (Fig. 1) [11]. The half-life of heroin is 5–9 min, between 38 and 45 min for 6-monoacetylmorphine, and between 90 and 180 min for morphine. Heroin is rarely detected in biological samples except for those collected immediately after ingestion. Heroin ingestion is usually evidenced by the detection of morphine. However, codeine, which is often used as a cough medicine, can also be metabolised to morphine. Thus, the use of heroin can only be proved by detecting the presence of 6-monoacetylmorphine, the characteristic metabolite of heroin, in addition to morphine.

Gas chromatography coupled to mass spectrometry (GC–MS) is the most common method used to determine

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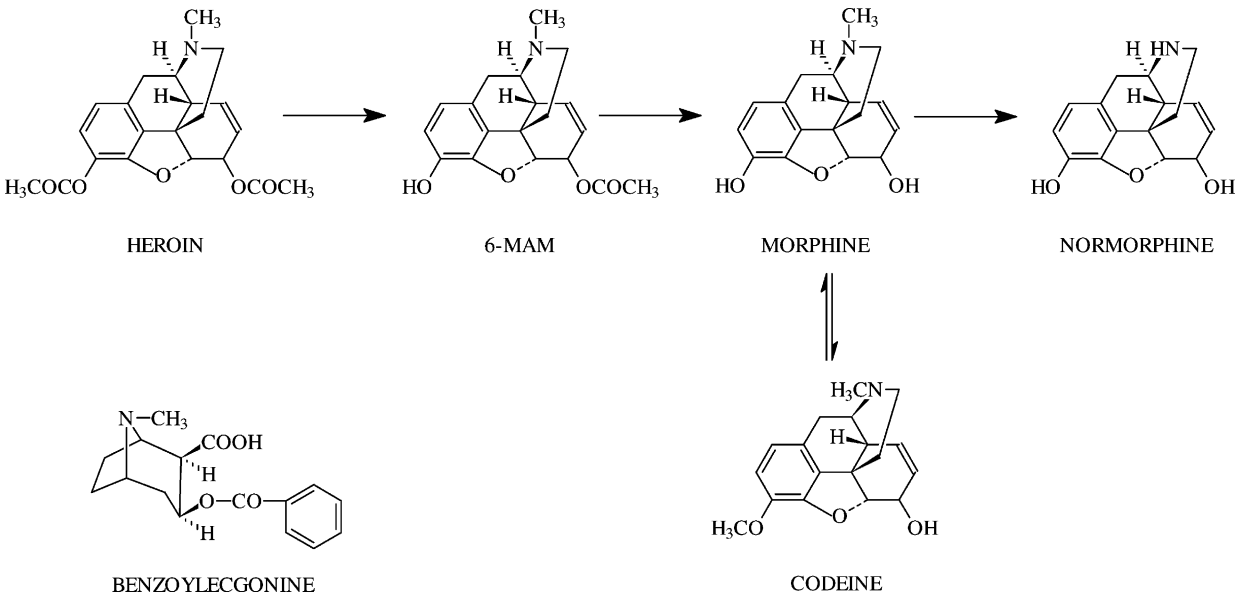

Fig. 1. Metabolic pathway of heroin and chemical structures of the compounds studied. 6-MAM is 6-monoacetylmorphine.

these drugs in different biological samples, including blood [12–15], saliva [14,15], urine [15,16] or human hair [13,15]. Other methods used to determine benzoylecgonine, heroin and its metabolites are high performance liquid chromatography (HPLC) [17,18], radioimmunoassay [17] or zonal capillary electrophoresis (CE) [19,20], electrochromatography [21], and electrospray ionisation mass spectrometry [22]. Most published procedures use HPLC with fluorescence [23], electrochemical [24], UV [25,26] or diode array detection [18,27]. GC, HPLC and CE methods for the analysis of biological samples like serum, blood, plasma, or urine require a previous step to eliminate proteins and other interferences from the matrix before injection into the column, which usually involves solid-phase or liquid–liquid extraction. This extraction step makes the procedure long, tedious and poorly reproducible.

Micellar liquid chromatography (MLC) allows biological samples to be analysed without needing to eliminate proteins and other interfering substances, thus considerably reducing the cost and analysis time. In MLC, the retention behaviour of compounds can be predicted with high accuracy [28]. This fact simplifies the optimisation of the mobile phase composition. In addition, one of the main applications of MLC is the possibility of direct sample injection of biological material into the column due to the ability of micellar aggregates to dissolve sample proteins and other compounds. MLC technique has proved to be a useful technique in the determination of diverse groups of drugs in serum and urine samples [29–34].

