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Determination of morphine and its 3- and 6-glucuronides, codeine, codeine-glucuronide and 6-monoacetylmorphine in body fluids by liquid chromatography atmospheric pressure chemical ionization mass spectrometry

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Abstract

A selective assay of morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), morphine, codeine, codeine-6-glucuronide (C6G) and 6-monoacetylmorphine (6-MAM) based on liquid chromatography atmospheric pressure chemical ionization mass spectrometry (LC–APCI–MS) is described. The drugs were extracted from serum, autopsy blood, urine, cerebrospinal fluid or vitreous humor using C_{18} solid-phase extraction cartridges and subjected to LC–APCI–MS analysis. The separation was performed on an ODS column in acetonitrile–50 m*M* ammonium formate buffer, pH 3.0 (5:95), using a flow-rate gradient from 0.6 to 1.1 ml/min (total analysis time was 17 min). The quantitative analysis was done using deuterated analogues of each compound. Selected-ion monitoring detection was applied: *m/z* 286 (for morphine, M3G-aglycone and M6G-aglycone), 289 (for morphine-d_3, M3G-d_3-aglycone and M6G-d_3-aglycone), 300 (for codeine and C6G-aglycone), 303 (for C6G-d_3-aglycone), 306 (for codeine-d_6), 328 (for 6-MAM), 334 (for 6-MAM-d_6), 462 (for M3G and M6G), 465 (for M3G-d_3 and M6G-d_3), 476 (for C6G) and 479 (for C6G-d_3). The limits of quantitation were: 1 μg/l for morphine, 2 μg/l for 6-MAM, 5 μg/l for M3G, M6G and codeine and 200 μg/l for C6G. The recovery ranged from 85 to 98% for each analyte. The method appeared very selective and may be used for the routine determination of opiates in body fluids of heroin abusers and patients treated with opiates. © 1997 Elsevier Science B.V.

Keywords: Morphine; Morphine glucuronide; Codeine; Codeine-6-glucuronide; 6-Monoacetylmorphine

1. Introduction

The simultaneous determination of opiates and their glucuronides in body fluids is of great practical value in clinical and forensic toxicology. In the case of morphine, this drug is metabolized mainly to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). M6G shows high affinity for the opioid receptor and exerts corresponding analgesic activity [1–10]. During chronic medication with morphine, M6G may accumulate in the body of patients with renal failure and precipitate symptoms of morphine overdose [11–14].

In the case of suspected heroin abuse or overdose, the differentiation between heroin and morphine intake can be unequivocally done on the basis of 6-monoacetylmorphine (6-MAM) identification in

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blood and urine, usually with gas chromatography–mass spectrometry (GC–MS) [15–21].

The concentration of 6-MAM in body fluids, as well as the ratio of free morphine to its glucuronides may give some clues in the assessment of survival time after acute heroin overdose [22–27].

The relevance of determination of all heroin metabolites became even more evident after a recent report hypothesized that heroin, 6-MAM and M6G probably act through a unique receptor mechanism, different from that of morphine [28].

In the case of codeine intake, assessment of the metabolic profile requires the determination not only of the parent drug, but also of codeine-6-glucuronide (C6G), as well as morphine, M3G and M6G [29].

For the simultaneous determination of parent opiates (morphine or codeine) and their glucuronides, solid-phase extraction with high-performance liquid chromatographic (HPLC) separation and UV absorbance [30], electrochemical [31,32], fluorimetric [33] detection or a combination of these techniques [34,35] was reported. The advent of liquid chromatography atmospheric pressure ionization mass spectrometry (LC–API–MS) brought an application of this technique (electrospray option) for morphine and its glucuronides [36,37].

In our previous study, morphine, M3G, M6G and 6-MAM were determined in body fluids of heroin victims by means of LC–APCI–MS, using two isocratic elution runs and morphine-d_3 as an internal standard [38].

The purpose of the present study was to develop an LC–APCI–MS procedure for the determination of morphine, M3G, M6G, codeine, C6G and 6-MAM in one chromatographic run, using separate deuterated internal standards for each compound involved.

2. Experimental

2.1. Reagents

Morphine, morphine-d_3, M3G, M6G, codeine and 6-MAM were obtained from Sigma-Aldrich (Deisenhofen, Germany). M3G-d_3, M6G-d_3, C6G and C6G-d_3 were purchased from Lipomed (Arlesheim, Switzerland), 6-MAM-d_6 was from Radian (Austin, TX, USA) and codeine-d_6 was from High Standard Products (Inglewood, CA, USA).

Ammonium carbonate buffer (0.01 *M*, pH 9.3) for extraction: to 900 ml of ammonium carbonate solution (0.96 g/l), ammonium hydroxide was added (at first concentrated, then a 1 *M* solution) to pH 9.3 (determined using a pH meter) and the solution was made up to 1000 ml with ammonium carbonate. Ammonium formate buffer stock solution (0.5 *M*, pH 3.0) for the HPLC mobile phase: to 400 ml of 500 m*M* ammonium formate solution (15.765 g/500 ml), formic acid was added (at first concentrated, then 1 *M* solution) to pH 3.0 (determined using a pH meter) and the solution was made up to 500 ml with 500 m*M* ammonium formate.

Ammonium formate buffer working solution (0.05 *M*, pH 3.0) for HPLC was prepared from stock solution by dilution (1:10, v/v) with water.

Solid-phase extraction (SPE) cartridges, Bond Elut C_{18} (200 mg), were supplied by ICT (Bad Homburg, Germany). The cartridges were rinsed with 1 ml of methanol, 1 ml of water and 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) before use.

2.2. Biological samples

The serum used for method validation was obtained from a local blood bank and was preliminarily screened for the absence of drugs using an immunochemical procedure (EMIT).

Blood and urine samples taken from living persons suspected of driving under the influence of drugs (over 80 cases), as well as blood, urine, cerebrospinal fluid (CSF) and vitreous humor samples taken during autopsy from seven victims of suspected heroin overdoses, were analyzed. Urine samples were also taken from a volunteer after oral intake of 60 mg of codeine.

