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Routine determination of morphine, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide in human serum by liquid chromatography coupled to electrospray mass spectrometry

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Abstract

A robust liquid chromatographic mass spectrometric method capable of quantifying morphine, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide down to 1.0 ng/ml, 5.0 ng/ml and 2.0 ng/ml respectively in human serum is presented. The method was validated over linear ranges of 1.0 to 20.0 ng/ml for morphine, 5.0 to 500.0 ng/ml for morphine 3-β-D-glucuronide and 2.0 to 100.0 ng/ml for morphine 6-β-D-glucuronide using deuterated morphine as internal standard. In tandem mass spectrometry conditions, the product ions of morphine-3-glucuronide and morphine-6-glucuronide were the ion *m/z* corresponding to the morphine moiety. By contrast morphine which presented numerous product ions after collision did not allowed a tandem methodology. Compounds were extracted on 100 mg C_{18} columns and analysed on the PE Sciex API 300 system equipped with a C_{18} column and electrospray ionisation interface. The interrun precision of quality controls (1.0, 2.0, 10.0, 20.0 ng/ml for morphine, 5.0, 10.0, 250.0, 500.0 ng/ml for morphine 3-β-D-glucuronide and 2.0, 4.0, 50.0, 100.0 ng/ml for morphine 6-β-D-glucuronide) was ≤9.3% and accuracy was between 97.9 and 109.8% for each analyte. Sample stabilities in biological matrix were also investigated. This method has been applied to pharmacokinetic analysis of morphine, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide in human serum. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Morphine; Morphine glucuronides

1. Introduction

Morphine is metabolized by conjugation to morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide (Fig. 1) [5]. Morphine and its two metabolites are present in blood and urine.

Many methodologies were developed for determining morphine and its metabolites [1–4,7–9] but liquid chromatography (LC) appeared to be the technique that can separate both these lipophilic and the hydrophilic analytes, while mass spectrometry (MS) using an atmospheric pressure electrospray interface proved useful to achieve the required sensitivities [5,6,10].

Here is presented a LC–tandem MS method for simultaneous quantitation of morphine (LC–MS), morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide (LC–MS–MS) in human serum, using [$^{2}H_3$]morphine (morphine-d_3) as internal standard.

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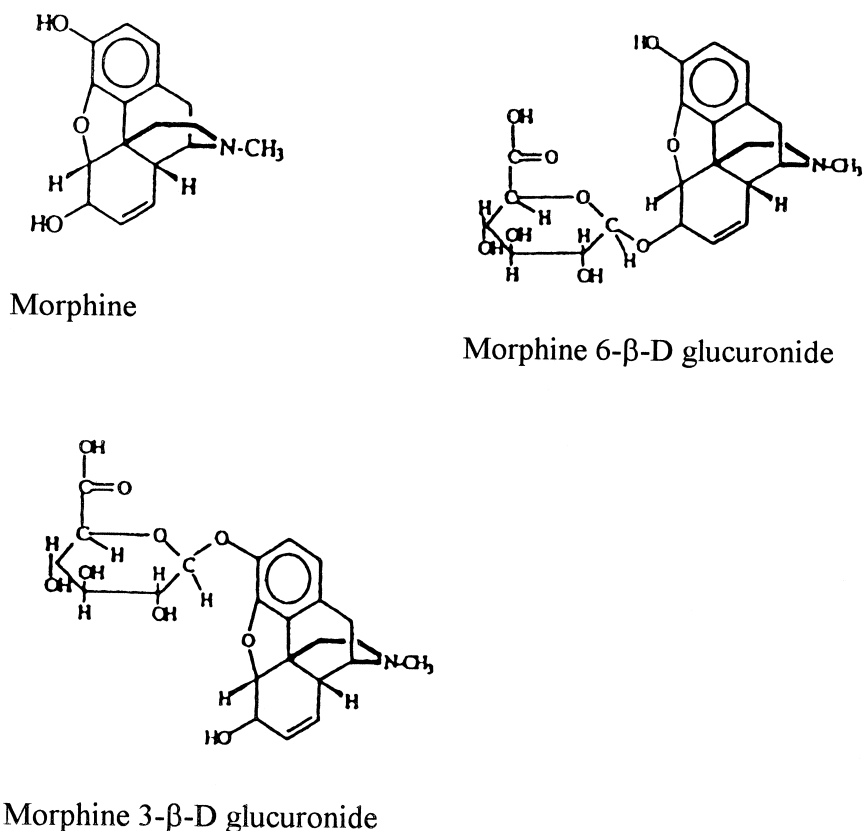

Fig. 1. Structure of studied analytes.

