ELSEVIER

Journal of Chromatography B, 661 (1994) 245–253

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of 6-monoacetylmorphine and morphine in plasma, whole blood and urine using high-performance liquid chromatography with electrochemical detection

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First received 8 April 1994; revised manuscript received 5 July 1994

Abstract

6-Monoacetylmorphine and morphine were determined simultaneously in plasma, whole blood and urine, after solid-phase extraction, by high-performance liquid chromatography using amperometric detection at 600 mV oxidation potential. The recoveries ranged from 92 to 99%. The reproducibility study indicated that the coefficients of variation were less than 11% for morphine and 12.4% for 6-monoacetylmorphine. The determination limits were 1 ng/ml for morphine and 4 ng/ml for 6-monoacetylmorphine. The method had a good selectivity towards opiate and nonopiate analgesics and other drugs. The stability of the analytes in methanol (standard solutions), in samples (plasma, whole blood and urine) at −20°C and at 20°C, and in samples after enzymatic hydrolysis at 37°C, was also studied. For samples containing 6-monoacetylmorphine, inadequate storage or hydrolysis could lead to overestimation of morphine or its conjugates. The technique described can be applied for the study of the pharmacokinetics of heroin; it is also available for forensic toxicology to distinguish heroin use from medical prescription of morphine and other opiate drugs.

1. Introduction

In man diacetylmorphine (heroin) is rapidly metabolised to 6-monoacetylmorphine (6MAM) and further to morphine (M). Due to the unstability of heroin, most of the analysis procedures are directed to the determination of morphine. Other opioid drugs, such as codeine, pholcodine, ethylmorphine, (prescribed as antitussive), are also metabolised to morphine [1–3]; therefore determination of morphine alone is not sufficient to distinguish heroin use from medical prescription of opiates. Thus the analysis of both 6MAM and M in biological fluids has been proposed to confirm heroin use. The ester compound 6MAM is known to be unstable in aqueous solution [4], but it appears to be stable in human plasma [5,6]. Barrett et al. [7] recently studied the stability of 6MAM as a function of pH, temperature and storage conditions of the sample. The authors affirmed that the short

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0378-4347/94/$07.00
SSDI 0378-4347(94)00345-9

contact of 6MAM with alkaline medium during the extraction stage (pH 9) and during the chromatographic analysis caused only negligible hydrolysis; on the other hand, hydrolysis was complete under strongly alkaline conditions and at high temperature. As for plasma, breakdown of 6MAM during sample collection or storage, which would lead to overestimation of morphine, was not significant at room temperature (20°C) for up to 3 h or at −30°C for up to one week.

Numerous methods were described for the determination of M, but only few reported the determination of both 6MAM and M in biological matrices: they used gas chromatography–mass spectrometry (GC–MS) [8–11] and high-performance liquid chromatography (HPLC) with fluorescence detection [7,12–14]. Recently a procedure for the determination of 6MAM in urine using electrochemical detection was published [15].

The present study was undertaken to develop a HPLC method using amperometric detection, for the simultaneous determination of 6MAM and M in plasma, whole blood and urine, after solid-phase extraction and cleanup.

2. Experimental

2.1. Reagents and standards

All reagents were of analytical grade. Ammonium acetate (RPE, Reagente Puro Erba), diethyl-ether (RPE), chloroform (RPE), isopropanol (RPE), methanol (RS = Reagente Speciale for HPLC) were from Carlo Erba (Milano, Italy). Ammonium phosphate (Rectapur) and sodium sulphate (Rectapur) were from Prolabo (Paris, France). C_{18} Sep-Pak Vac cartridges (1 g packing material, 6 ml cartridge size) and vacuum manifold were from Waters Millipore (Saint-Quentin-en-Yvelines, France). β-Glucuronidase (*Escherichia coli*, type IX A), pH 6.8 phosphate buffer, morphine hydrochloride, 6-monoacetylmorphine hydrochloride and nalorphine hydrochloride (I.S. = internal standard) were obtained from Sigma (La Verpillière, France).

Stock solutions of tested compounds (M, 6MAM and I.S.) were prepared in methanol at 1 μg/μl and stored at −20°C. The working solutions were diluted with methanol before use for preparation of calibration standards.