2.3. Sample preparation

A 1.5-ml volume of each sample was centrifuged for 5 min at 14 000 *g*, to remove cell debris. A 1-ml volume of supernatant was vortex-mixed with 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) and with the internal standard mixture (morphine-d_3, M3G-d_3, M6G-d_3, codeine-d_6, C6G-d_3 and 6-MAM-d_6, 100 ng each). After a 10 min centrifugation at

5000 *g*, which removed all particles, 2 ml of clear supernatant were applied on the SPE cartridge and slowly passed through it (ca. 5 min). The SPE cartridge was rinsed with 2 ml of 0.01 *M* ammonium carbonate buffer (pH 9.3) and vacuum dried for 5 min. The retained drugs were eluted with 0.5 ml of methanol–0.5 *M* acetic acid (9:1, v/v) under gravity force. The eluates were dried under nitrogen, reconstituted in 100 μl of HPLC mobile phase and centrifuged for 4 min at 14 000 *g* and, finally, 10–20 μl of supernatant were injected manually into the LC–MS system.

2.4. Liquid chromatography

A Merck–Hitachi Model 2000 gradient pump with a Type 8125 Rheodyne injection valve (20 μl loop) was used. The chromatographic separation was performed in the isocratic mode with a Superspher RP 18 column (125×3 mm I.D., 4 μm particle size; Merck, Darmstadt, Germany). The mobile phase consisted of acetonitrile–50 m*M* ammonium formate buffer, pH 3.0 (5:95, v/v). The flow-rate was programmed as follows: 0.6 ml/min for 4 min, increased to 1.1 ml/min in 3 min, 1.1 ml/min for 10 min.

2.5. APCI–MS

A SSQ 7000 single quadrupole instrument (Finnigan MAT, San Jose, CA, USA), equipped with an APCI source, was used. The APCI inlet conditions were as follows: sheath gas (nitrogen) pressure, 70 p.s.i.; auxiliary gas (nitrogen), 20 ml/min; heated vaporizer temperature, 400°C; heated capillary temperature, 170°C; corona current, 5 μA. Mass spectra of substances involved were taken between 100 and 500 u at an octapole offset of 10 V (positive ions). Based on the mass spectra in full scan mode and on the observed retention times, a procedure was written for the selected-ion monitoring (SIM) detection of a number of precursors in one chromatographic run. Time windows and ions monitored were: time (*t*) 0–5 min, *m/z* 286, 289, 462 and 465; *t*=5–11 min, *m/z* 300, 303, 306, 476 and 479; *t*=11–17 min, *m/z* 328 and 334. The scan time was 0.5 s.

3. Results and discussion

3.1. APCI mass spectra

Figs. 1 and 2 show the mass spectra of the compounds examined, taken in full scan mode (*m/z* 100–500 u). In the case of morphine and morphine-d_3, only protonated molecules, $(M+H)^+$ at *m/z* 286 and 289 and corresponding isotopic peaks $(M+1+H)^+$ were observed (Fig. 1a). Besides its molecular peak, codeine showed a loss of an hydroxyl group (Fig. 1c). 6-MAM and 6-MAMd_6 showed mainly protonated molecular ions and the fragments *m/z* 268 and 271, respectively. For 6-MAMd_6, an acetonitrile adduct (*m/z* 375) was also observed (Fig. 1b,d).

Morphine and codeine glucuronides underwent distinct fragmentation to the corresponding aglycones (morphine, codeine or their deuterated analogues). In the cases of M3Gd_3 and M6Gd_3, a fragment at *m/z* 271 was also observed (Fig. 2).

It must be stressed that the extent of fragmentation of M3G and M6G depended greatly on the composition of the mobile phase, i.e. increased with an increasing amount of acetonitrile (Fig. 3). This phenomenon may be caused by at least two variables; the percentage of organic modifier (acetonitrile) or the ionic strength of the mobile phase. Control experiments showed that the other variable, i.e. a change in the flow-rate (in the range of 0.3–0.7 ml/min) did not exert any visible influence on mass spectra. The ratios of drug to internal standard in the two phases were practically unaffected; for M3G, they were 0.85 and 0.87, for M6G, they were 1.99 and 1.88 and for morphine, they were 0.46 and 0.47, respectively. The fragmentation was virtually unaffected by changes in the heated vaporizer (in the range of 400–550°C) or in the temperature of the heated capillary (in the range of 170–200°C).

The influence of different batches of the same mobile phase on the fragmentation of LSD using electrospray (ESI) LC–MS was reported by Webb et al. [39]. Although the primary ionization mechanisms in APCI and ESI are not identical, the fragmentation (collision-induced dissociation) in both techniques occurs in the octapole region and, therefore, the mass spectra obtained with APCI and ESI are very similar. The problem of reproducibility of