This study aims to develop a rapid, simple and selective MLC procedure for the screening of benzoylecgonine, heroin and two of its metabolites (6-monoacetylmorphine and morphine) in serum. The main advantage of the method lies in the direct injection of the serum samples, which largely simplifies the determination of these compounds.

2. Experimental

2.1. Reagents

Benzoylecgonine, heroin, 6-monoacetylmorphine and morphine were purchased from Sigma-Aldrich (Steinheim, Germany). The structures of these drugs are shown in Fig. 1. The mobile phases were prepared with the surfactant sodium dodecyl sulphate (99% purity, Merck, Darmstadt, Germany) and the alcohols: 1-propanol, 1-butanol or 1-pentanol (HPLC grade, Scharlab, Barcelona, Spain). The pH was buffered at 7 with disodium hydrogen phosphate (Panreac, Barcelona). Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout. Methanol (HPLC grade, Scharlab) was used to clean the column.

2.2. Apparatus and chromatographic conditions

An Agilent Technologies 1100 chromatograph (Palo Alto, CA, USA) equipped with a quaternary pump (Model G1311A), an autosampler with 2 mL vials (Model G1313A), a thermostated column compartment (Model G1316A) and a UV–vis detector (Model 1321A) was used. A PC workstation with HP^{3D} software was employed for instrumental control, acquisition of the chromatographic data and to measure peak properties, i.e. retention factor (k), efficiency (N) and asymmetry (B/A).

The analytical separation was accomplished using a Kromasil C18 column (Scharlab, 5 μm particle size, 250 mm × 4 mm i.d.). The flow-rate was 1 mL min^{-1} and the injection volume, 20 μL. The chromatographic runs were carried out at 25 ± 0.2 °C. Monitoring was performed at 230 nm. The column was washed with water and methanol before changing the mobile phase. The new mobile phase was then

flushed at the working flow-rate until stability of the base-line was achieved. The chromatographic system was usually washed weekly.

The optimum mobile phase composition was 0.1 mol L^{-1} SDS–4% (v/v) butanol–phosphate buffer 0.01 mol L^{-1} at pH 7. The pH of the mobile phase was measured before the addition of the modifier with a Crison GLP 22 potentiometer (Barcelona), equipped with a combined Ag/AgCl/glass electrode. UV spectra of the drugs were obtained with a Perkin Elmer UV–vis spectrophotometer Lambda 19 (Norwalk, CT, USA). The mobile phases were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA). The dead volume was measured as the mean value of the first significant deviation of the base-line in the chromatograms. Optimisation of the mobile phase composition was assisted by Michrom software [28].

2.3. Standard solutions and serum samples

Stock standard solutions containing 100 μg mL^{-1} of the drugs were prepared by dissolving the compounds in a few mililiters of methanol with the aid of an ultrasonic bath (Selecta, Mixtasel, Barcelona) and then diluted to the convenient amount with 0.1 mol L^{-1} SDS–4% butanol at pH 7. Working solutions were always filtered directly into the autosampler vials through 0.45 μm nylon membranes of 12.5 mm diameter (Micron Separations).

Serum samples were diluted in a 1:10 factor with the selected mobile phase before injection. Serum solutions were spiked with known amounts of the drugs and injected into the chromatographic system without any other treatment than filtration. The optimisation of the procedure was performed with spiked serum samples. The blood was preserved with fluoride after sampling to avoid hydrolysis. All the solutions and blood samples were stored at 4 °C in glass light-resistant bottles.

3. Results and discussion

3.1. Optimisation of the mobile phase composition

3.1.1. Selection of the pH

The equilibria between the protonated and non-protonated morphine-like compounds ($\log K_1 = 9.19 - 9.53$ and $\log K_2 = 7.6 - 7.9$) take place outside the working pH range of a non-modified C18 column (3–7). Moreover, in micellar media these constants increase in two units and this behaviour guarantees that the equilibrium is outside the working range of pH. For these compounds, the retention was thus the same using mobile phases of SDS, SDS–propanol, SDS–butanol or SDS–pentanol at pH 3, 5 and 7. The following steps were made at pH 7 which was the best suited for the maintenance of the column. Since benzoylecgonine has a free-carboxylic group, it will be partially dissociated and negatively charged at pH 7, thus eluting faster than at an acidic pH.

