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# Method for quantification of morphine and its 3- and 6-glucuronides, codeine, codeine glucuronide and 6-monoacetylmorphine in human blood by liquid chromatography–electrospray mass spectrometry for routine analysis in forensic toxicology

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**Abstract**

Simultaneous determination of opiates and their glucuronides in body fluids has a great practical interest in the forensic assessment of heroin intoxication. A selective and sensitive method for quantification of morphine and its 3- and 6-glucuronides, codeine, codeine glucuronide and 6-monoacetylmorphine (6-MAM) based on liquid chromatography–electrospray ionisation mass spectrometry is described. The drugs were analysed in human autopsy whole blood after solid-phase extraction on a $C_8$ cartridge. The separation was performed on an ODS column in acetonitrile (analysis time 15 min). For the quantitative analysis, deuterated analogues of each compound were used as internal standards. Selected-ion monitoring was applied where the molecular ion was chosen for quantification. The limits of quantification were 0.5 ng/ml for morphine and 6-MAM and 1 ng/ml for the 6-glucuronide of morphine, codeine-6-glucuronide and codeine and 5 ng/ml for the 3-glucuronide of morphine. 

*Keywords:* Forensic analysis; Morphine; Codeine; Glucuronides; Monoacetylmorphine

## 1. Introduction

The simultaneous determination of opiates and their glucuronides in body fluids has a great practical interest in forensic toxicology. It can help to understand the mechanism of intoxication by heroin or other opiates, and to find out markers for characterising the gravity of intoxication. First, heroin is metabolized to 6-monoacetylmorphine (6-MAM), which is then deacetylated in morphine [1]. Morphine itself is either conjugated at the 3 or 6 positions giving, respectively, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), or it is *N*-demethylated into normorphine. In the case of codeine intake, the products of metabolism are codeine-6-glucuronide (C6G), morphine and its glucuronides (Fig. 1).

Each of these metabolites possess specific pharmacological activities and display different relative concentrations in each body fluid [2–5].

Until now, most analytical toxicologists routinely measured free and total morphine and codeine in blood by gas chromarography–mass spectrometry (GC–MS) to assess the toxicological effects of

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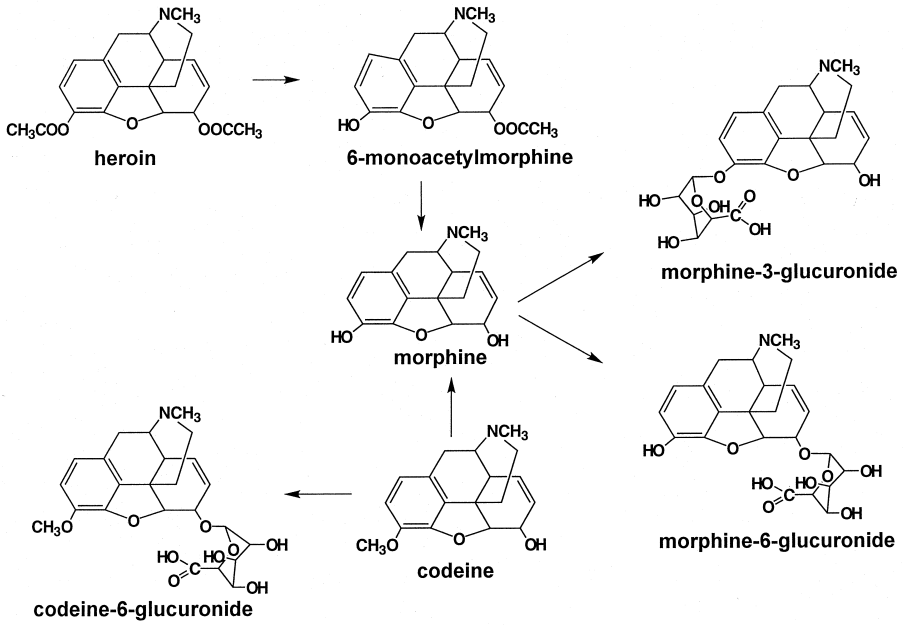


Fig. 1. Main metabolism products of heroin, morphine and codeine.

opiates. 6-MAM is used to characterise a heroin intake [6].

The value of the ratio of free to total morphine as obtained by GC–MS is often used to estimate the elapsed time since injection of heroin [7]. In recent studies, where HPLC was used for the analyses of morphine and its metabolites, it has been proposed that the molar ratios M6G/morphine and M3G/morphine in blood correlate with the survival time [8–10].