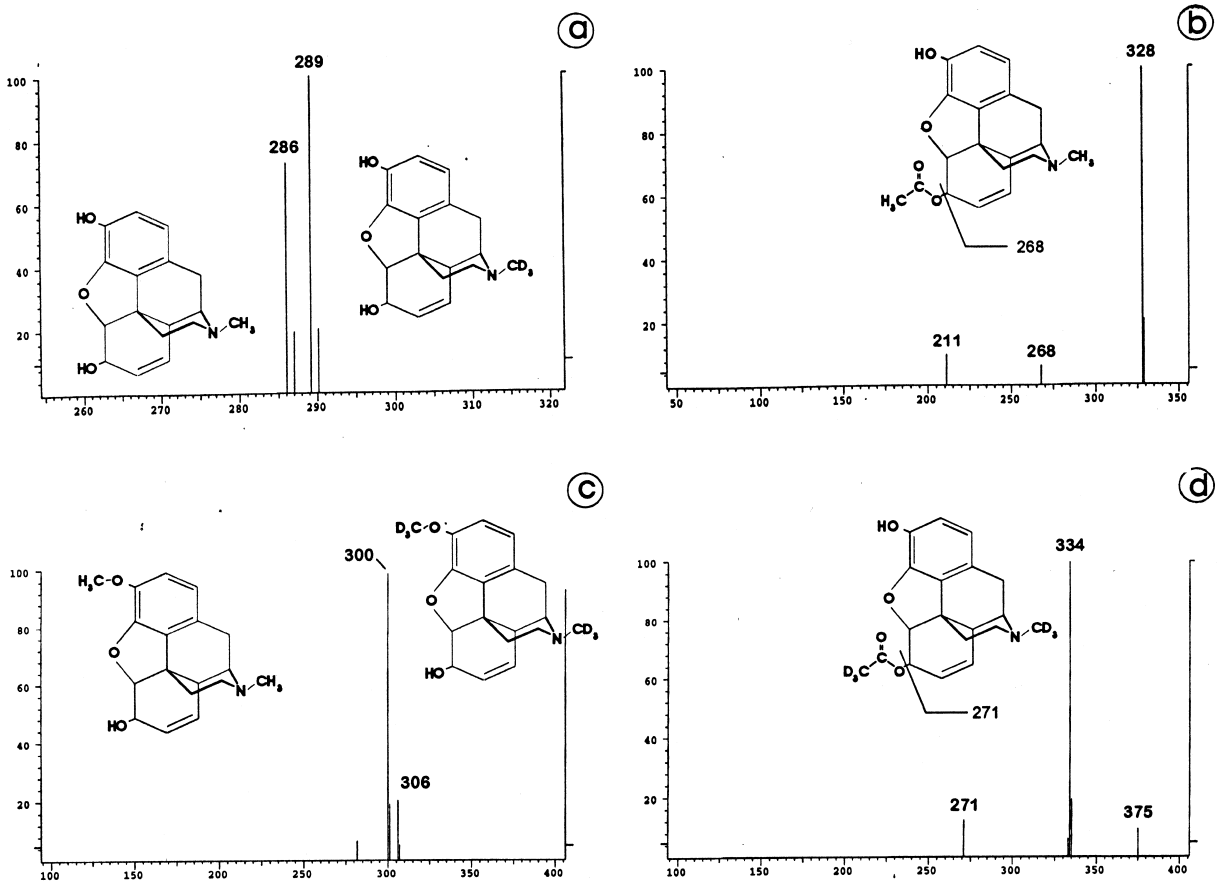

Fig. 1. Mass spectra of morphine and morphine-d_3 (a), 6-MAM (b), codeine and codeine-d_6 (c) and 6-MAM-d_6 (d).

APCI mass spectra is of critical importance and a systematic inter-laboratory study on this topic is already in progress.

3.2. Separation and validation

In pilot experiments on chromatographic separation, several acetonitrile gradient elution programs were tried. It was observed that, despite satisfactory separation, the background noise in the gradient elution gradually increased, affecting the detection limits of later-eluting drugs. Also, the application of an acetonitrile gradient, which is never totally reproducible due to technical reasons, may influence the degree of fragmentation, as was mentioned above. Therefore, a gradient of flow-rate was applied instead. All examined substances were fully separated under the chromatographic conditions used. Typical retention times (in min) were as follows: for M3G, 2.2; for M6G, 3.3; for morphine, 4.1; for C6G, 7.8; for codeine, 9.5 and for 6-MAM, 14.5 (Fig. 4 Fig. 5). All deuterated analogues eluted slightly earlier. This had been observed previously for amphetamines, examined by LC–APCI–MS [40].

The results of the fragmentation study, together with the chromatographic behavior of all compounds observed in full scan runs, served as a basis for time-scheduled SIM conditions. From 0 to 5 min, the molecular and fragment ions of M3G, M3G-d_3, M6G, M6G-d_3, morphine and morphine-d_3 were registered; from 5 to 11 min, the molecular and