3.1.2. Selection of the concentration of surfactant and modifier

The retention of heroin, 6-monoacetylmorphine, and morphine in a C18 column with pure micellar eluents (without modifier) was high (>30 min). Thus, it was deemed advisable to add a small amount of an organic solvent to decrease the retention times. Propanol, butanol and pentanol were studied. The retention times of the drugs decrease progressively as the alcohol chain increases. The use of hybrid micellar mobile phases of variable concentrations of surfactant and modifier usually produces changes in the retention factors, efficiencies and asymmetries of the chromatographic peaks. Adequate control of the concentrations of both additives is therefore necessary to achieve chromatograms showing good resolution and sufficient elution strength.

In liquid chromatography, interpretive optimisation strategies are more efficient and reliable than sequential approaches. The methodology followed by chromatographers can be mimicked by these strategies that can be assisted by computer simulation, thus reducing the amount of time and effort required. MLC is capable of predicting the retention of compounds using simple equations. Different mathematical models can be used to describe the retention of analytes. The model employed in this work was as follows [28]:

$$k = \frac{K_{AS}((1 + K_{SD}\varphi)/(1 + K_{AD}\varphi))}{1 + K_{AM}[M]((1 + K_{MD}\varphi)/(1 + K_{AD}\varphi))} \quad (1)$$

where $[M]$ and φ are the concentrations of surfactant and modifier; K_{AS}, K_{AM}, K_{MD}, K_{SD}, K_{AD} and K_{AD} correspond to the equilibria between the solute (A) in the stationary phase (S), micelle (M), or bulk water (D). This equation was non-linearly fitted according to the Powell method [35] using the retention data from injections of the drug solutions in several mobile phases. To avoid inconsistent results, the experimental design should have at least one mobile phase more than parameters in the equation, and in this case consisted of seven mobile phases in the following concentration ranges: 0.05–0.15 mol L^{-1} SDS, 2.5–12.5% (v/v) for propanol; 1–7% (v/v) for butanol; and 2–6% for pentanol (v/v).

The accurate prediction of the retention according to Eq. (1) allowed to apply an interpretive procedure to predict the optimum mobile phase, following a criterion that uses the valley-to-peak ratios [36]. Incorporation of the peak shape in the optimisation procedure improves the results. The reliable simulation of peak shape for any mobile phase of the variable space was carried out with an asymmetrical Gaussian function where the standard deviation is a first-degree polynomial function [37]. Using Eq. (1) and the mathematical treatment described here, the relative global error in the prediction of retention factors was below 5% for all drugs studied.

Three optimisation procedures were carried out using the alcohols: propanol, butanol and pentanol as modifiers in each case. The elution order of the drugs was always the same in the mobile phases of SDS, regardless of the alcohol used. Complete resolution of the compounds was always achieved using

