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Rapid, highly sensitive method for the determination of morphine and its metabolites in body fluids by liquid chromatography–mass spectrometry

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Abstract

A rapid, highly sensitive method for the determination of morphine and its metabolites morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and normorphine has been developed using high-performance liquid chromatography–electrospray mass spectrometry, with the deuterated analogues as internal standards. The analytes were extracted automatically using end-capped C_2 solid-phase extraction cartridges. Baseline separation of morphine, M3G and M6G was achieved on a LiChrospher 100 RP-18 end-capped analytical column (125×3 mm I.D., 5 μm particle size) with water–acetonitrile–tetrahydrofuran–formic acid (100:1:1:0.1, v/v) as the mobile phase. Morphine and normorphine coeluate and were separated mass spectrometrically. The mass spectrometer was operated in the selected-ion monitoring mode using *m/z* 272 for normorphine, *m/z* 286 for morphine, *m/z* 462 for morphine-6-glucuronide. Due to an interfering peak, M3G was measured by tandem mass spectrometry in the daughter-ion mode. The limits of quantitation achieved with this method were 1.3 pmol/ml for morphine, 1.5 pmol/ml for normorphine, 1.0 pmol/ml for M6G and 5.4 pmol/ml for M3G in serum or cerebrospinal fluid. The limits of quantitation achieved in urine were 10 pmol/ml for morphine, 20 pmol/ml for normorphine and M6G and 50 pmol/ml for M3G using a sample size of 100 μl. The method described was successfully applied to the determination of morphine and its metabolites in human serum, cerebrospinal fluid and urine in pharmacokinetic and drug interaction studies.

Keywords: Morphine; Morphine glucuronide; Normorphine

1. Introduction

Morphine is a potent opioid analgesic used traditionally for the short-term treatment of surgical pain and for the long-term treatment of moderate-to-severe cancer pain. In human subjects, morphine is metabolized primarily via conjugation with glucuronic acid to form morphine-3-glucuronide

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(M3G) and morphine-6-glucuronide (M6G). The marked analgesic potency of M6G implies a contribution to the analgesic effect of morphine [1–3]. The principal metabolite M3G has no opioid action but seems to be a functional antagonist of morphine and M6G [4,5]. Some studies indicated that M3G might be responsible for side-effects after morphine-treatment [6]. The *N*-demethylated metabolite, normorphine is also active, but is formed to a minor extent [7]. For an assessment of the pharmacodynamics and pharmacokinetics of morphine and its metabolites, we intended to determine the concentrations of the parent compound and its metabolites in serum and cerebrospinal fluid (CSF) of patients or volunteers receiving morphine during a clinical study.

There are several methods for the simultaneous determination of morphine and its metabolites in biological fluids reported. Various assays use solid-phase extraction and high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [8–10], UV detection [11,12] and fluorescence detection [13–15], or a combination of different detectors [16].

More recently the application of liquid chromatography–mass spectrometry (LC–MS) [17–19] and LC–MS–MS [20] as been described for morphine and its glucuronides.

As in our pharmacokinetic studies, extremely low levels were expected in CSF and in serum up to 36 h after morphine administration, HPLC methods were not sensitive enough.

We have developed a specific and sensitive LC–MS method which enables the quantification of morphine and its metabolites in serum and urine up to 36 h after a single oral dose of 10 mg of morphine sulfate [21], or in serum and CSF up to 36 h after a single morphine dose of 0.4 mg/kg. The achieved limits of quantitation of 1.3 pmol/ml for morphine, 1.5 pmol/ml for normorphine, 1.0 pmol/ml for M6G and 5.4 pmol/ml for M3G are a significant improvement over current published LC–MS assays. Tyrefors et al. [18] achieve limits of quantification (LOQ) of 4.4 pmol/ml for morphine, 11 pmol/ml for M6G and 16 pmol/ml for M3G. Bogusz et al. [19] report a limit of detection with the same value as our LOQ for morphine and M3G, but validation data are given for much higher concentrations (between 100 and 200 pmol/ml). Zheng et al. [20] have validated their method for 91 pmol/ml morphine, 58 pmol/ml M3G, 63 pmol/ml M6G and 74 pmol/ml normorphine using 100 μl of rat plasma.