For the simultaneous quantification of parent opiate compounds (morphine and codeine) and their glucuronides, HPLC separation with UV [11,12], electrochemical [13] or fluorimetric [14] detection have been already reported. These detectors are quite sensitive but offer most of the time insufficient selectivity when biological samples are analysed. In order to overcome these difficulties, liquid chromatography–atmospheric pressure ionisation mass spectrometry (LC–API-MS) was introduced. Some methods for the quantification of morphine and its glucuronides by LC–atmospheric pressure chemical ionisation (APCI) MS and liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS) were published [15–17], but we found these procedures too labourious and time consuming to be applied to our laboratory routine.

The goal of the present study was to develop a more efficient and rapid LC–MS method for the direct quantitative measurement of the main active metabolites of heroin, morphine and codeine by using the Turbo-Ionspray interface. The corresponding deuterated opiate analogues were used as internal standards.

## 2. Experimental

### *2.1. Reagents*

Codeine phosphate and morphine hydrochloride were obtained from Siegfried (Zofingen, Switzerland), morphine-3-D-glucuronide and morphine-6-D-glucuronide were from Cambridge Isotope Labs. (Innerberg, Switzerland) and 6-monoacetylmorphine hydrochloride was from SALARS (Como, Italy). [$^2H_3$]Morphine (M-$d_3$) and [$^2H_3$]codeine (C-$d_3$) were purchased from Radian (Promochem SARL, Molsheim, France). Codeine-6-D-glucuronide, [$^2H_3$]codeine-6-D-glucuronide (C6G-$d_3$),

[$^2H_3$]morphine-3-D-glucuronide (M3G-$d_{3)}$ and [$^2H_3$]morphine-6-D-glucuronide (M6G-$d_3$) were from Lipomed (Arlesheim, Switzerland). Ammonium carbonate, ammonium formate and formic acid were from Fluka (Buchs, Switzerland). Solid-phase extraction (SPE) cartridges, Bond Elut $C_8$ (500 mg) were obtained from Varian (Basel, Switzerland). Sylon BFT was supplied by Supelco (Bellefonte, PA, USA).

### 2.2. Instrumentation

The LC–MS system (Perkin-Elmer Biosystem Europe, Rotkreuz, Switzerland) consisted of two high-pressure pumps and an autosampler, connected to a PE SCIEX API 150EX Serie 200 single quadrupole instrument with a Turbo-IonSpray interface used as the electrospray ionisation source.

The GC–MS system (Hewlett-Packard, Palo Alto, CA, USA) consisted of an HP 7376A automatic liquid injector and an HP 5980 Serie II Plus gas chromatograph coupled directly to an HP 5970 mass-selective detector.

### 2.3. Biological samples

The serum used for the method validation was obtained from the local hospital blood bank. Blood samples taken from living persons suspected of driving under the influence of drugs and blood samples taken during autopsy in our Institute from victims of suspected heroin overdose were collected in 10 ml EDTA tubes and immediately frozen. These tubes were kept at temperature below −20°C until analysed.

### 2.4. Sample preparation

A 1-ml sample of serum or blood containing 100 ng of each deuterated standard was mixed with 3 ml of 10 m*M* ammonium carbonate buffer, pH 9.3, vortexed and centrifuged for 30 min at 4000 *g* at 4°C. The supernatant was applied onto an activated SPE column (Bond Elut $C_8$, 500 mg). After all the extract had passed through, the column was rinsed with 4 ml of 10 m*M* ammonium carbonate buffer pH 9.3 and dried for 5 min under vacuum. The sample was eluted with 2×1 ml of methanol, dried under $N_2$ and reconstituted in 100 μl of 1 m*M* ammonium formate buffer solution pH 3.0.

### 2.5. Liquid chromatography

The chromatographic separation was performed on an Inertsil ODS-3 column (150×3 mm I.D., 5 μm particle size; Chrompack, The Netherlands). The mobile phase consisted of a mixture of 1 m*M* ammonium formate solution at pH 3.0 and acetonitrile. The flow-rate was kept constant at 0.4 ml/min. The following step-wise gradient elution program was used: the acetonitrile concentration was maintained at 5% (v/v) for 5 min, then increased to 20% in 5 min and remained constant for the last 5 min.