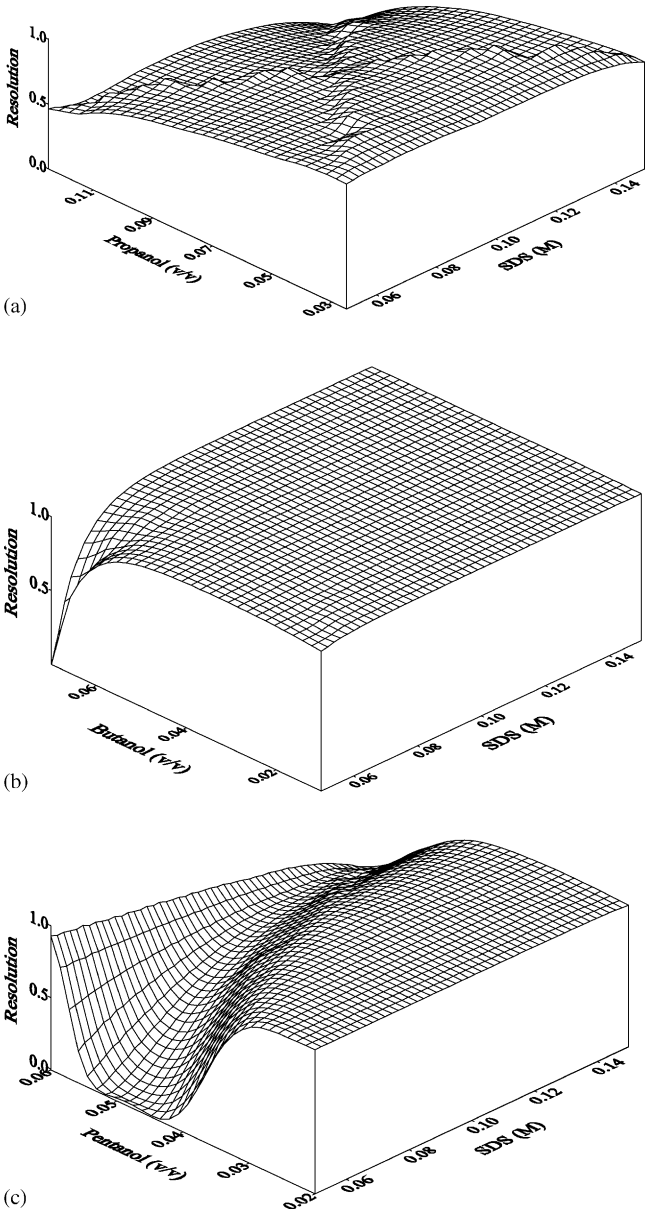

Fig. 2. Global resolution diagram according to the peak-to-valley optimisation criterion for the separation of the drugs studied, eluted with mobile phases containing SDS and the modifiers: (a) propanol; (b) butanol; and (c) pentanol.

any modifier. Global resolution diagrams obtained with the modifiers are depicted in Fig. 2. The resolution diagram of butanol (Fig. 2b) provides the broadest surface where complete resolution is achieved. The four compounds can be separated almost in the whole space. For propanol (Fig. 2a), the best resolutions are only attained at modifier concentrations below 9%; and for pentanol (Fig. 2c), complete resolution is only achieved in a narrow region (below 3%). In addition, higher efficiencies were obtained when butanol (3000–4000) or pentanol (1500–3000) were employed, compared with propanol (around 1000), but benzoylecgonine was eluted at the dead volume using pentanol. For these reasons, butanol was finally selected to carry out the determination of the drugs.

Table 1 shows the coefficients fitted for Eq. (1) for each drug, thus allowing the prediction of the mobile phase composition for any desired retention time and a simple way to optimise the separation of mixtures. It must be noted that, in the analysis of physiological fluids, the retention time of the endogenous compounds and the protein band at the head of the chromatogram should be considered when selecting the mobile phase.

As can be seen in Fig. 2b, maximum resolutions can be reached using mobile phases containing butanol at a concentration below 5% (v/v). The best resolution was for the composition 0.1 mol L^{-1} SDS–4% butanol ($r = 0.99$), which was finally selected to perform the analysis of serum samples. The analysis time was below 18 min. Fig. 3 shows the simulated and experimental (serum spiked) chromatograms for a mixture of the four drugs in the optimum mobile phase. The agreement between both chromatograms is excellent. The retention times were (min): benzoylecgonine (3.9), morphine (8.1), 6-monoacetylmorphine (11.2) and heroin (16.3).

3.2. Validation of the method

3.2.1. Linearity

The study of linearity was estimated by the analysis of aqueous solutions of the analytes and aliquots of serum spiked samples diluted in a 1:10 factor. Calibration curves using the areas of the chromatographic peaks were constructed. Triplicate injections of five solutions at increasing concentrations, in the 0.02–10 μg mL^{-1} range, were made. The slopes of the calibration curves in the absence and presence of serum matrix were similar, the intercepts were usually statistically zero, and the regression coefficients, $r > 0.999$. Table 2 summarises the parameters of the calibration curves obtained for each drug in serum matrix.