2. Experimental

2.1. Chemicals and reagents

Morphine sulphate pentahydrate, morphine-3-glucuronide, morphine-6-glucuronide and deuterated morphine hydrochloride were obtained from Sigma (St. Quentin Fallavier, France). The concentrations in the text given as ng/ml refer to the concentration of the compounds as free base. All chemicals were analytical reagent grade and were used without further purification.

2.2. Solid-phase extraction

After thawing, the calibration standard samples and the quality control (QC) samples were homogenised by shaking on a vibration shaker for a few seconds. After centrifugation at 4000 rpm for 5 min, aliquots of 1 ml were pipetted into 10 ml polypropylene tubes. Serum sample was spiked with the deuterated internal standard, morphine-d_3, (10 ng) while a blank serum was spiked with morphine solution, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide to be in the range 1–20 ng/ml for morphine, 5–500 ng/ml for morphine 3-β-D-glucuronide and 2–100 ng/ml for morphine 6-β-D-glucuronide. After addition of 1 ml Tris buffer, pH 7.5, the sample was submitted to solid–liquid extraction using 100 mg C_{18} columns (Bond Elut, Varian, Les Ullis, France). The columns were conditioned with 1 ml methanol, 1 ml bidistilled water and 1 ml Tris buffer pH 7.5. Serum samples were loaded onto the column, washed with 1 ml bidistilled water, eluted

with 1 ml acetonitrile–bidistilled water (80:20, v/v) containing 1% formic acid. The elute was evaporated to dryness under nitrogen at 40°C. Residue was redissolved in 100 μl acetonitrile–bidistilled water (6:94, v/v) containing 1% formic acid. After centrifugation for 5 min at 2800 *g*, a 10 μl aliquot was injected onto the HPLC column

2.3. LC

HPLC was carried out using a 100-5 RP 18 e.c. LiChrospher 30×4 mm, without precolumn. The mobile phase consisted of acetonitrile–10^{-3} *M* ammonium formate (6:94, v/v) with 1% (v/v) formic acid. Separation was achieved by isocratic solvent elution at a flow-rate of 0.3 ml/min using a split of 1:19 into the electrospray source.

2.4. MS

An API 300 PE Sciex was used for signal detection in the following optimized conditions (see Table 1):

Morphine	MS first quadrupole (precursor ion) *m/z*: 286.0 $[M+H]^+$ MS–MS third quadrupole *m/z*: 286.0
Morphine 3-β-D-glucuronide	MS first quadrupole (precursor ion) *m/z*: 462.0 $[M+H]^+$ MS–MS third quadrupole *m/z*: 286.0
Morphine 6-β-D-glucuronide	MS first quadrupole (precursor ion) *m/z*: 462.0 $[M+H]^+$ MS–MS third quadrupole *m/z*: 286.0
Morphine-d_3 internal standard	MS first quadrupole (precursor ion) *m/z*: 289.0 $[M+H]^+$ MS–MS third quadrupole *m/z*: 289.0

Table 1
Ions monitored under SIM mode and MRM mode on LC–MS–MS

Analyte	Molecular mass (*M*)	Q1 *m/z*	Q3 *m/z*
Morphine	285.3	286.0	286.0
Morphine 3-β-D-glucuronide	461.5	462.0	286.0
Morphine 6-β-D-glucuronide	461.5	462.0	286.0
Morphine-d_3	288.3	289.0	289.0

Mass spectra are presented in Figs. 2 and 3 for morphine, Figs. 4 and 5 for morphine 3-β-D-glucuronide and Figs. 6 and 7 for morphine 6-β-D-glucuronide.

3. Results and discussion

This methodology was developed to determine morphine, morphine-3-glucuronide and morphine-6-glucuronide in human serum for a routine assay in large series, i.e, hundreds of samples analysed in sequential series with interruption between series for pharmacokinetic purposes.

The main target was robustness. Three steps had to be optimised: chromatographic selectivity, ionisation conditions and mass spectrometry conditions.