2. Experimental

2.1. Materials

Solvents used were of HPLC quality; chemicals were of analytical grade. Morphine hydrochloride trihydrate was obtained from Merck (Darmstadt, Germany), morphine-d_3 hydrochloride trihydrate, normorphine hydrochloride, M3G and M6G dihydrate were obtained form Sigma (Deisenhofen, Germany). 2′,3′,4′-[$^2H_3$]Morphine-3-glucuronide (M3G-d_3) and 2′,3′,4′-[$^2H_3$]morphine-6-glucuronide dihydrate (M6G-d_3) were prepared by Koenigs-Knorr synthesis using 2,3,4-[$^2H_3$]-2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronic acid methyl ester. Synthesis of M3G-d_3 started from morphine in the presence of LiOH. M6G-d_3 was prepared from 3-acetylmorphine using Ag_2CO_3 as catalyst, cleavage of the protective groups was carried out in methanolic sodium hydroxide. Solid-phase extraction (SPE) cartridges, IST C_2 (EC) (100 mg) were supplied by ICT (Bad Homburg, Germany).

2.2. Preparation of standard solutions

Stock standard solutions (1 mg/ml) of morphine, morphine-d_3, normorphine, M3G, M3G-d_3, M6G and M6G-d_3 were prepared in water from the respective salts (see Section 2.1). Working standard solutions were prepared from the stock solutions. All standard solutions were kept at −30°C.

2.3. Sample preparation

Samples (1 ml of serum or 0.5 ml of CSF, diluted with 0.5 ml of water or 0.1 ml of urine, diluted with 0.9 ml of water) were spiked with 25 μl of internal standard mix (8.7 pmol/μl M3G-d_3, 2 pmol/μl M6G-d_3, 2.7 pmol/μl morphine-d_3). Sample work-up was performed automatically on an ASPEC XL system (Gilson, Abimed, Langenfeld, Germany) using C_2 SPE columns. Each column was first conditioned with 2 ml of methanol and 1 ml of water

followed by 2 ml of 10 m*M* ammonium hydrogen carbonate buffer (10 m*M*, pH 9.3) at a flow rate of 10 ml/min. Sample was loaded onto the column at a flow rate of 1 ml/min, then washed with 3 ml of ammonium hydrogen carbonate buffer at a flow rate of 5 ml/min, dried by applying 2 ml of air and eluted with 1 ml of methanol at a flow rate of 2 ml/min. The eluate was evaporated to dryness under a stream of nitrogen and redissolved in 125 μl of mobile phase A. After centrifugation (1100 *g* for 10 min) 100 μl of the supernatant was transferred to autosampler vials and subjected to LC–MS analysis.

For recovery experiments the peak areas obtained with SPE from serum samples were compared to peak areas obtained from the same amounts directly injected into the LC–MS system without extraction.

2.4. HPLC conditions

A TSP 4100 MS gradient pump with a TSP AS 300 autosampler (Thermo Separation Products, Egelsbach, Germany) was used. Chromatographic separation was performed on a LiChrospher 100 RP-18 end-capped column (125×3 mm I.D., 5 μm particle size; Merck, Darmstadt, Germany) at a flow-rate of 1 ml/min. The mobile phases were: (A) water–acetonitrile–tetrahydrofuran–formic acid (100:1:1:0.1, v/v) and (B) water–acetonitrile–formic acid (20:80:0.1, v/v). Gradient runs were programmed as follows: 100% A for 5 min, increase from 0 to 100% B in 2 min, 100% B for 3 min, then re-equilibration with 100% A for 5 min, until the next sample was injected.

2.5. Mass spectrometry

A TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT, Bremen, Germany), equipped with a Finnigan electrospray source was used. Electrospray parameters were: spray voltage, 4.0 kV; heated capillary temperature, 215°C; sheath gas pressure, 50 p.s.i.; auxiliary gas, 20 ml/min. For the first 2.4 min the mass spectrometer was operated in MS–MS mode with an argon collision cell pressure of 1.6 mTorr and a collision energy of 40 eV; for selected reaction monitoring (SRM) of M3G, *m/z* 462 was used as parent ion and *m/z* 286 as daughter ion, and for M3G-d_3 the respective ions were *m/z* 465 and *m/z* 286. Normal MS mode with selected ion monitoring (SIM) mode was used for the other analytes. The following ions were used: *m/z* 272 for normorphine, *m/z* 286 for morphine, *m/z* 289 for morphine-d_3, *m/z* 462 for M6G and *m/z* 465 for M6G-d_3.

2.6. Standardization

Calibration samples were prepared by adding increasing amounts of M3G, M6G, morphine and normorphine to control serum or urine. For the determination of CSF samples serum was used as matrix. Standard curves were evaluated by linear regression analysis based on internal standard calibration and were obtained by plotting peak–area ratios against the amount of the substance. The respective deuterated substances were used as internal standards, except for normorphine, where morphine-d_3 was used.