3.2.2. Limits of detection and quantification

The limits of detection (LODs) and quantification (LOQs) were determined in serum samples by an empirical method that consists in analysing a series of serum samples containing decreasing amounts of the analytes [38]. LOD was the

Table 1
Coefficients of Eq. (1) used to predict the chromatographic behaviour of the substances studied

Compound	K_{AS}	K_{AM}	K_{MD}	K_{AD}	K_{SD}
Benzoylecgonine	40×10^4	7	21×10^6	11×10^6	56
Heroin	422	200	4.3	70	6.7
6-Monoacetylmorphine	79×10^3	65×10^3	4.2	32×10^3	4.2
Morphine	17×10^6	50	67×10^6	72×10^6	88

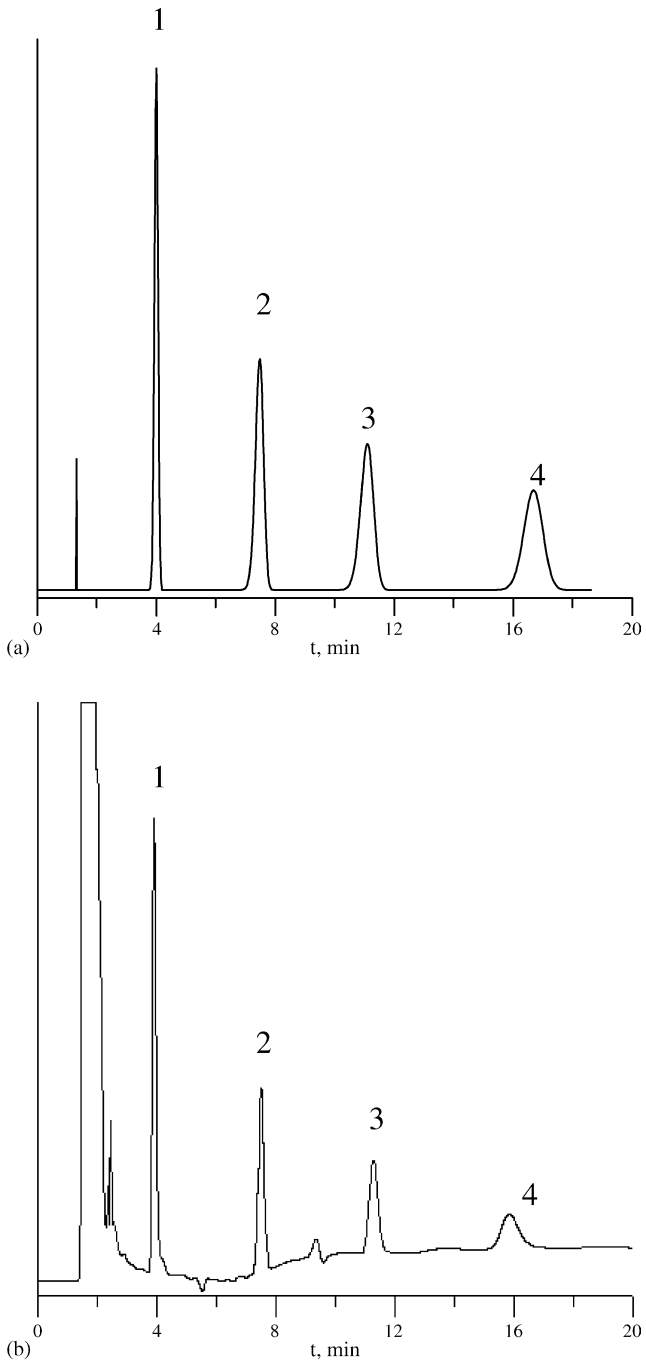

Fig. 3. Simulated (a) and experimental (spiked serum sample) (b) chromatograms of the drugs: benzoylecgonine (1); morphine (2); 6-monoacetylmorphine (3); and heroin (4), using the optimum mobile phase: 0.1 mol L^{-1} SDS–4% (v/v) butanol at pH 7. Concentration drugs in serum samples were 150 ng mL^{-1}, except for 6-monoacetylmorphine that was 200 ng mL^{-1}.

lowest concentration that presented a coefficient of variation (CV, %) that did not exceed 20% and the LOQ was taken as the lowest concentration that presented a CV that did not exceed 10% (Table 2). As can be seen, the LODs obtained are appropriate to monitor the drugs at therapeutic levels.