### 2.6. LC–MS analyses

The LC–MS instrument equipped with a Turbo-IonSpray source was used for ESI-MS in positive ion mode. The Turbo-IonSpray conditions were the following: temperature 475°C, heater gas flow 7 l/min, ionspray voltage 4500 V, nebuliser gas position 13 and curtain gas position 11. Selected ion monitoring (SIM) was applied for quantification: 286.1 (morphine); 289.1 (M-$d_3$); 300.2 (codeine); 303.2 (C-$d_3$); 328.2 (6-MAM); 462.2 (M3G, M6G); 465.2 (M3G-$d_3$, M6G-$d_3$); 476.2 (C6G) and 479.2 (C6G-$d_3$). The voltages on the curtain plate orifice and the ring were optimised for each ion separately (OR: 31–41V; RNG: 200–230 V) in order to achieve highest signal-to-noise ratio.

### 2.7. GC–MS analyses

Free opiates fractions were obtained from 1 ml of whole blood using a three-step base–acid–base extraction method. For the determination of total morphine and total codeine, 1 ml of the blood was hydrolyzed with 1 ml of HCl (32%) at 100°C during the 30 min before extraction [18]. Dried extracts were derivatised with Sylon BFT [bis(trimethylsilyl)trifluoroacetamide+1% trimethylchlorosilane] in pyridine at 60°C during 30 min. Quantification was performed with the GC–MS operating in the SIM mode. Two ions were selected for each molecule. C-$d_3$ was used as the internal

standard for 6-MAM and codeine quantification and M-$d_3$ was used for the morphine determination.

## 3. Results and discussion

### *3.1. Mass spectra*

The mass spectra of the examined opiate compounds are shown in Fig. 2. They were recorded in full scan mode ($m/z$ 100–500 amu). For morphine, M-$d_3$, codeine and C-$d_3$, only protonated molecules $(M+H)^+$ at $m/z$ 286, 289, 300 and 303, respectively, were observed. 6-MAM gave mainly a protonated molecular ion ($m/z$ 328) and two fragment ions at $m/z$ 268 and 211.

Morphine- and codeine glucuronides fragmented into the corresponding aglycones (morphine, C). The extent of such fragmentation was different for the three glucuronides. M3G showed the most abundant aglycon ion intensity (about 30%) and C6G the least (about 10%). Variation in pH had little influence on the extent of the fragmentation pattern. Control experiments showed that the fragmentation was