2.7. Assay validation

To determine assay variability, quality control samples were prepared by adding known amounts of M3G, M6G, morphine and normorphine to 25 ml of drug-free serum, which was divided into 1.1-ml aliquots and stored at −20°C. Quality control samples were analysed always together with the serum samples.

3. Results and discussion

3.1. Solid phase extraction and HPLC separation

Sample extraction was performed automatically by a modification of the method of Pacifici et al. [22]. Different cartridges were tested for serum, and the best results with respect to recovery were obtained with C_2 (EC) cartridges (Table 1). The same extraction procedure optimized for serum samples could be used for urine. To avoid an overload of the cartridge only 100 μl of urine were used. With automated sample processing recoveries were slightly higher than with manual SPE and showed a better reproducibility.

Morphine and its glucuronides are usually sepa-

Table 1
Recoveries from different SPE cartridges ($n=4$) using 1 ml of serum spiked with 266 pmol/ml morphine, 307 pmol/ml normorphine, 217 pmol/ml M3G, and 201 pmol/ml M6G

Cartridge	Recovery (mean±S.D.) (%)			
	M3G	M6G	Morphine	Normorphine
C_{18}	43±4.2	46±4.7	70±3.6	54±4.6
C_8	49±9.2	57±6.1	73±5.3	56±5.3
Phenyl	5.8±5.3	12±4.9	64±3.8	60±6.8
CN	—	—	15±3.7	13±9.7
C_2 (EC)	65±4.4	71±3.0	88±2.9	72±4.1

rated by ion-pair reversed-phase HPLC. For LC–MS ion-pair additives and nonvolatile buffers should be avoided. Mobile phases usually employed in ESI-MS result in a poor retention for the morphine glucuronides. In preliminary experiments several HPLC columns were tested. Using a LiChrospher RP18 end-capped column and water–acetonitrile–tetrahydrofuran–formic acid (100:1:1:0.1, v/v) as the mobile phase, baseline separation was achieved for M3G, M6G and morphine with retention times of 1.7, 3.2 and 3.7 min, respectively. Normorphine coeluted with morphine but could be separated mass spectrometrically (Fig. 2). Signal reduction of normorphine due to morphine was not observed; in quality control samples increasing amounts of morphine showed no influence on precision and accuracy. To prevent column contamination with late-eluting compounds, a short gradient was employed after elution of the analytes. With this wash step and regularly changing the precolumn after about 80 samples, the HPLC column could be used for more than 500 samples. Typical chromatograms of 1 ml serum extracts are shown in Figs. 1–3. In the blank serum no interfering peaks can be detected (Fig. 1). The small peak for M3G represents the amount of undeuterated M3G present in the deuterated internal standard (0.4%). Chromatograms of a serum sample spiked with M3G, M6G, morphine and normorphine and of a serum sample after treatment with morphine are shown in Figs. 2 and 3.

3.2. Mass spectrometry

With electrospray ionization (ESI) usually the protonated molecular ions are observed as base peak. For the morphine glucuronides a minor fragmentation to protonated morphine (m/z 286) occurred. Mass spectrometric parameters (capillary voltage, heated capillary temperature, gas flows) were adjusted to get a maximum signal for the MH^+ ion. Under these conditions m/z 286 showed an abundance below 8% of the base peak (m/z 462) resulting in better sensitivity than with atmospheric pressure chemical ionization (APCI), where the fragmentation is at least 25–30% [19].

In some serum samples an interfering peak with the same mass and retention time of M3G-d_3 was observed. Therefore we used MS–MS for the detection of M3G and M3G-d_3. The respective MH^+ ions (m/z 462 for M3G and m/z 465 for M3G-d_3) were used as parent ions. After collision-induced dissociation (CID) with argon, both compounds showed the same daughter ion of m/z 286, which results from abstraction of the glucuronosyl moiety. These ions were used for detection in the selected-reaction monitoring (SRM) mode.

3.3. Validation

The method has good linearity over the entire range measured: 1.3–530 pmol/ml for morphine, 1.5–610 pmol/ml for normorphine, 1.0–400 pmol/ml for M6G and 5.4–1730 pmol/ml for M3G. Reproducibility was determined by repeatedly analyzing aliquots of serum or urine samples spiked with known amounts of analytes. The intra- and inter-assay variabilities for serum are given in Tables 2 and 3. Intra-assay reproducibility was better than 9%. The day-to-day variation was less than 7% for M3G and M6G. For morphine and normorphine the day-to-day variation was less than 9% over a period of 2 weeks.