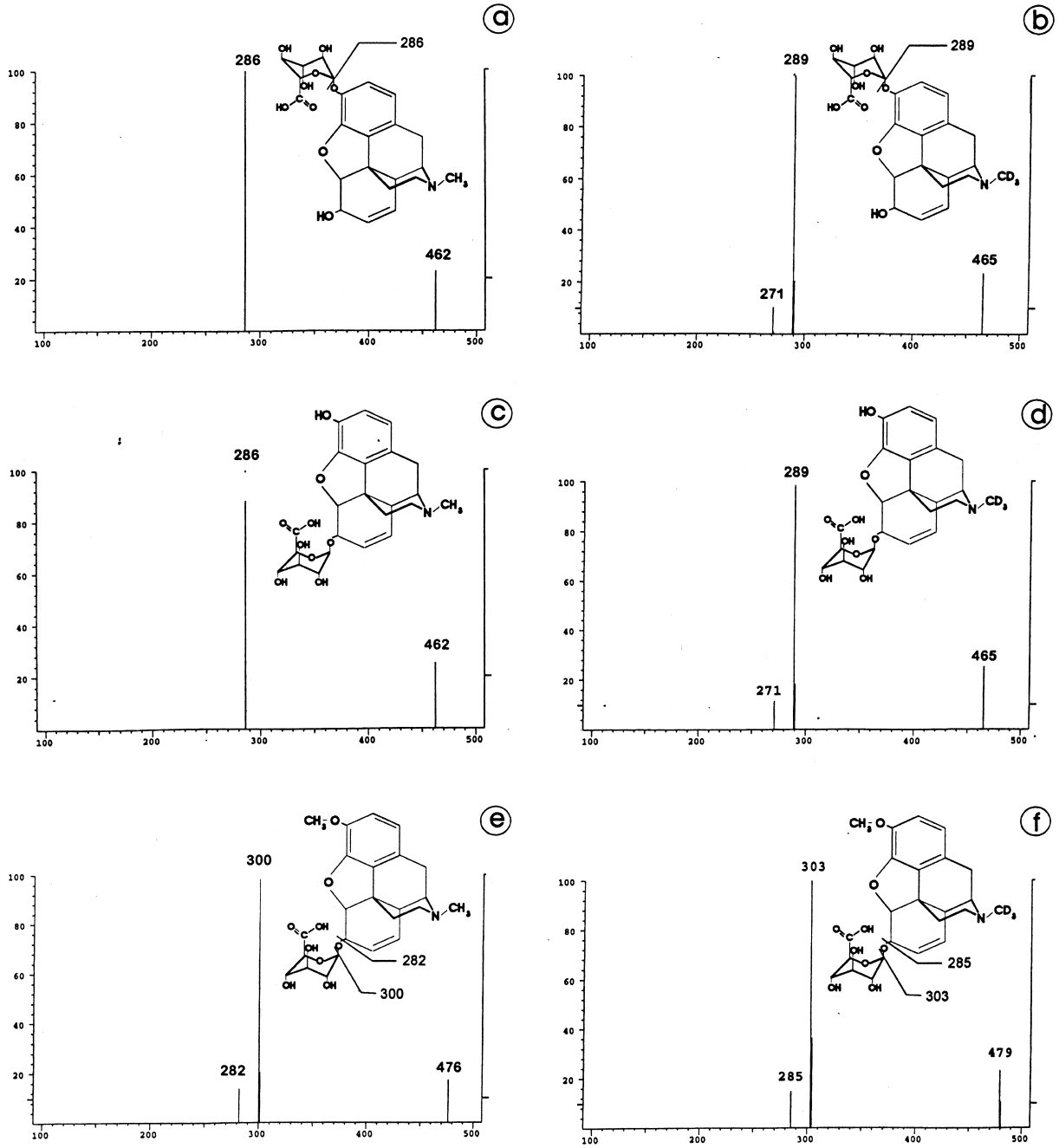

Fig. 2. Mass spectra of M3G (a), M3G-d_3 (b), M6G (c), M6G-d_3 (d), C6G (e) and C6G-d_3 (f).

fragment ions of C6G, C6G-d_3, codeine and codeine-d_6 were monitored and from 11 to 14 min, the molecular ions of 6-MAM and 6-MAM-d_6 were monitored. For quantitation, the protonated molecular ions of analytes and their deuterated analogues were used.

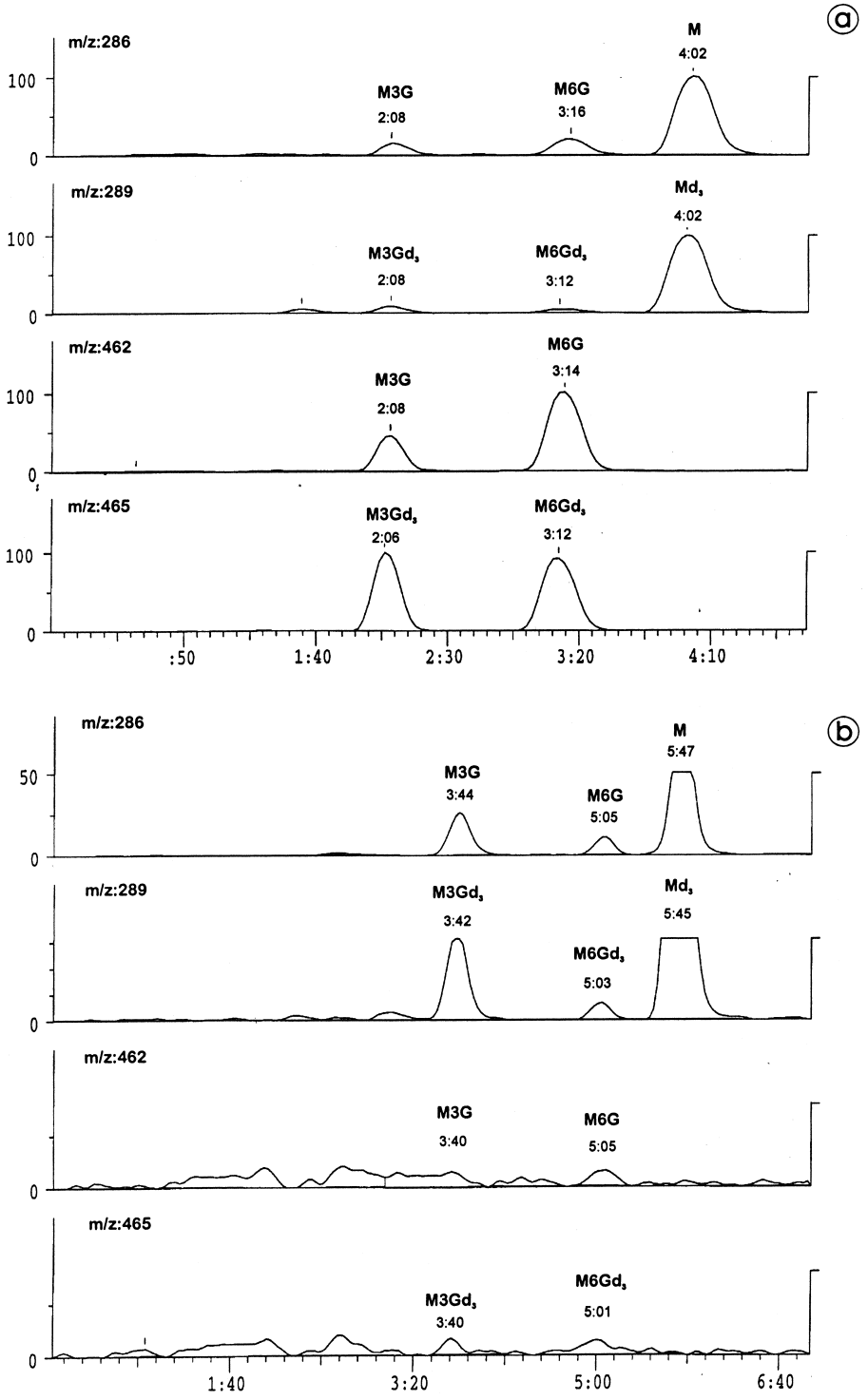

Fig. 3. Influence of mobile phase composition on the fragmentation of M3G and M6G. Both chromatograms were obtained in acetonitrile–50 m*M* ammonium formate buffer m*M* (pH 3.0) mixtures. (a) With 5% acetonitrile and a flow-rate of 0.6 ml/min. (b) With 7% acetonitrile and a flow-rate of 0.3 ml/min.