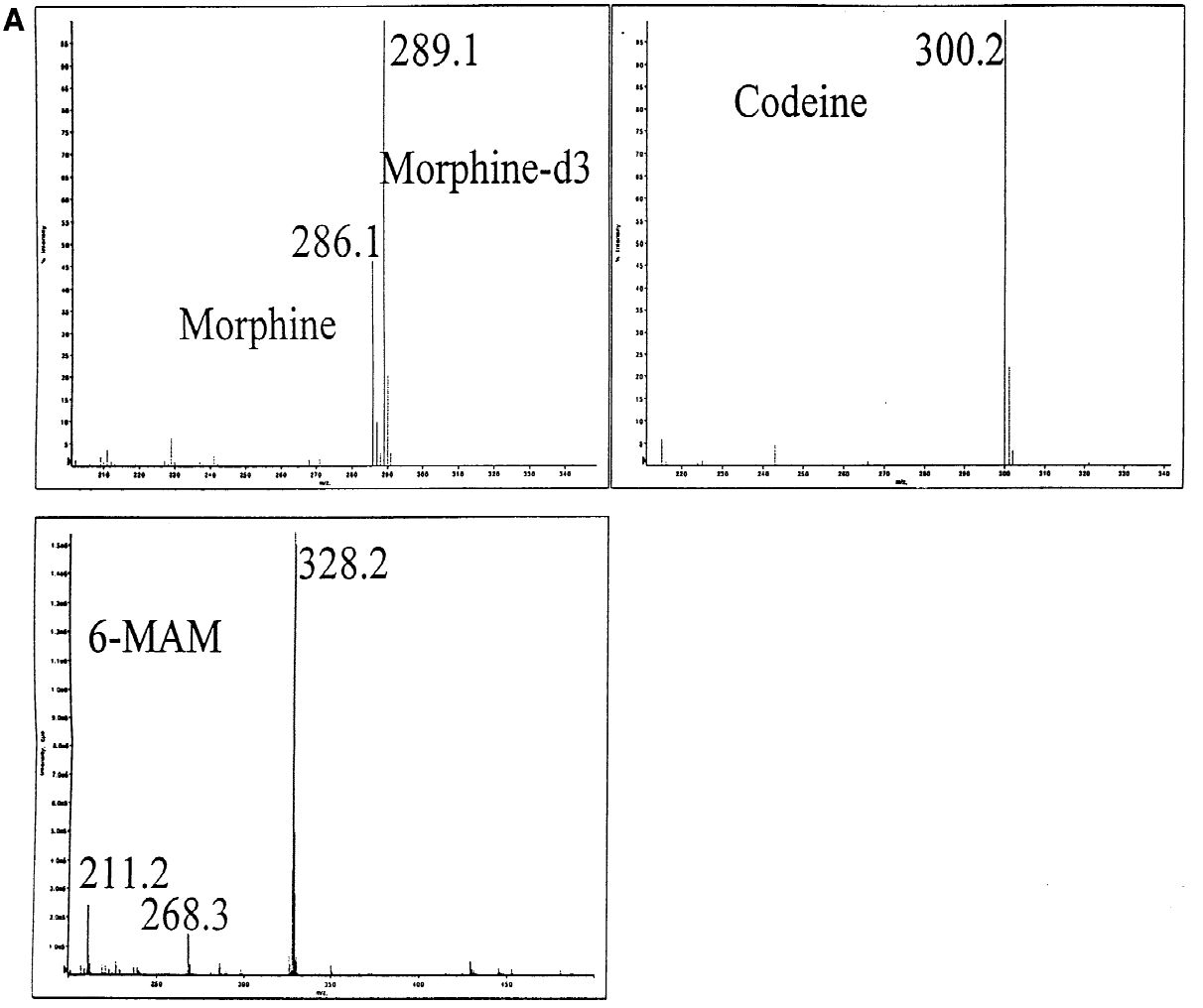


Fig. 2. (A) Mass spectra of both morphine and morphine-$d_3$, codeine and 6-MAM; (B) mass spectra of M3G, M3G-$d_3$, M6G, M6G-$d_3$, C6G and C6G-$d_3$.