Intra-assay variability for urine is given in Table 4. Using 100 μl of urine nearly the same LOQ per sample can be achieved as in serum (5 pmol for M3G, 2 pmol for M6G and normorphine and 1 pmol for morphine). At the LOQ the variation was less than 15% for morphine and M3G and less than 11% for M6G and normorphine. For higher concentrations the variation was less than 5%

Due to the small amounts of CSF available, serum had to be used as matrix for calibration and quality control samples. The variability was evaluated by re-assaying some of the samples on another day and comparing the results (Table 5).

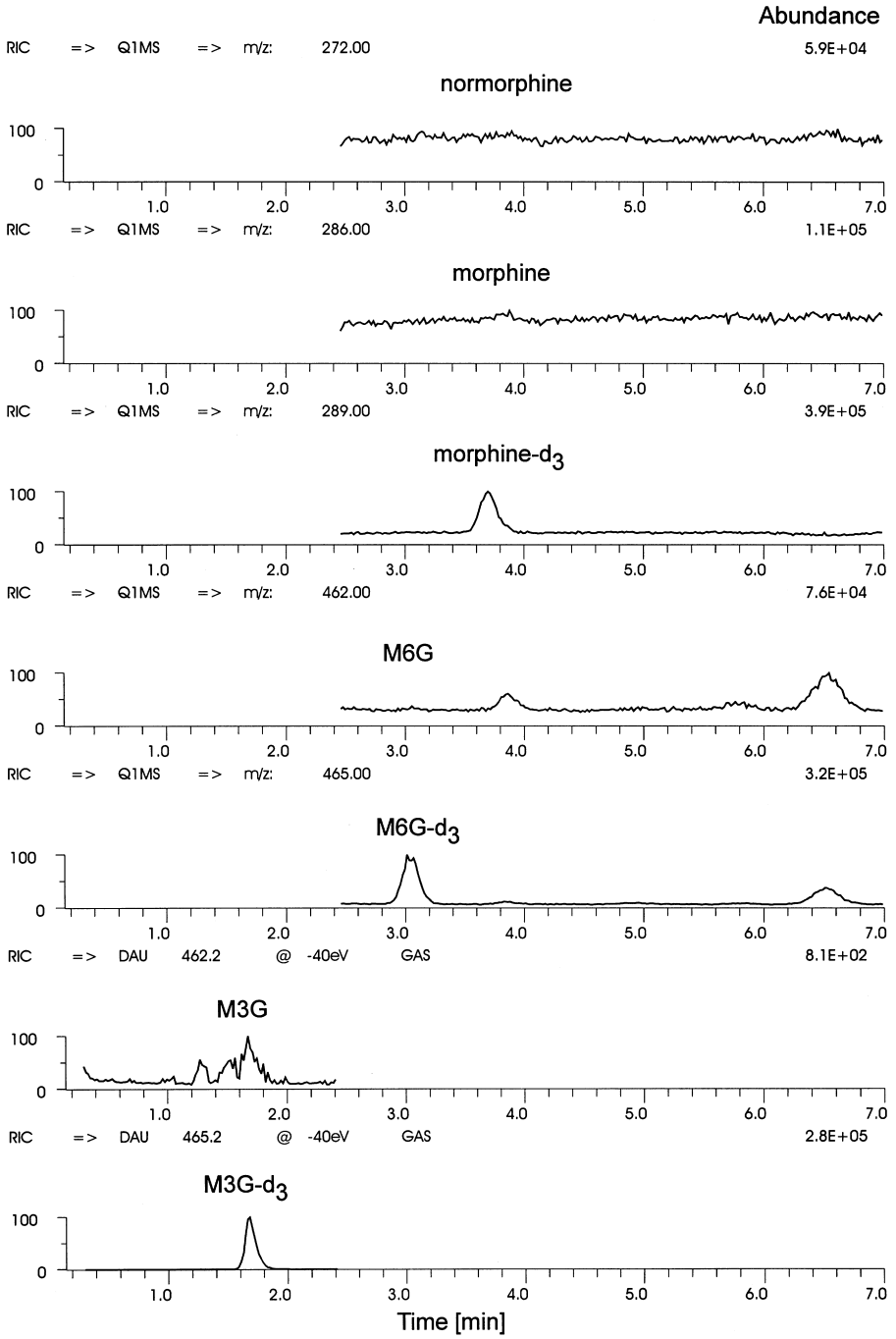

Fig. 1. Mass chromatograms of an extract of 1 ml of blank serum spiked with internal standards (217.5 pmol/ml M3G-d_3, 50 pmol/ml M6G-d_3, 67.5 pmol/ml morphine-d_3).