All glassware was first washed with a 3% RBS 25 biodegradable alkaline solution from Biolyon (Dardilly, France) containing a mixture of anionic and nonionic detergents and then rinsed with distilled water and dried before use.

2.2. Apparatus and chromatographic parameters

Chromatographic analysis was performed on a LDC Analytical system (Orsay, France) consisting of an A 1000 autoinjector, a 3500 constametric pump, an M400 EG and G (Princeton, NJ, USA) electrochemical detector (with a glassy carbon working electrode and an Ag/AgCl reference electrode), a 3200 Spectromonitor UV detector (monitored at 220 nm, and possibly connected in series to confirm the electrochemical detection) and an Epson (Levallois-Perret, France) A X 2e Computer with a LX 850 printer. A Waters Millipore μBondapak Phenyl column (300 × 3.9 mm I.D., particle size 10 μm) connected to a μBondapak Phenyl Guard Pak column (5 × 6 mm I.D.) was used. The mobile phase was 0.025 *M* ammonium acetate–acetonitrile (72 : 28, v/v) pH 6.5; the flow-rate was 1 ml/min. 6MAM and M were both detected at a 600 mV oxidation potential with a full scale deflection of 10–20 nA. The determination was carried out at room temperature.

2.3. Procedure

Sample collection and storage

All samples (plasma obtained by centrifugation for 10 min at 2800 *g* and 4°C from blood drawn into heparinized tubes, whole blood and urine) were kept frozen at −20°C until analysis.

Sample preparation

The C_{18} Sep Pak cartridge was conditioned by flushing with 2 × 6 ml of methanol followed by 2 × 6 ml distilled water. The sample (1–2 ml) was pipetted into a 20-ml glass tube. Four ml of 0.2 *M* ammonium phosphate solution (pH 8.5) and 20 μl of nalorphine (I.S.) at 5 ng/μl in

methanol were added. The mixture was vortex-mixed and passed through the conditioned cartridge via the vacuum manifold at a negative pressure not higher than 5 mmHg. The cartridge was then washed by passing 2 × 6 ml of distilled water. The undesired polar compounds in the sample were removed by passing successively 2 × 6 ml of 0.1 *M* sodium sulphate (pH 6), 2 × 6 ml of distilled water and 2 × 6 ml of 0.2 *M* ammonium phosphate (pH 8.5) through the cartridge. Finally 1.5 ml of chloroform–isopropanol (9 : 1, v/v) was passed through the cartridge and the eluate collected in a 5-ml glass tube. The aqueous layer from the void volume of the cartridge was drawn off and discarded. The organic layer was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 100 μl of methanol. After vortex-mixing and centrifuging for 10 min at 2800 *g* and 4°C, 20 μl of this solution were injected onto the chromatographic system.

Calibration curve and quantitation

Standard calibration curves were established by spiking blank samples with M and 6 MAM at increasing concentration (2–100 ng/ml) and proceeding as described above. The curve was constructed by plotting the peak-area ratio to internal standard (drug/I.S.) against the spiked concentrations. The concentrations of the analytes were calculated after incorporating their peak-area ratio to internal standard into the curve equation.

3. Results and discussion

Under the conditions described, the capacity factors (k') were 0.75 for M, 2.13 for MAM and 1.40 for nalorphine (I.S.).

Fig. 1 shows the voltammograms of M and 6 MAM. Figs. 2, 3 and 4 show examples of chromatograms from plasma, whole blood and urine specimens respectively.

3.1. Linearity

Within the concentration range 2–100 ng/ml the relations were linear. The equations of the regression curves and their correlation coefficients (r) were as follows: M in plasma: $y = 0.023x - 0.016$ ($r = 0.998$); M in whole blood: $y = 0.020x - 0.019$ ($r = 0.998$); M in urine: $y = 0.022x + 0.015$ ($r = 0.999$); 6MAM in plasma: $y = 0.006x + 0.063$ ($r = 0.998$); 6MAM in whole blood: $y = 0.006x - 0.012$ ($r = 0.998$); 6MAM in urine: $y = 0.006x + 0.008$ ($r = 0.999$) where y is the ratio of analysed compound to internal standard and x is the quantity of spiked compound.