Table 2
Calibration parameters, limits of detection (LODs, ng mL^{-1}) and quantification (LOQs, ng mL^{-1}) for the substances eluted in the mobile phase 0.10 mol L^{-1} SDS–4% butanol at pH 7

Compound	Slope	Intercept	r	LOD	LOQ
Benzoylecgonine	33.8 ± 0.8	0.03 ± 0.06	0.9996	4	17
Heroin	12.8 ± 0.3	0.01 ± 0.02	0.9991	23	36
6-MAM	6.1 ± 0.4	0.05 ± 0.01	0.9998	15	28
Morphine	16.1 ± 0.3	0.005 ± 0.013	0.9992	11	23

Table 3
Intra- and inter-day assay (CV, %; $n = 10$) values for the substances in serum samples

Compound	Intra-assay precision[a]			Inter-assay precision[a]		
	0.05	5	10	0.05	5	10
Benzoylecgonine	1	0.6	0.3	1.7	1.3	1
Heroin	2	1.1	1.2	3	2.4	2.2
6-MAM	1.4	0.8	0.7	2.2	1.9	1.4
Morphine	1.3	0.5	0.4	1.8	1.6	1.3

Concentrations are given in μg mL^{-1}.
[a] Ten replicates for each concentration were performed for the analyses.

3.2.3. Intra- and inter-assay precision

Precision, defined as the relative standard deviation or coefficient of variation, was determined by intra- and inter-assay. Following the ICH Harmonised Tripartite Guideline, three test solutions were prepared in the serum matrix to assess the repeatability and intermediate precision of the assay for the four analytes. Repeatability was determined by assaying the three test solutions 10 times in the same day. The intermediate precision was the average of ten measurements of intra-assay values taken on 10 days over a three-month period and made by different analysts using different equipment, at three different drug concentrations, within the therapeutic ranges. They were performed by analysing spiked serum samples at concentrations of 0.05, 5 and 10 μg mL^{-1} for all analytes (Table 3). Ten replicates for each concentration were performed for the analyses. The coefficients of variation were always below 2% and 3% for the intra- and inter-day assays, respectively.

Table 4
Determination of the substances expressed as concentration (μg mL^{-1}) of added analyte and found from spiked serum samples

Compound	Added	Found	Recovery (%)
Benzoylecgonine	0.05	0.0521 ± 0.0012	104.2
	1	1.011 ± 0.002	101.1
	5	4.999 ± 0.004	99.98
Heroin	0.05	0.045 ± 0.003	90.0
	1	0.977 ± 0.014	97.7
	5	4.846 ± 0.017	96.9
6-MAM	0.05	0.049 ± 0.002	98.0
	1	1.012 ± 0.012	101.2
	5	4.993 ± 0.008	99.9
Morphine	0.05	0.0496 ± 0.0019	99.2
	1	0.9891 ± 0.0012	98.9
	5	5.016 ± 0.003	100.3

3.3. Analysis of serum samples

Serum samples contain a large amount of proteins and other interfering endogenous compounds, which may destroy the packing material of the column, when used for a long period of time. In addition, the peak of the protein band may interfere with the compound of interest and may hinder the detection of the drugs. In order to overcome this problem and to increase the life of the column, it was finally decided to dilute the serum samples in the ratio of 1:10 with the mobile phase being used. This reduces the protein band peak, thus avoiding the interference with the benzoylecgonine peak, which is the first eluting compound. The sensitivity achieved after dilution of the serum samples was adequate for the detection of the compounds. The state of the column was tested by checking the sensitivity and the retention time after 250 injections. No change was observed for either parameter.