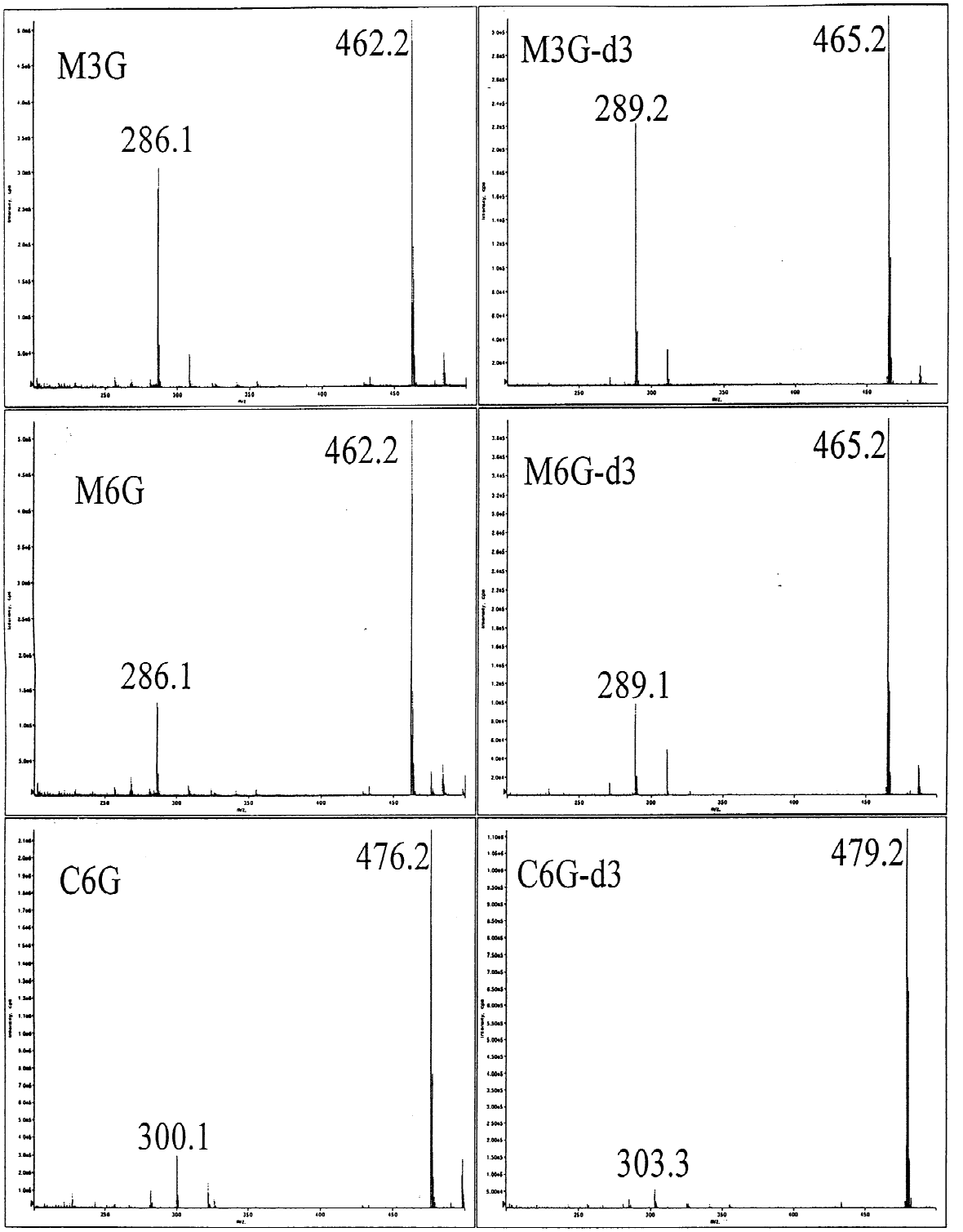


Fig. 2. (*continued*)

stable and independent from the composition of the mobile phase (in the range of 0–90% of acetonitrile–1m*M* ammonium formate at pH 3). Also, when using the chromatographic conditions described here, we did not find any effect of increasing the buffer concentration (1–50 m*M* ammonium formate at pH 3), but we observed a diminution of the signal intensities. This contrasts with the observation reported earlier [17] that the mobile phase composition (i.e. percentage of acetonitrile) influences the fragmentation patterns of the M3G and M6G using an APCI source and a 50 m*M* ammonium formate buffer solution. Otherwise, the mass spectra obtained with the two different ionisation modes were very similar. This might reflect the rapid and decisive improvement in LC–ESI-MS interface technology provided by the Turbo-IonSpray device.

### *3.2. Separation*

The separation of the opiates was performed in 15 min on a ODS reversed-phase column using a linear gradient of ammonium formate and acetonitrile. All examined substances were separated with the typical retention time of 2.2 min for M3G, 2.7 min for morphine, 4.3 min for M6G, 7.3 min for codeine, 10.8 min for C6G and 11.2 min for 6-MAM. As expected, the deuterated substances were eluting on the same time or a little bit earlier (Fig. 3).

In preliminary experiments using several ammonium formate concentrations, we observed strong modifications on the retention of 6-glucuronides. Using the mobile phase with ammonium formate concentration less than 5 m*M*, 6-glucuronides were less hydrophilic than the corresponding aglycon. On increasing the concentration, they became more hydrophilic (Fig. 4). This change can be related to the steric conformation of the molecules. Carrupt et al. [19] reported that morphine-glucuronides behave as ''molecular chameleons'', meaning that they adapt their polarity to that of the medium. They present two spatial forms: extended and folded conformations, in polar and apolar media, respectively. A theoretical conformation study showed [19] that M3G prefer the extended form more than M6G. From the present results, we can hypothesize that the M6G molecule changes from the folded conformation to the extended form when the ionic strength of the solvent increases.

### *3.3. Calibration*

Referring to the results of our scan experiments, base peak ions for each opiate and their deuterated analogues were selected when the LC–MS was operating in the SIM mode. Since the fragmentation of the deuterated analogues was similar to that of the opiate-glucuronides, we used them as internal standards to assure accurate measurements whatever the composition of the mobile phase and the ionisation conditions were.

The validation experiments of the SIM method were performed with serum spiked with morphine, M3G, M6G, codeine and C6G in the concentration range 1–500 ng/ml (1, 5, 50, 100, 250 and 500 ng/ml). A separate calibration was made for the 6-MAM in the same concentration range. The internal standards, morphine-$d_3$, M3G-$d_3$, M6G-$d_3$, C-$d_3$ and C6G-$d_3$, were spiked at the concentration of 100 ng/ml each. For the 6-MAM quantifications, we used C-$d_3$ as the internal standard because deuterated 6-MAM was not available to us. As the solvent composition had no effect on the fragmentation pattern, the use of C-$d_3$ as the internal standard for the quantification of the 6-MAM was acceptable. The results of that validation are summarised in Table 1.

It is important to note that 6-MAM is not stable in buffer solution, as it slowly decomposes to morphine. We measured as much as 10% morphine in our 6-MAM stock solution. Therefore, the precise calibration of 6-MAM and morphine simultaneously is impossible. In order to achieve accurate determinations of both morphine and 6-MAM, as well of all other opiates, two separate calibration curves, one for 6-MAM and the second one for the other targeted compounds were performed. Consequently, we can estimate that the calculated value of 6-MAM in the samples should be at the most about 10% too high.