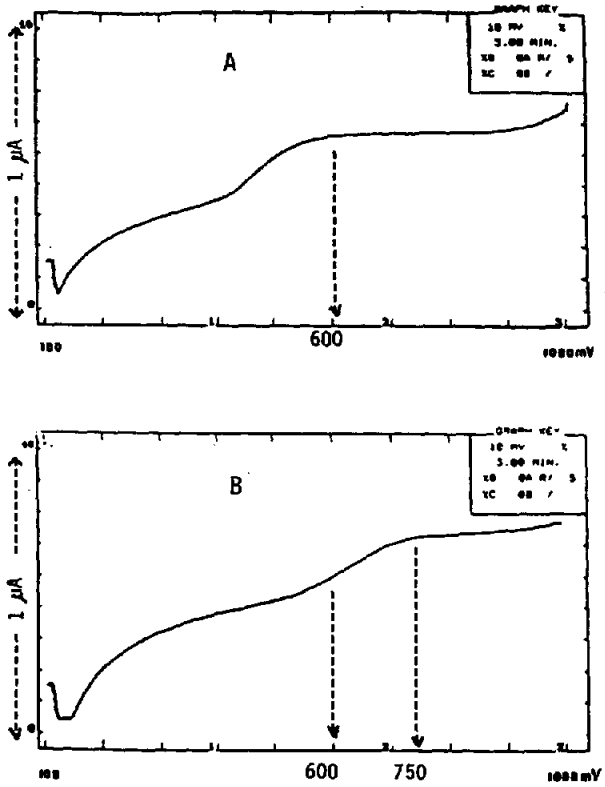

Fig. 1. Voltammograms obtained for solutions of (A) M: 5 ng/μl, (B) 6MAM: 20 ng/μl in mobile phase. Scanning potentials were from 100 mV to 1000 mV at 1 μA with 5 mV per second during 180 s.

3.2. Recovery

Blank samples (1 ml) (spiked with 10, 20 and 50 ng of each substance) were extracted as described above. Finally the internal standard (100 ng in 100 μl of methanol) was added to each residue. Peak-area ratios of the extracts were compared with those obtained from direct