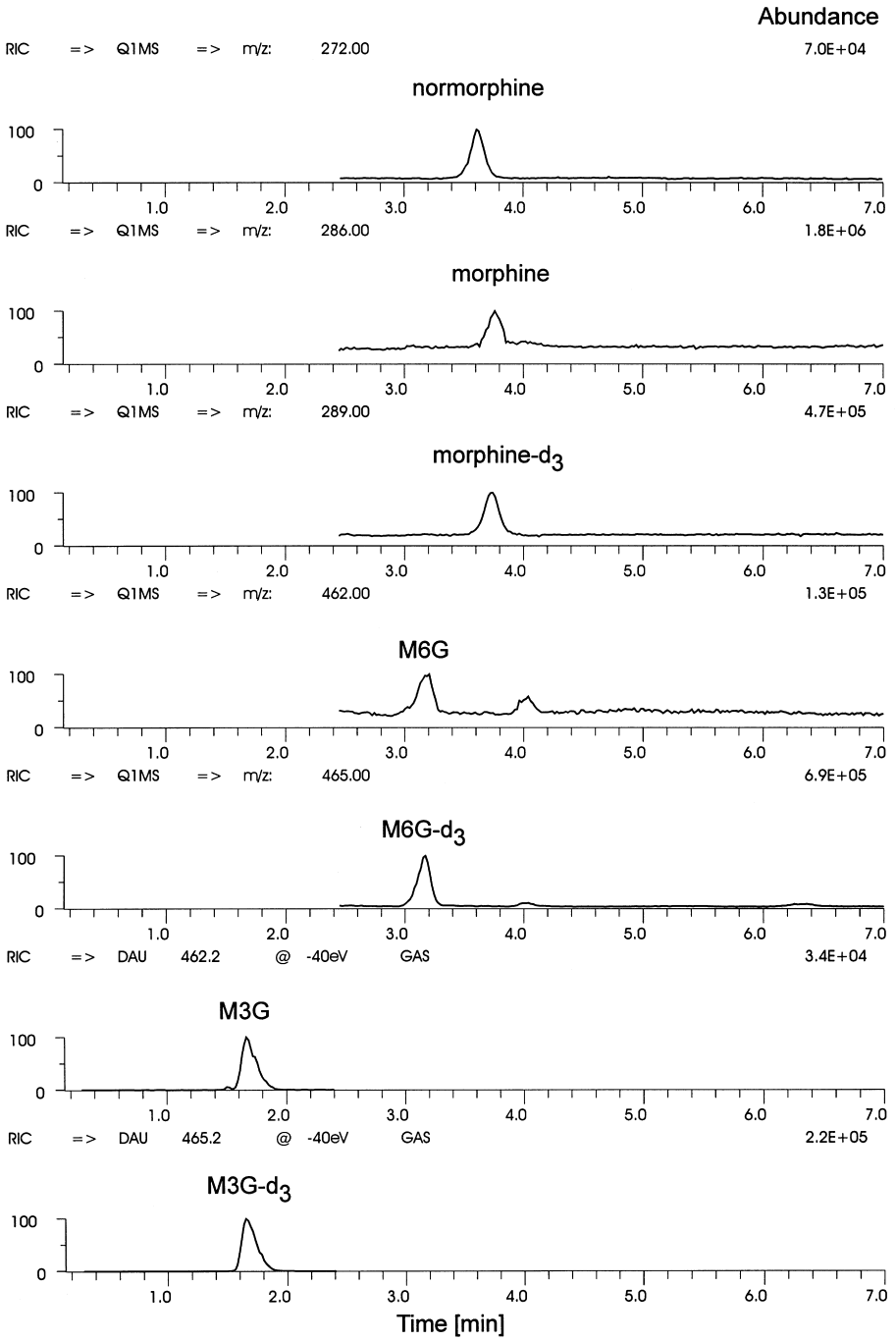

Fig. 2. Mass chromatograms of an extract of 1 ml of serum spiked with 10 pmol/ml morphine, 20 pmol/ml of normorphine and M6G and 80 pmol/ml of M3G.