In the blank serum, no interferent peaks could be detected at the expected retention times of each opiate. The calibration curves were linear between 1 and 500 ng/ml for all opiates with a correlation coefficient, $r^2$, higher than 0.99.

The precision of the method was determined in

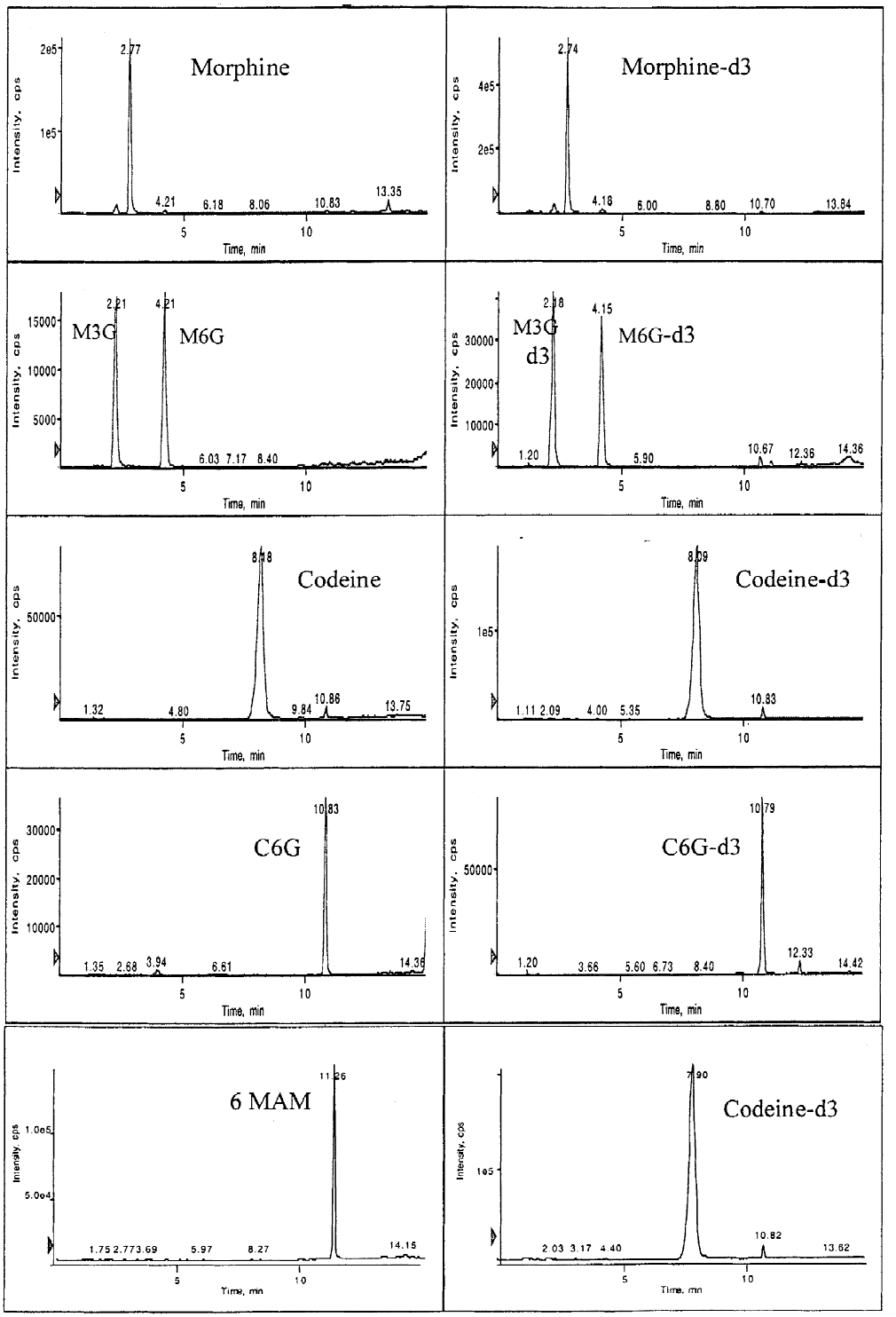


Fig. 3. LC–ESI-MS chromatograms of serum spiked with 50 ng/ml of morphine, morphine-3-glucuronide, morphine-6-glucuronide, codeine, codeine-6-glucuronide, 6-MAM and 100 ng/ml of the corresponding deuterated standards.