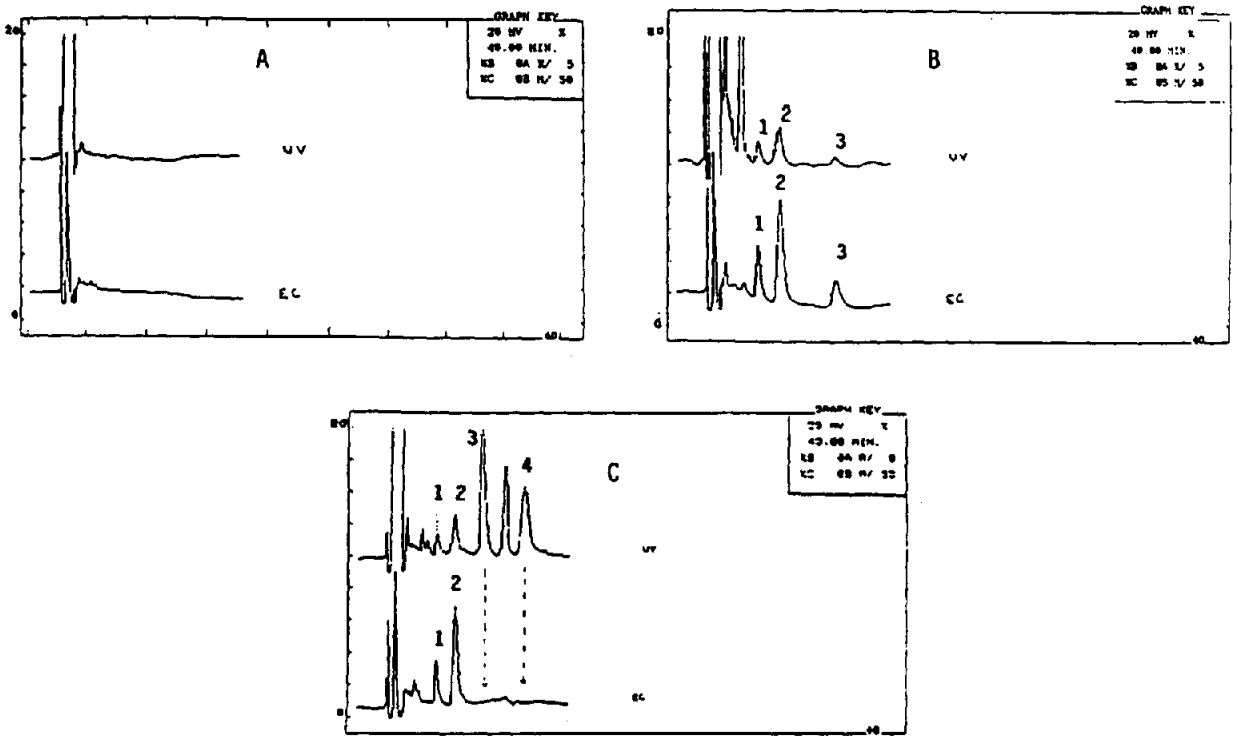

Fig. 2. Chromatograms from plasma samples obtained with both UV detection at 220 nm (upper chromatograms) and electrochemical detection at 600 mV (lower chromatograms). (A) Blank plasma (2 ml); (B) plasma (1 ml) of an heroin addict. Peaks: 1 = M, 22 ng/ml, 2 = I.S., 100 ng added, 3 = 6MAM, 32 ng/ml; (C) plasma (2 ml) of a patient treated with codeine plus ethylmorphine. Peaks: 1 = M, 9 ng/ml, 2 = I.S., 100 ng added. Peaks of codeine (3) and ethylmorphine (4) are only present in the chromatogram obtained by UV detection.

injection of standard solutions containing 10, 20 and 50 ng of M and 6MAM and 100 ng of I.S. in 100 μl of methanol. The recovery was tested with two assays. In the first assay M and 6MAM were extracted simultaneously, in the second the drugs were extracted separately to avoid possible

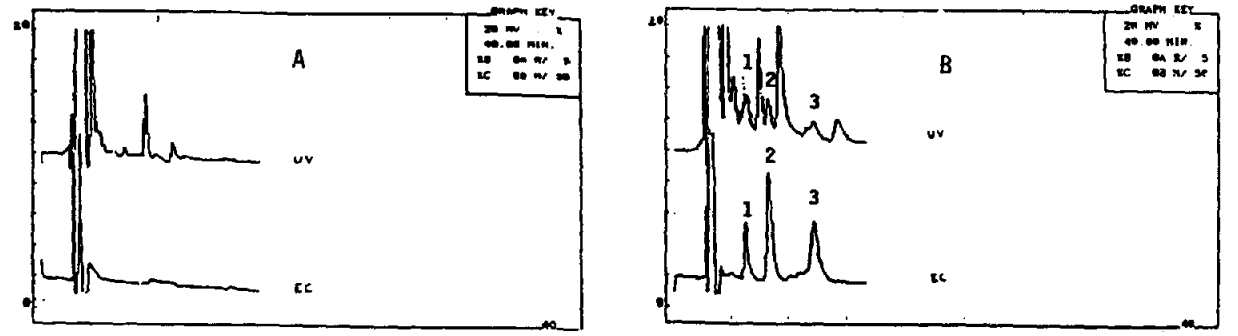

Fig. 3. Chromatograms from whole blood samples obtained with both UV detection at 220 nm and electrochemical detection. (A) Blank whole blood (2 ml); (B) whole blood (2 ml) of an heroin user. Peaks: 1 = M, 13 ng/ml, 2 = I.S., 100 ng added, 3 = 6MAM, 39 ng/ml.