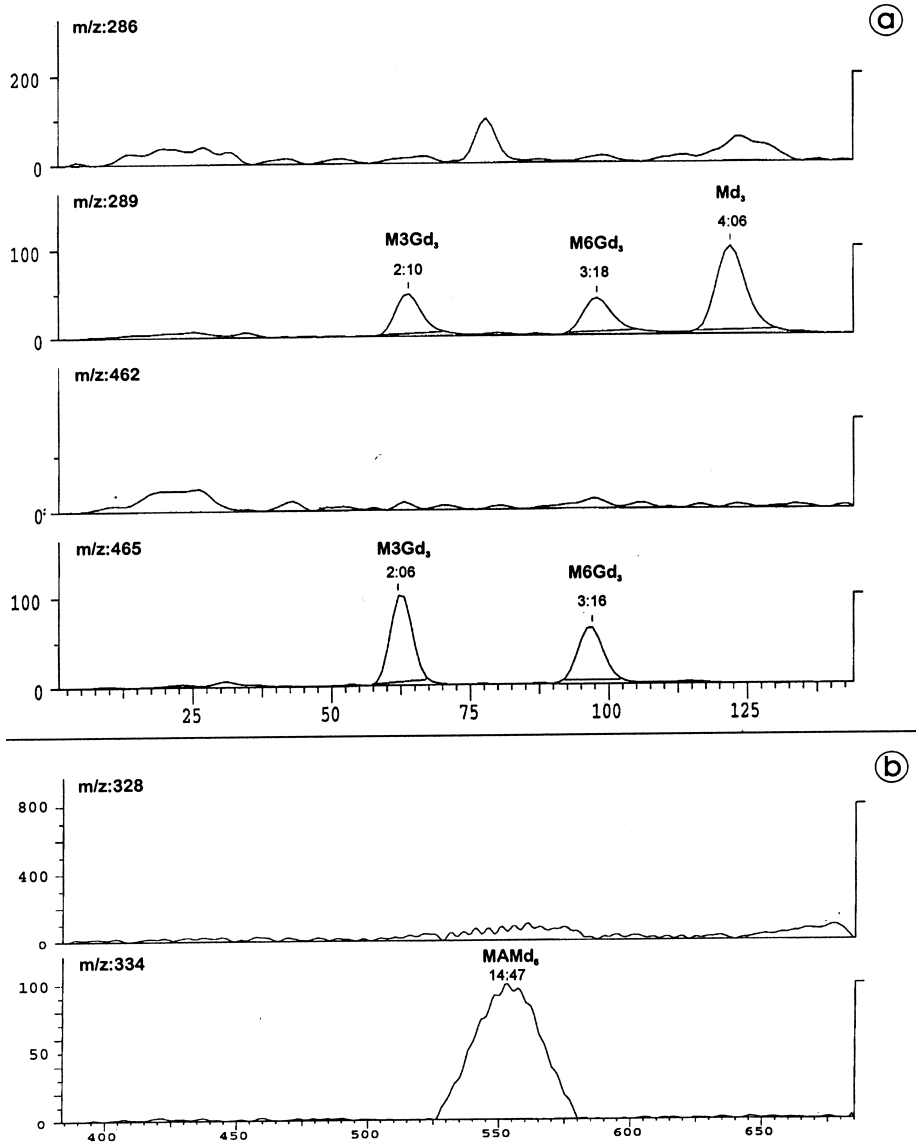

Fig. 4. Chromatogram of blank serum spiked with the mixture of deuterated internal standards.

In the preliminary study, blank serum, blood, urine and CSF samples were spiked with a mixture containing 100 ng/ml of M3G, M6G, morphine, codeine and 6-MAM and subjected to extraction and LC–MS analysis. The results of quantitative analysis showed virtually no differences between these matrices. Therefore, for further validation experiments, only serum standards were used. The validation was done in three series of serum standards, spiked with morphine, M3G, M6G, codeine, C6G and 6-MAM in a concentration range from 5 to 500 μg/l. The quantitation was performed against the respective deuterated analogues, which were used as internal standards. Table 1 shows the results of the validation. The within-day precision was measured in three series at the following concentrations: 50 μg/l for morphine and 6-MAM, 100 μg/l for M3G, M6G and codeine, and 500 μg/l for C6G. Limits of detection