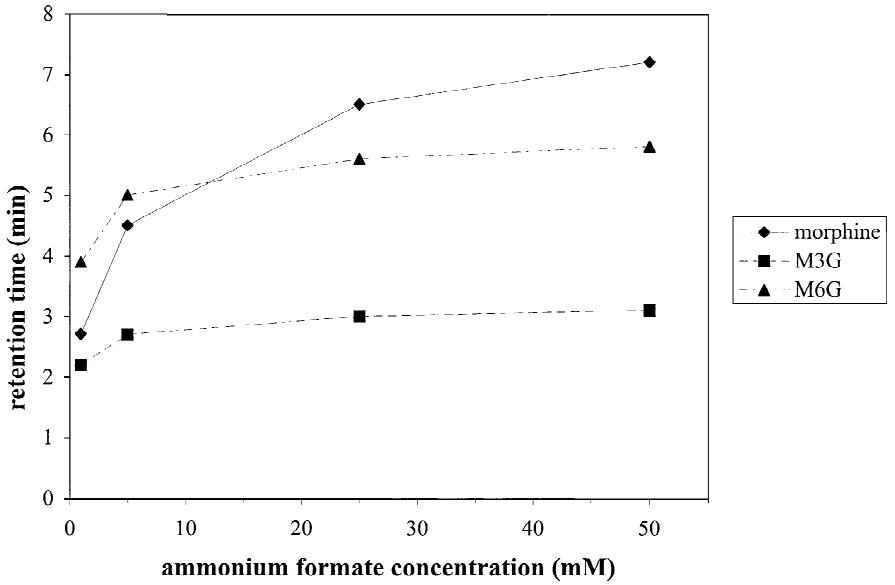


Fig. 4. The effect of ammonium formate concentration on the retention times of morphine, M3G and M6G. Elution condition: solvent A=acetonitrile; solvent B=ammonium formate solution, pH 3.0. Linear acetonitrile gradient: 10 min, 5% A; 5 min, linear gradient to 10% A; 10 min, 10% A.

three series with double injections of three spiked serums at 5, 50 and 250 ng/ml concentrations. The relative standard deviations calculated from these results for the examined compounds were between 4 and 7%. When the between-days precision was determined in three series at the 50 ng/ml medium concentration, the values were between 6 and 11%

The absolute recoveries were expressed as the peak area ratio (in percentage) of the corresponding amounts of extracted and non-extracted drugs injected into the LC–MS system. They were between 70 and 84%.

### *3.4. Analyses of forensic blood samples*

Our method was used for the quantitative de-

Table 1
Validation data

| Compounds | Retention time (min) | LOD[a] (ng/ml) | Linearity[b] (1–500 ng/ml) | $r^2$ | Recovery[c] (%) | Precision 24 h[d] 5 ng/ml | 50 ng/ml | 250 ng/ml | Precision day-to-day[d] (50 ng/ml) |
|---|---|---|---|---|---|---|---|---|---|
| M3G | 2.2 | 5.0 | $y=0.9578x$ | 0.9981 | 81 | 27.0 | 2.6 | 2.5 | 6 |
| Morphine | 2.7 | 0.5 | $y=0.7698x$ | 0.9981 | 79 | 5.2 | 1.9 | 3.0 | 6 |
| M6G | 4.3 | 1.0 | $y=1.0973x$ | 0.9951 | 83 | 16.0 | 11.0 | 11.0 | 11 |
| Codeine | 7.3 | 1.0 | $y=0.8464x$ | 0.9979 | 70 | 9.9 | 9.6 | 3.1 | 10 |
| C6G | 10.8 | 1.0 | $y=0.9307x$ | 0.9974 | 83 | 19.0 | 3.9 | 7.1 | 8 |
| 6-MAM | 11.2 | 0.5 | $y=0.4179x$ | 0.9967 | 84 | 5.9 | 4.7 | 3.9 | 8 |

[a] The limit of detection (LOD) is defined as the signal of the targeted ion giving a signal-to-noise ratio equal or higher than 3. The limit of quantification (LOQ) was considered as the double of the LOD.

[b] The calculated regression line, based on 5 points, was forced to pass through zero.

[c] Defined as the mean base peak areas ratio (in percentage) of the extracted and the corresponding amount of non-extracted drugs injected into the LC–MS system using identical instrumental settings.