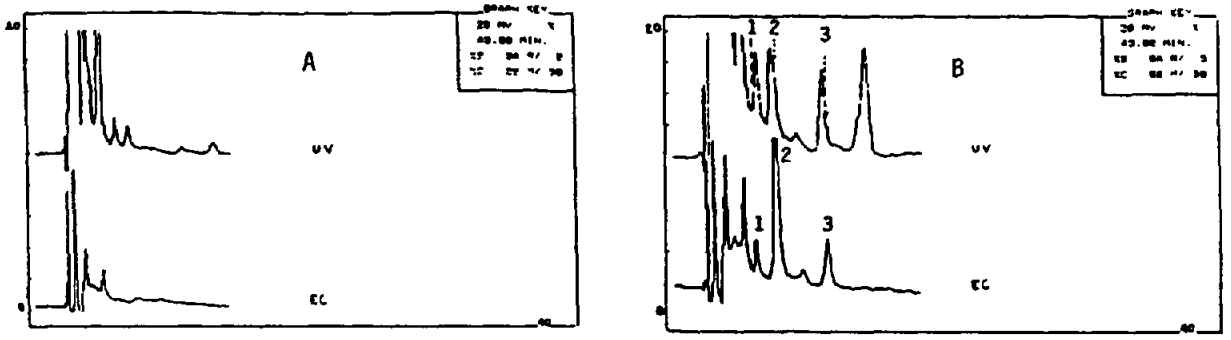

Fig. 4. Chromatograms from urine samples obtained with both UV detection at 220 nm and electrochemical detection. (A) Blank urine (2 ml); (B) urine (1 ml) of an heroin user. Peaks: 1 = M, 8 ng/ml, 2–I.S., 100 ng added, 3 = 6MAM, 43 ng/ml.

overestimation of morphine due to 6MAM hydrolysis. Each assay was carried out immediately after the addition of the drugs. The results are shown in Table 1. No significant differences were found between these two assays. No increase of M in assay 1 or the morphine peak in assay 2 were observed. Thus no conversion of 6MAM was demonstrated during the extraction procedure. The recoveries were in the range 92–99% for the two substances (Table 1). For M the extraction percentage was higher from urine than from plasma and whole blood. For 6MAM the recoveries were in the same range for the two assays.

3.3. Stability during storage

The stability of M and 6MAM was studied after storage at +20°C and −20°C for up to two weeks.

Stability in methanol (standard solutions)

The test was carried out after injection of 20 ng M and 80 ng 6MAM as standard solution onto the chromatograph. The analysis showed no degradation of M at +20°C and −20°C; however, at +20°C a decrease of 6MAM was observed: from 5%, after a 12-hour storage period, to 27% after fourteen days (Table 2). Thus the

Table 1
Recovery of morphine (M) and 6-monoacetylmorphine (6MAM).

Added (ng/ml)	Matrix[a]	Extraction percentage (mean ± S.D., $n = 6$)			
		Assay 1		Assay 2	
		M	6MAM	M	6MAM
10	P	95 ± 5	95 ± 5	95 ± 3	96 ± 5
	B	92 ± 5	95 ± 4	93 ± 5	95 ± 4
	U	96 ± 5	95 ± 2	96 ± 2	96 ± 3
20	P	95 ± 3	96 ± 4	95 ± 4	96 ± 4
	B	92 ± 4	96 ± 3	94 ± 5	95 ± 5
	U	97 ± 3	96 ± 2	97 ± 4	96 ± 3
50	P	96 ± 2	96 ± 3	97 ± 2	97 ± 2
	B	93 ± 3	96 ± 5	95 ± 2	97 ± 4
	U	99 ± 2	96 ± 4	98 ± 2	98 ± 3

[a] P = plasma, B = whole blood, U = urine.

Table 2
Stability of morphine (M) and 6-monoacetylmorphine (6MAM)

Time	Methanolic solution				Biological samples, storage at +20°C and −20°C					Sample after enzymatic hydrolysis (+37°C)	
	M (20 ng)		6MAM (80 ng)		M (20 ng/ml)		6MAM (80 ng/ml)			M (20 ng/ml)	6MAM (80 ng/ml)
	+20°C	−20°C	+20°C	−20°C	+20°C	−20°C	+20°C	−20°C	−20°C[a]		
0h	0	0	0	0	P B U	0	0	0	0		
2h	0	0	0	0	P B U	0	0	0			
4h	0	0	0	0	P B U	0	12 10 10	0		0	75 73 80
8h	0	0	0	0	P B U	0	50 47 52	0		0	100 100 100
12h	0	0	5	0	P B U	0	62 50 62	0		0	
1d	0	0	10	0	P B U	0	65 62 65	0		0	
4d	0	0	12	0	P B U	0	75 68 80	0			
7d	0	0	19	0	P B U	0	93 82 94	6 5 7			
14d	0	0	27	0	P B U	0	100 100 100	7 9 9	0		

Data expressed as percentage loss.
[a] Only thawing at 14d.
P = plasma, B = whole blood, U = urine.

standard solutions, particularly that of 6MAM, must be stored at −20°C.