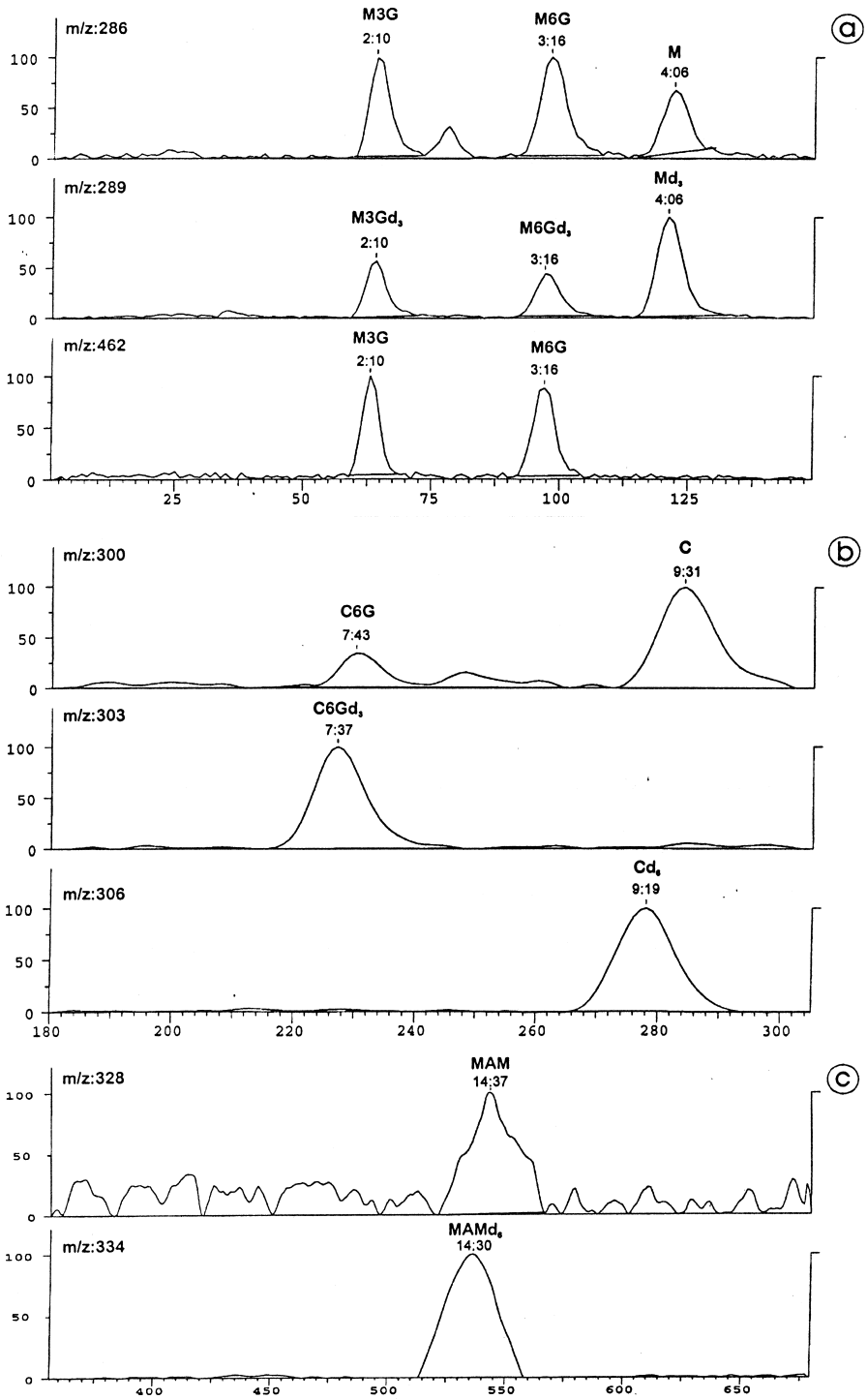

Fig. 5. Chromatogram of blank serum spiked with morphine (20 μg/l), M3G, M6G and codeine (100 μg/l), C6G (200 μg/l) and 6-MAM (5 μg/l).

(LOD) were defined as a signal-to-noise ratio of three. The limit of quantitation was taken to be twice the LOD. The absolute recoveries were expressed as the percentage peak area of non-extracted drugs (in amounts corresponding to 100% recovery) injected into the LC–MS system.

In several blank samples of serum, blood or urine, no peaks corresponding in mass profile and retention