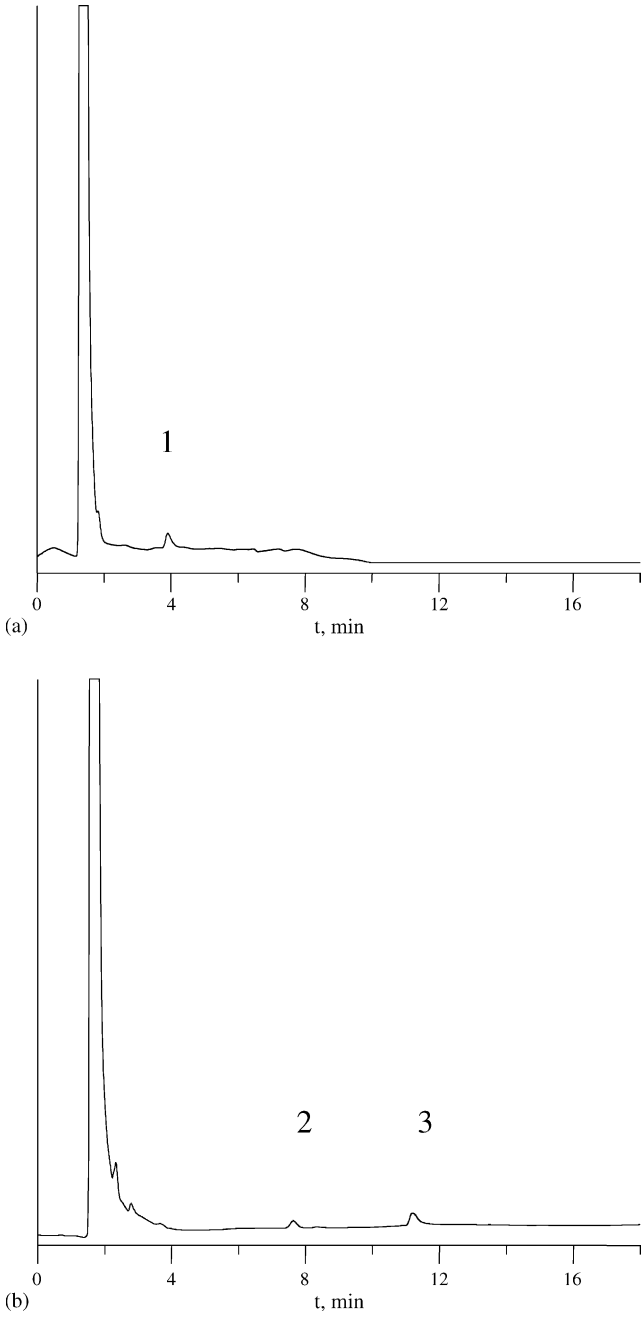

Fig. 4. Chromatograms of serum samples from patients that have been ingested cocaine (a) and heroin (b): benzoylecgonine (1); morphine (2); and 6-monoacetylmorphine (3). Mobile phase: 0.1 mol L^{-1} SDS–4% (v/v) butanol at pH 7.

To determine the specificity of the assay, blank serum samples collected from healthy adult volunteers were analysed using the reported procedure. No interfering peaks from endogenous compounds appeared at the same retention times of the drugs studied when the chromatograms were inspected. Thus, the procedure specificity was successfully checked in this assay.

The described method has an excellent sensitivity for serum samples. To demonstrate the usefulness of this procedure, blank serum samples were spiked with known amounts of each drug and then injected into the chromatographic system. Table 4 shows the good recoveries obtained (93–106%) for each drug at three different concentrations.

Finally, the applicability of the assay was proved by analysing serum samples provided by the Hospital Verge dels Lliris (Alcoi, Spain) from subjects that ingested cocaine and heroin (Fig. 4). Bezoylecgonine was detected in the subject that ingested cocaine (Fig. 4a), and morphine and 6-monoacetylmorphine were observed in the subject that has been intake heroin (Fig. 4b). Good correlations ($r > 0.996$) were obtained when the results obtained with our method and those provided by the hospital (for a total of 10 patients) were compared, giving slopes of 0.922 ± 0.006, 0.952 ± 0.004 and 0.894 ± 0.009, and y-intercepts of -0.004 ± 0.011, 0.005 ± 0.002 and 0.008 ± 0.002, for benzoylecgonine, morphine and 6-MAM, respectively. The approximate amounts found for the drugs were 8 ng mL^{-1} for benzoylecgonine, 14 ng mL^{-1} for morphine and 25 ng mL^{-1} for 6-MAM.

4. Conclusions

The analytical technique proposed for the determination of benzoylecgonine, heroin, 6-monoacetylmorphine and morphine in serum samples proved to be very precise, with analysis times usually below 18 min. The procedure is sensitive enough for the screening and routine analysis of the drugs studied at therapeutic serum levels, with LODs similar to those usually reported in the literature, taking into account that the serum samples were injected without any previous treatment in order to separate or concentrate the analytes. Good sensitivity and linearity were also obtained for all the analytes. The coefficients of correlation (r) were above 0.99 for the dynamic range studied, up to 10 μg mL^{-1}. The recoveries were very similar and varied between 90% and 104%. No interferences where found from the endogenous compounds of serum. This method also allows to follow the breakdown of heroin into 6-monoacetylmorphine and then

into morphine. This is important for forensic purposes because this procedure allows to detect some of the metabolites of heroin several hours after ingesting an overdose of the drug.

Acknowledgements

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