Stability in sample

Two aliquots of sample (plasma, whole blood and urine) were spiked each with 20 ng/ml of M and 80 ng/ml of 6MAM and frozen at −20°C until analysis. After thawing, the samples were treated according to the above extraction procedure. The results obtained are shown in Table 2. M was very stable in both cases (+20°C and −20°C), but 6MAM proved relatively unstable:

after four hours at +20°C a loss of 10–12% was observed and after seven days the compound was almost entirely hydrolysed (with a loss of 82–94%). These results showed that the storage of 6MAM at room temperature (+20°C) could be the origin of an overestimation of M. At −20°C, 6MAM remained stable up to one week, then its concentrations decreased slowly with a significant loss of 6–9% after fourteen days. Without successive freezing and thawing, 6MAM was stable up to 14 days. Thus after collection, the sample must be immediately frozen at −20°C until analysis. For control samples containing 6MAM, successive freezing and thawing must be avoided.

3.4. Stability in sample after enzymatic hydrolysis

For the determination of M-6-glucuronide enzymatic hydrolysis by β-glucuronidase at +37°C was often proposed. Under such conditions 6MAM could be converted to M. Thus 6MAM conversion could contribute to the total amount of M (free plus conjugated) and lead to an overestimation of conjugated M. The stability of M and 6MAM was studied separately after incubation with 10 000 I.U. β-glucuronidase per ml of sample, at pH 6.8 (phosphate buffer) (37°C). Every four hours one ml of hydrolysate was treated as described above. For 6MAM, a 73–80% loss was observed after a 24-h incubation period.

3.5. Reproducibility

Reproducibility was tested on a pool of blank samples spiked with 5, 10, 20, 50 and 100 ng/ml of each compound. M and 6MAM were then determined simultaneously. Within-day coefficients of variation (C.V. %) were less than 10.5% for M and 11% for 6 MAM (Table 3). Day-to-day coefficients of variation were less than 11% for M and 12.4% for 6MAM over a period of two weeks (the samples were frozen at −20°C until analysis).

Table 3
Reproducibility of morphine (M) and 6-monoacetylmorphine (6MAM) determination

Added (ng/ml)	Matrix	Within day C.V. ($n = 8$) (%)		Day-to-day C.V. ($n = 8$) (%)	
		M	6MAM	M	6MAM
5	P	9.50	10.20	10.20	12.40
	B	10.50	11.00	11.00	12.00
	U	7.84	8.10	9.80	9.80
10	P	9.50	9.50	9.50	10.50
	B	10.20	10.80	10.80	11.40
	U	7.80	9.60	8.20	9.20
20	P	8.20	7.90	9.10	10.30
	B	8.00	7.50	9.60	10.20
	U	7.30	7.06	8.90	9.40
50	P	6.70	7.10	8.20	9.50
	B	7.20	7.80	9.00	10.00
	U	6.80	8.00	8.10	8.60
100	P	3.20	5.60	6.85	8.80
	B	4.30	7.00	6.50	9.50
	U	3.90	6.90	6.03	9.05

P = plasma, B = whole blood, U = urine.

3.6. Sensitivity

To assess the optimal detection potential for M and 6MAM the voltammograms of the two compounds were established. They were obtained after filling the detector cell with standard solution of M or 6MAM (in mobile phase, pH 6.5) and scanning from 100 mV to 1000 mV. A plateau was reached at 600 mV for M and 750 mV for 6MAM (Fig. 1), indicating the optimal potentials for the detection of M and 6MAM respectively. A 600-mV potential was selected because, when 750 mV was used, other unknown peaks from oxidisable substances in the samples were often observed. In this study at 600 mV, after injection of equal quantities of the standard solutions, the M peak was four-fold higher than the 6MAM peak.

According to O'Connor et al. [16], morphine is the most electrochemically labile among the opiate drugs. This property could be attributed to the two oxidisable hydroxyl groups present in its structure. The other compounds would be less electrochemically active because they carry substitutions at these positions. According to Tagliaro et al. [17] the only phenolic group is oxidisable; in morphine an electron oxidation seems to occur at the 3-hydroxy group, prior to its ionization, followed by dimerization to pseudomorphine. Jordan and Hart [18] demonstrated the mechanism of electrochemical activity by comparing morphine and codeine at 450 mV (where morphine shows a good response). The only difference between the morphine and codeine molecule is that the 3-phenolic hydroxyl in codeine is blocked by a methyl group. After analyzing codeine, no response was observed at 450 mV; this confirmed that morphine oxidation can be attributed to the phenolic group. Moreover the authors affirmed that oxidation occurs in three steps: first oxidation of the phenolic group, followed by oxidation of the product, pseudomorphine, and finally oxidation of the tertiary amine group (at a high potential).