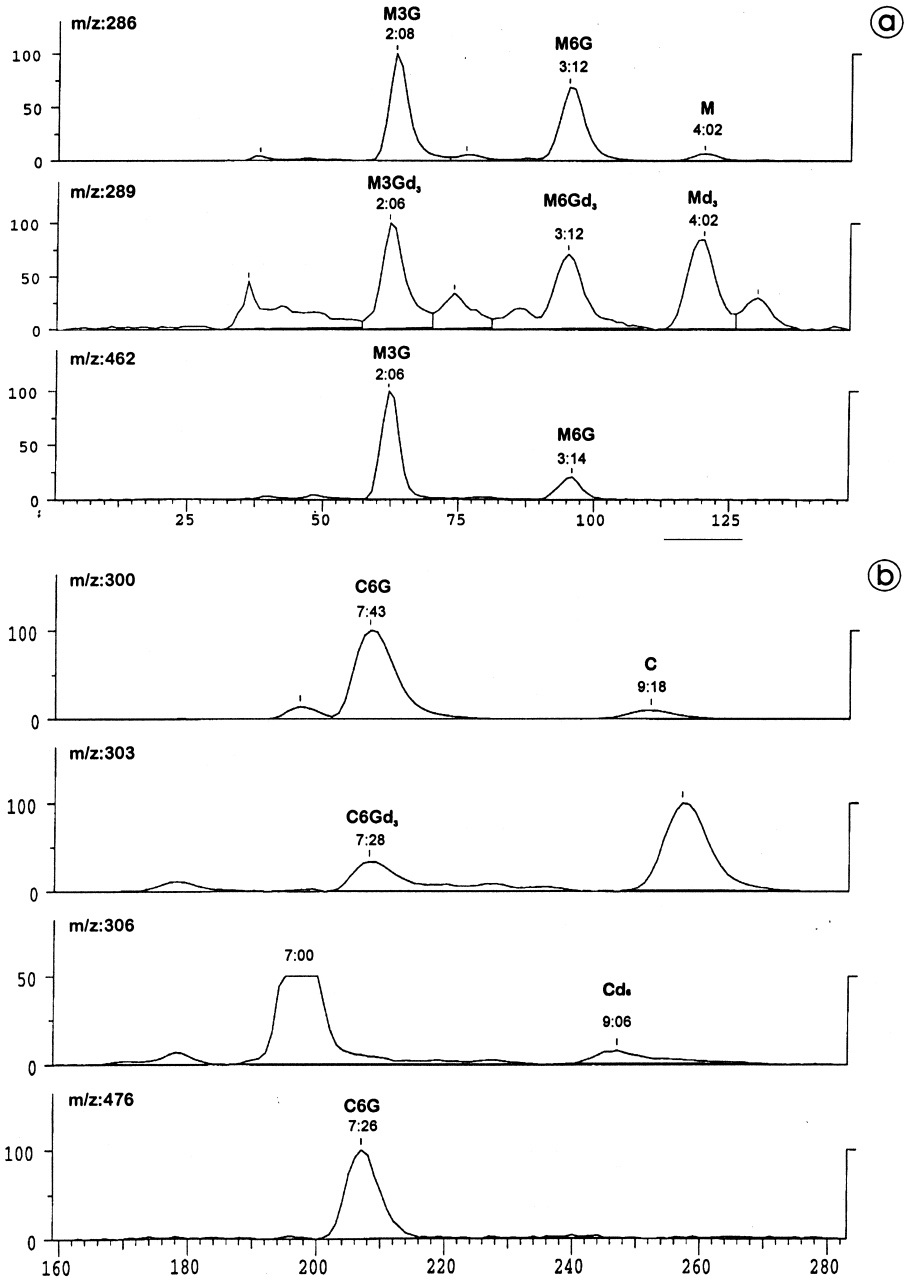

Fig. 6. Chromatogram of a urine extract, collected 0–6 h after oral intake of 60 mg of codeine. The following concentrations were found (in μg/l): M3G, 945; M6G 930; morphine, 47; C6G, 18 100 and codeine, 4900.

Table 1
Validation data

Compound	Retention time (min)	LOD[a] (μg/l)	Linearity	r^2	Recovery[b] (%)	Precision[c] (% R.S.D.)
M3G	2.2	2.5	$y=0.0087x+0.12$	0.9948	94	7
M6G	3.3	2.5	$y=0.011x+0.13$	0.9998	97	5.5
Morphine	4.1	0.5	$y=0.0152x+0.05$	0.9955	98	3
C6G	7.8	100	$y=0.0252-4.35$	0.9574	90	10
Codeine	9.5	2.5	$y=0.0044-0.31$	0.9973	91	5
6-MAM	14.5	1	$y=0.0115x-0.02$	0.9937	85	5

[a]Defined as 3× a signal-to-noise ratio of three. Twice the LOD was taken to be the limit of quantitation.
[b]Defined as the percentage peak area of corresponding amounts of non-extracted drugs injected into the LC–MS system.
[c]Calculated in three series (day-to-day) at the following concentrations: 50 μg/l for morphine and 6-MAM, 100 μg/l for M3G, M6G and codeine and 500 μg/l for C6G.

time to morphine, M3G, M6G, codeine or MAM, were observed. In the case of C6G, the background noise was particularly high at m/z 476. Also, a peak of m/z 300, corresponding to codeine (C6G aglycone), eluted from some serum extracts at a retention time corresponding to that of C6G. For this reason, the detection limit of this compound was as high as 100 μg/l. This drawback is of minor relevance, since free codeine, originating from acetylcodeine, was usually detected in urine samples from heroin addicts. In the case of codeine intake, C6G was easily detected. The determination of opiates in urine, collected 0–6 h after codeine intake (30 mg orally), is illustrated in Fig. 6. Also, plasma concentrations of C6G, observed after an oral dose of codeine are much higher than the LOD. The oral intake of 25 mg of codeine was associated with maximal plasma concentrations of C6G ranging from 700 to 1670 μg/l [29]. Therefore, the LOD for C6G of the present method, although much higher than for other analytes, seems to be sufficient for clinical and forensic purposes.

It must be stressed that, in the case of M3G, M6G and C6G, two masses were used for identification, i.e. molecular ion mass and aglycone.

On the basis of the successful validation, the

Table 2
Concentrations of opioids (μg/l) found in blood and urine samples of car drivers that had been arrested

Case no.	Material	M3G	M6G	Morphine	C6G	Codeine	6-MAM
1	B	190	32	5	n.d.	n.d.	n.d.
	U	463	146	19	n.d.	6	n.d.
2	B	32	15	4	n.d.	n.d.	Trace
	U	89	60	18	n.d.	n.d.	4
3	B	84	16	9	n.d.	n.d.	n.d.
	U	2600	1280	290	n.d.	48	32
4	B	153	120	25	530	95	3
	U	9900	2500	1600	2600	450	320
5	B	281	199	52	n.d.	6	n.d.
6	B	287	93	5	n.d.	n.d.	n.d.
7	B	397	117	153	n.d.	17	n.d.
8	B	696	169	95	n.d.	11	n.d.
9	B	286	136	34	n.d.	n.d.	n.d.
10	B	142	90	47	n.d.	Trace	Trace

Abbreviations: B=blood, U=urine, n.d.=not detected, trace=between the LOD and the LOQ.