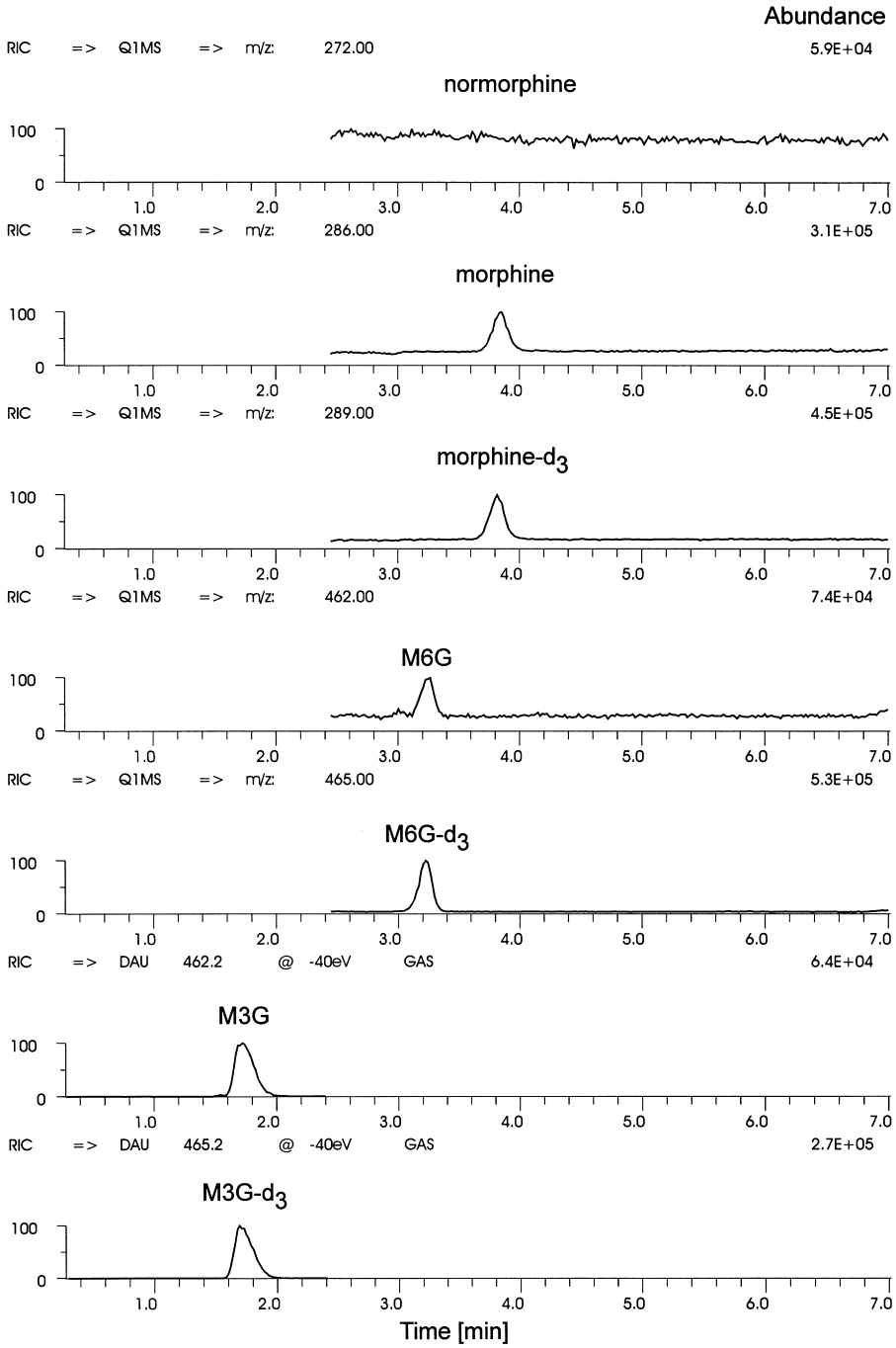

Fig. 3. Mass chromatograms of an extract of 1 ml of serum after treatment with morphine. The following concentrations were found: 128 pmol/ml M3G, 11.1 pmol/ml M6G and 12.3 pmol/ml morphine.