6MAM contains the same oxidisable group. However its optimal detection potential is ca. 150 mV higher than that of morphine. This difference could be attributed to the presence of an acetyl substituent on the 6-hydroxy group.

The lower limit of detection was found to be 100 pg for M and 400 pg for 6MAM (injected directly onto the chromatographic systems). These amounts gave a signal-to-noise ratio of 3 for M and 3.5 for 6MAM at 10 nA full scale deflection. In samples spiked with 100 pg of M and 400 pg of MAM, no peaks were observed. In such a case the determination limits were 1 ± 0.4 ng/ml ($n = 6$) for M and 4 ± 0.5 ng/ml ($n = 6$) for 6MAM, using 2 ml of plasma or urine. When 2 ml of whole blood was used, the cartridge was often clogged; in this case two cartridges were required for an assay and the collected eluates had to be pooled before evaporation. Under these conditions the detection limits for whole blood were about the same as those for plasma or urine using 2 ml of sample.

3.7. Selectivity

Comparison with UV detection at 220 nm confirmed that the used electrochemical detection at 600 mV potential gave a better sensitivity and selectivity in real matrices (Figs. 2–4). Chromatograms of blank samples showed no background interference from endogenous constituents. Several drugs were tested for possible interferences (Table 4). M was well separated from normorphine. Opiate drugs, e.g. heroin, codeine, norcodeine, ethylmorphine, pholcodine, buprenorphine, naltrexone, naloxone, did not interfere with M and 6MAM because of their different capacity factors (k') or absence of electrochemical response.

Nalorphine, which has been used as an antagonist in the treatment of heroin or morphine poisoning, has now been replaced by naloxone. Thus its use as internal standard can be maintained.

Non-opiate analgesics, such as pentazocine, pethidine, methadone, dextromoramide, dextropropoxyphene and nordextropropoxyphene, were resolved from M and 6MAM. Non-narcotic analgesics, e.g. acetaminophen, acetylsalicylic acid, glafenine and other drugs (listed in Table 4), were also tested. No interferences were observed.

On the basis of these results the proposed method seems to be specific for M and 6MAM.

Table 4
Capacity factor (k') and quality response of drugs tested for possible analytical interference

Drug (200 ng/ml)	Electrochemical response (at 600 mV) and (k')	
Opiate analgesics		
Buprenorphine	–	none
Codeine	–	
Ethylmorphine	–	
Heroin	–	
6-Monoacetylmorphine (6MAM)[a]	2.30	good
Morphine (M)[a]	0.75	good
Nalorphine (I.S.)[a]	1.40	good
Naloxone	1.48	medium
Naltrexone	1.64	medium
Normorphine	0.38	medium
Norcodeine	–	none
Pholcodine	1.32	medium
Non-opiate analgesics		
Dextromoramide	–	none
Dextropropoxyphene	–	
Methadone	–	
Nordextropropoxyphene	–	
Pentazocine	7	poor (tailing)
Pethidine	–	none
Other drugs		
Acetaminophen	0.30	good
Acetylsalicylic acid	–	none
Amitriptyline	–	
Amphetamine	–	
Benzoylecgonine	–	
Bupivacaine	–	
Caffeine	–	
Clomipramine	–	
Cocaine	–	
Diazepam	–	
Glafenine	–	
Imipramine	–	
Lidocaine	–	
Lorazepam	–	
Methamphetamine	–	
Nicotine	–	
Oxazepam	–	

[a] Compound analysed in present study.

Although electrochemical detection represents a very sensitive and specific technique, problems of sample preparation still remain, especially when complex or putrified materials have to be investigated.

4. Conclusions

The proposed procedure for the simultaneous determination of morphine and 6-monoacetylmorphine in whole blood, plasma and urine by high-performance liquid chromatography, using amperometric detection at 600 mV, is reproducible, sensitive and selective. It can be applied to pharmacokinetic studies of heroin. It is also suitable for forensic toxicology to distinguish heroin use from medical prescription of morphine and other opiate drugs.

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