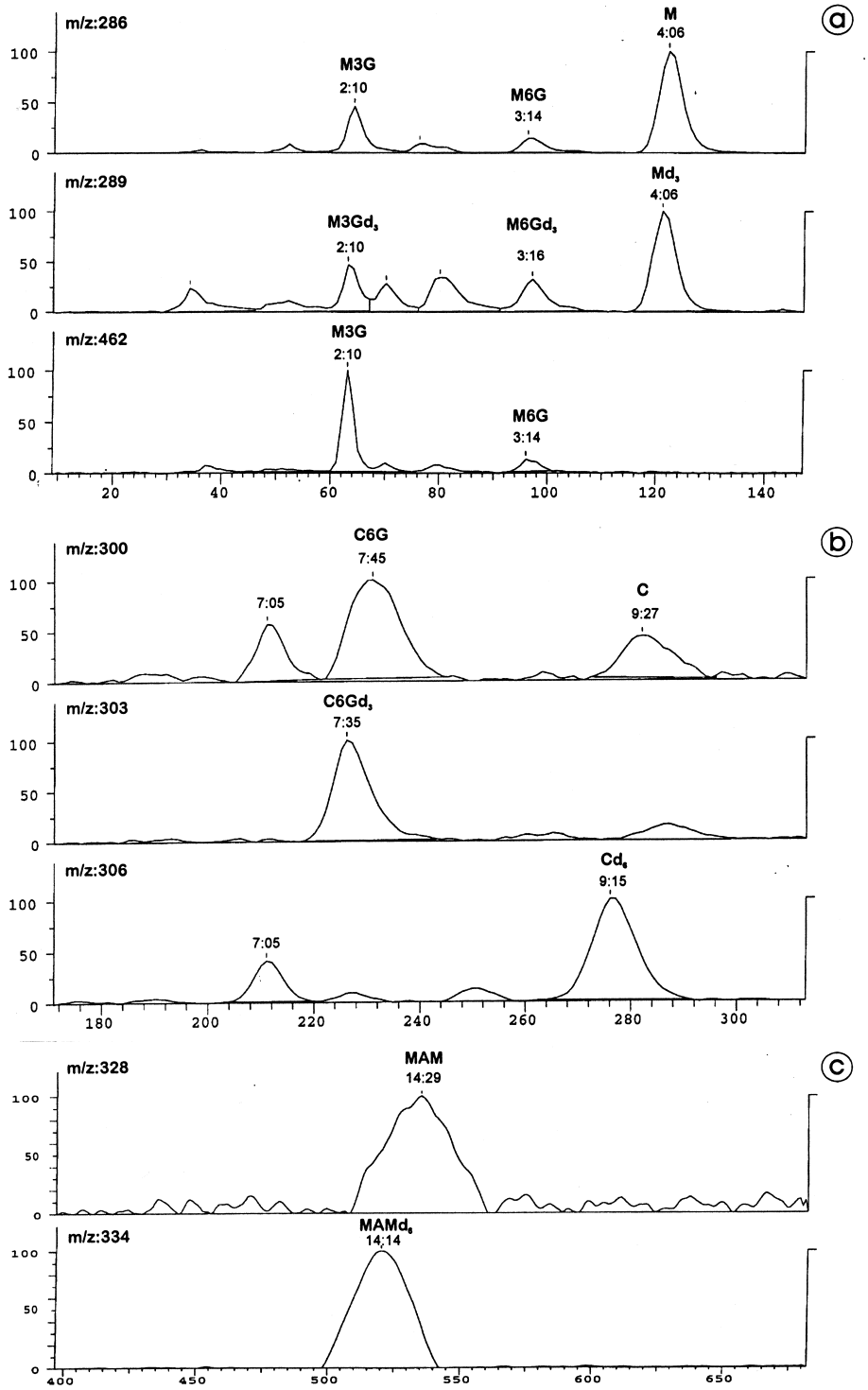

Fig. 7. Chromatogram of a femoral vein blood extract from a case of fatal heroin overdose. The following concentrations were found (in μg/l): M3G, 432; M6G, 165; morphine, 245; C6G, 333; codeine, 19 and 6-MAM 33.

method was applied to the routine determination of opiates in blood, urine and other body fluids in forensic cases (road traffic offences, intoxications, etc). Along with examined samples, serum calibrators spiked with 50 μg/l morphine, 100 μg/l M3G, 100 μg/l M6G, 200 μg/l codeine, 500 μg/l C6G, 50 μg/l 6-MAM, and urine calibrators, spiked with 100 μg/l morphine, 200 μg/l M3G, 200 μg/l M6G, 500 μg/l codeine, 500 μg/l C6G and 100 μg/l 6-MAM, were extracted. Also, blank serum and urine samples, spiked with the mixture of deuterated internal standards, were analyzed. In the case of high concentrations of opiates, exceeding the calibration range (which occurred in urine samples), the analysis was repeated using 0.1 ml of sample instead of 1 ml. The results obtained in some blood and urine samples taken from arrested car drivers are given Table 2.

Fig. 7 shows the results of femoral vein blood analysis in a typical case of fatal heroin overdose. The concentrations were (in μg/l): M3G, 432; M6G, 165; morphine, 245; C6G, 333; codeine, 19 and 6-MAM, 33.

Selected extracts of spiked and authentic samples were stored at −20°C and analyzed several times. Practically identical results (the differences were less than 5%) were observed after up to 30 days of storage.

The method appeared very robust in everyday use. For chromatographic separations, the original column is still in use, showing no deterioration in selectivity after thirteen months. Also, tuning of the instrument was required no more than every six months.

4. Conclusion

It may be stated that the application of LC–APCI–MS allowed the determination of morphine and its glucuronides, codeine and its glucuronide and monoacetylmorphine in one analytical run in a specific manner. LC–APCI–MS offers a new quality in comparison with HPLC with electrochemical, spectrophotometric or fluorimetric detection and may be regarded as a method of choice for opiate determination of biological material.

However, in our experience, some drawbacks of the LC–APCI–MS determination of opiates should be mentioned:

– As was previously stated, the extent of fragmentation depends on the composition of the mobile phase. Therefore, the method should be very carefully standardized and the use of individual deuterated internal standards for each compound is highly recommended.

– The fragmentation may depend on the composition of the sample being examined. This was observed in the case of commercially available control serum, when the peak m/z 286 was observed, eluting at a retention time corresponding to that of codeine. This may suggest that codeine underwent fragmentation to morphine [41]. In authentic serum and urine samples and in laboratory-prepared serum samples (serum spiked with codeine), such fragmentation was not observed.

– The use of deuterated internal standards with only three deuterium atoms (d_3) may contribute to some systematic error in quantitation. In the case of high concentrations of analytes, the isotope contribution of mass m/z (M+3) may be high enough to influence the ratio of drug to internal standard (I.S.), e.g., the isotopic contribution for C_{16}-hydrocarbons (corresponding to morphine) was estimated at 0.1% for the abundance (M+3) [42]. Assuming that the apparent concentration ratio of sample to I.S. is 100, the isotope peak of morphine-d_3 may diminish the ratio to 90.9. In the case of a ratio of 200, the value would be reduced to 166.6. Therefore, the use of internal standards with deuterium labels >3 is recommended [43]. Unfortunately, in the case of morphine, M3G, M6G and C6G, only d_3-deuterated analogues are commercially available at present.

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