Table 2
Intra-assay precision and accuracy for the determination of morphine and its metabolites in serum

	Concentration added (pmol/ml)	*n*	Concentration found (pmol/ml)	Bias (%)	C.V. (%)
M3G	5.41	10	5.22	−3.6	7
	54.2	9	54.1	−0.2	4
	867	6	897	3.5	2
M6G	1.01	10	0.99	−2.0	9
	20.1	9	20.8	3.4	6
	201	6	204	1.5	5
Morphine	1.33	10	1.25	−6.0	8
	26.6	9	27.4	3.0	6
	266	6	288	8.4	5
Normorphine	1.53	10	1.44	−6.0	9
	30.7	9	30.1	1.8	7
	307	6	317	3.3	8

Table 3
Inter-assay precision and accuracy for the determination of morphine and its metabolites in serum

	Concentration added (pmol/ml)	*n*	Concentration found (pmol/ml)	Bias (%)	C.V. (%)
M3G	5.41	9	5.22	−3.6	4
	54.2	8	54.1	−0.1	4
	867	6	918	6.0	4
M6G	1.01	9	0.98	−2.0	7
	20.1	8	21.0	4.4	4
	201	6	215	7.1	2
Morphine	1.33	9	1.41	6.0	5
	26.6	8	26.4	−0.7	9
	266	6	282	6.1	3
Normorphine	1.53	9	1.35	−12.0	8
	30.7	8	31.6	2.8	6
	307	6	342	11.4	7

Table 4
Intra-assay precision and accuracy for the determination of morphine and its metabolites in urine using a sample size of 100 μl

	Concentration added (pmol/ml)	*n*	Concentration found (pmol/ml)	Bias (%)	C.V. (%)
M3G	50	7	42.9	−14.3	13
	100	6	1046	4.6	4
	2500	6	2668	6.7	4
	12 500	6	12 250	−2.0	1
M6G	20	5	21.6	8.2	7
	500	6	539	7.9	4
	2500	6	2710	8.3	2
Morphine	10	7	9.6	−4.2	8
	20	6	21.0	4.8	14
	500	6	536	7.1	4
	2500	6	2460	−1.5	2
Normorphine	20	5	20.8	3.9	11
	500	6	499	−0.1	3
	2500	6	2600	39	4

Table 5
Values of M3G and M6G determined on two different days in CSF samples from patients administered 0.4 mg/kg morphine sulfate intravenously

Sample	Time (h)	M3G (pmol/0.5 ml)		M6G (pmol/0.5 ml)	
		Assay 1	Assay 2	Assay 1	Assay 2
1	1	3.66	3.71	1.03	0.98
2	1.5	7.80	8.68	2.13	1.81
3	3	20.1	20.8	6.07	5.50
4	4	25.5	27.7	5.60	7.35
5	12	40.5	44.7	9.9	10.4
6	20	35.0	28.6	6.52	6.19
7	32	23.8	18.9	3.60	3.52
8	36	17.7	17.0	3.30	4.30
9	20	19.7	21.3	4.99	5.24
10	36	35.8	34.2	10.9	8.2

3.4. Assay application

The sensitivity achieved is better than with previously published methods [17–20]. It is appropriate for the measurement of morphine glucuronides in serum up to 36 h after 0.4 mg/kg morphine administered intravenously in 30 min and in CSF after 24 h. A representative serum concentration–time curve is