[d] Precision (RSD,%) determined in three series at 5, 50 and 250 ng/ml concentrations for each compound.

termination of opiates in whole blood in forensic cases for routine analysis. Each series of samples included a blank serum, a blank serum spiked with internal standards, blank serums spiked with opiates at four concentration levels (5, 50, 250 and 500 ng/ml) and blank serums spiked with 6-MAM at two levels (5 and 50 ng/ml). The calibration curves were calculated from these data. In the case of very high concentrations of opiates in the sample (more than 500 ng/ml), the first determination was repeated using 5–10 times less blood volume. Typical results obtained with our LC–ESI-MS method were compared with those obtained by GC–MS (see Table 2).

The correlation of the results obtained with the two methods (GC–MS and LC–ESI-MS) was good. For codeine, we found nearly the same values at all concentration levels. We observed a significant difference in the results of total morphine concentration. When the concentrations were less than 600 ng/ml, the values obtained by LC–ESI-MS were somewhat lower than that those obtained by GC–MS. This apparent discrepancy could be explained as follows: results obtained by GC–MS included the concentration of morphine 3,6-glucuronide and morphine-3-ethereal sulfate, whereas the LC–MS results did not. Other differences could be explained by incomplete hydrolysis of the morphine conjugates, which might occur at high concentrations (e.g. case six) [18]. Cases five and six demonstrate the good reproducibility of our LC–MS method.

## 4. Conclusion

LC–ESI-MS was successfully applied for the simultaneous quantification of opiates and their glucuronides. This method was found to be reasonably rapid, selective, sensitive and sufficiently robust for routine analysis in forensic toxicology.

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Table 2
Quantitative analyses of forensic whole blood samples by GC–MS and LC–ESI-MS

| Sample | Method | Free morphine (ng/ml) | M3G (ng/ml) | M6G (ng/ml) | Total morphine[b] (ng/ml) | Free codeine[c] (ng/ml) | C6G (ng/ml) | Total codeine (ng/ml) | 6–MAM (ng/ml) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | GC–MS | n.d.[a] | – | – | 10.0 | n.d. | – | n.d. | n.d. |
| 1 | LC–ESI-MS | 0.2 | n.d. | n.d. | 0.2 | n.d. | 1.5 | 1.0 | n.d. |
| 2 | GC–MS | 10.0 | – | – | n.d. | 50.0 | – | 90.0 | 0.0 |
| 2 | LC–ESI-MS | 0.5 | n.d. | n.d. | 0.5 | 41.0 | 66.0 | 84.0 | 2.2 |
| 3 | GC–MS | 30.0 | – | – | 160.0 | n.d. | – | 0.0 | n.d. |
| 3 | LC–ESI-MS | 37.0 | 126 | 19 | 126.0 | n.d. | 0.4 | 0.3 | n.d. |
| 4 | GC–MS | 1.0 | – | – | 580.0 | n.d. | – | 40.0 | n.d. |
| 4 | LC–ESI–MS | 16.0 | 630 | 91 | 462.0 | 6.0 | 70.0 | 50.0 | 1.0 |
| 5 | GC–MS | 1390.0 | – | – | 1950.0 | 190.0 | – | 230.0 | 10.0 |
| 5 | LC–ESI-MS | 710.0 | 768 | 163 | 1284.0 | 84.0 | 58.0 | 121.0 | n.d. |
| 5[d] | LC–ESI-MS | 797.0 | 797 | 163 | 1390.0 | 84.0 | 62.0 | 122.0 | 26.0 |
| 6 | GC–MS | 920.0 | – | – | 870.0 | 90.0 | – | 120.0 | 10.0 |
| 6 | LC–ESI-MS | 896.0 | 438 | 101 | 1229.0 | 106.0 | 26.0 | 122.0 | 20.0 |
| 6[e] | LC–ESI-MS | 1062.0 | 435 | 100 | 1393.0 | 100.0 | 29.0 | 119.0 | 12.0 |

[a] n.d.=Not detected.

[b] Total morphine concentration was calculated for LC–ESI-Ms with the equation: $C_{\text{Free morphine}}[\text{ng/ml}] + (C_{\text{M3G}}[\text{ng/ml}] + C_{\text{M6G}}[\text{ng/ml}]) \cdot 0.62$.

[c] Total codeine concentration was calculated also as: $C_{\text{Free codeine}}[\text{ng/ml}] + (C_{\text{C6G}}[\text{ng/ml}]) \cdot 0.63$.

[d] Analysis was repeated using 0.1 ml of sample instead of 1 ml.

[e] Analysis was repeated using 0.2 ml of sample instead of 1 ml.

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