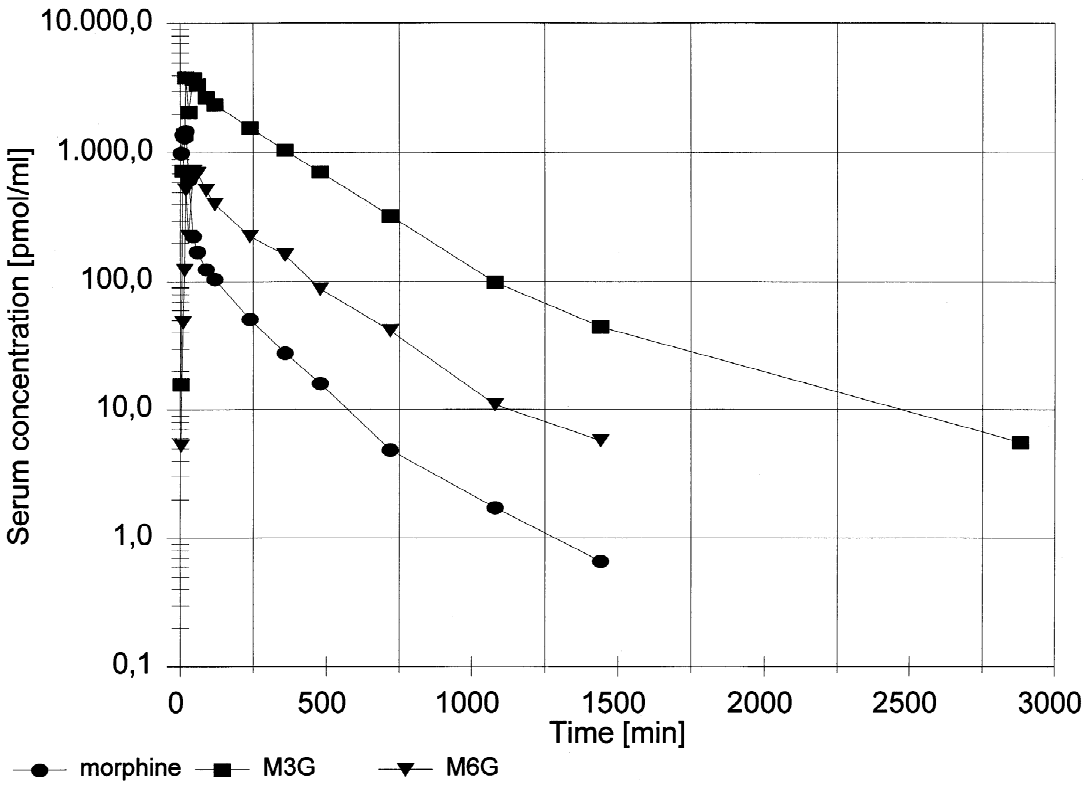

Fig. 4. Typical serum concentration–time curves for morphine, M3G and M6G after 0.4 mg/kg morphine administered intravenously in 30 min to one subject.

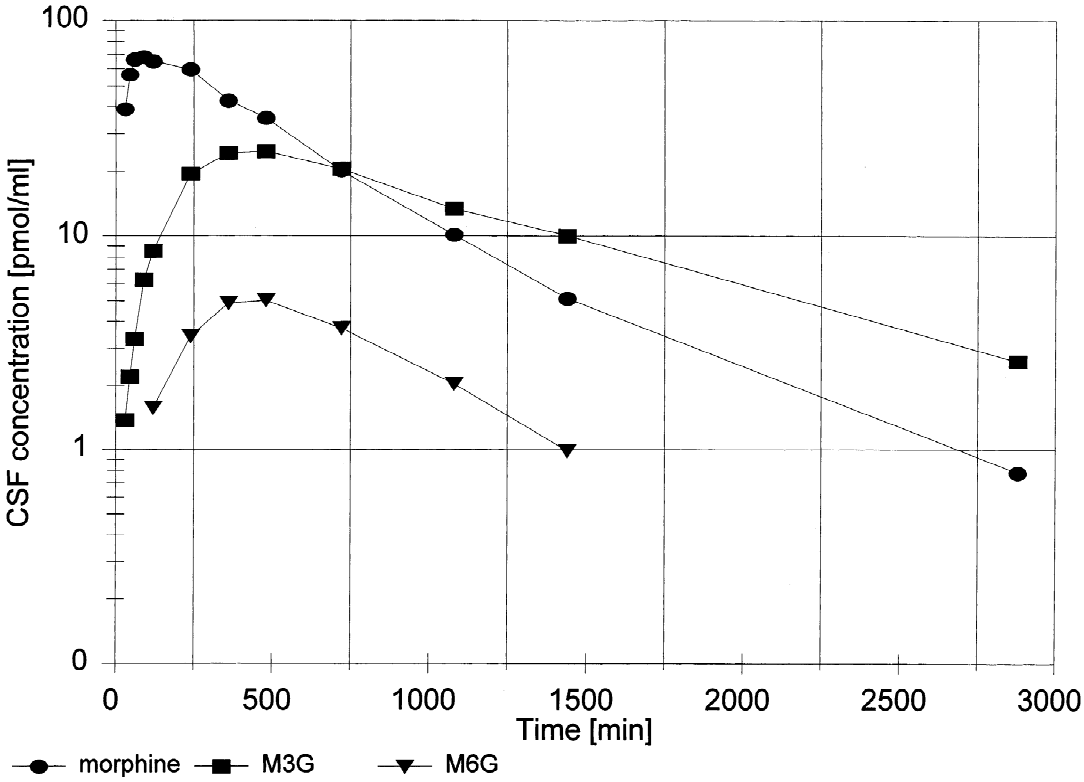

Fig. 5. Typical CSF concentration–time curves for morphine, M3G and M6G after 0.4 mg/kg morphine administered intravenously in 30 min to one subject.

shown in Fig. 4 and the CSF concentration–time curve of the same person is shown in Fig. 5. T_{max} values for the glucuronides are much higher in CSF than in serum compared to morphine. The assay presented here has meanwhile been used for the determination of morphine and its metabolites in several pharmacokinetic studies, for example to determine serum and urine concentrations in a drug interaction study with rifampin after a single oral dose of 10 mg of morphine sulfate [21].

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