3.1. Selectivity

Considering the chromatographic behaviour of the three components, the chromatographic selectivity which allows full separation of morphine-3-glucuronide and morphine-6-glucuronide had to be done since both have the same mother ion and the same product ion. About 30 HPLC reversed-phase columns were tested including $C_{18,}$ $C_{8,}$ and C_2 phases. Three of them gave enough separation for morphine-3-glucuronide and morphine-6-glucuronide, but only the 100-5PR 18 end-capped LiChrospher 30×4 mm brought the required selectivity all over runs of more than 100 injected samples.

3.2. Ionisation conditions

Since the mobile phase under isocratic conditions, comprises a high proportion of water (94%, v/v), containing 10^{-3} *M* ammonium formate, addition of

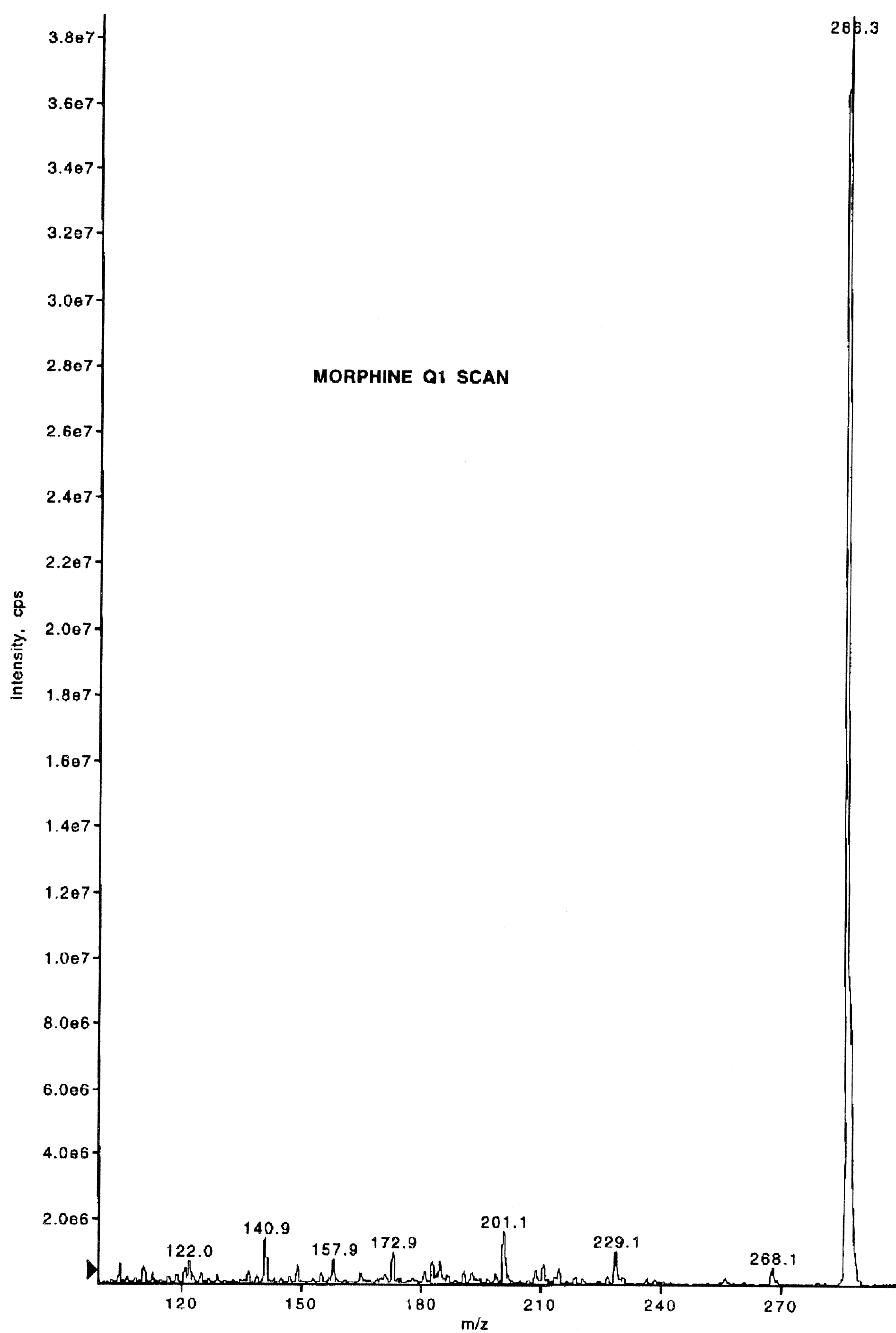

Fig. 2. Mass spectrum of morphine — Q1 scan.

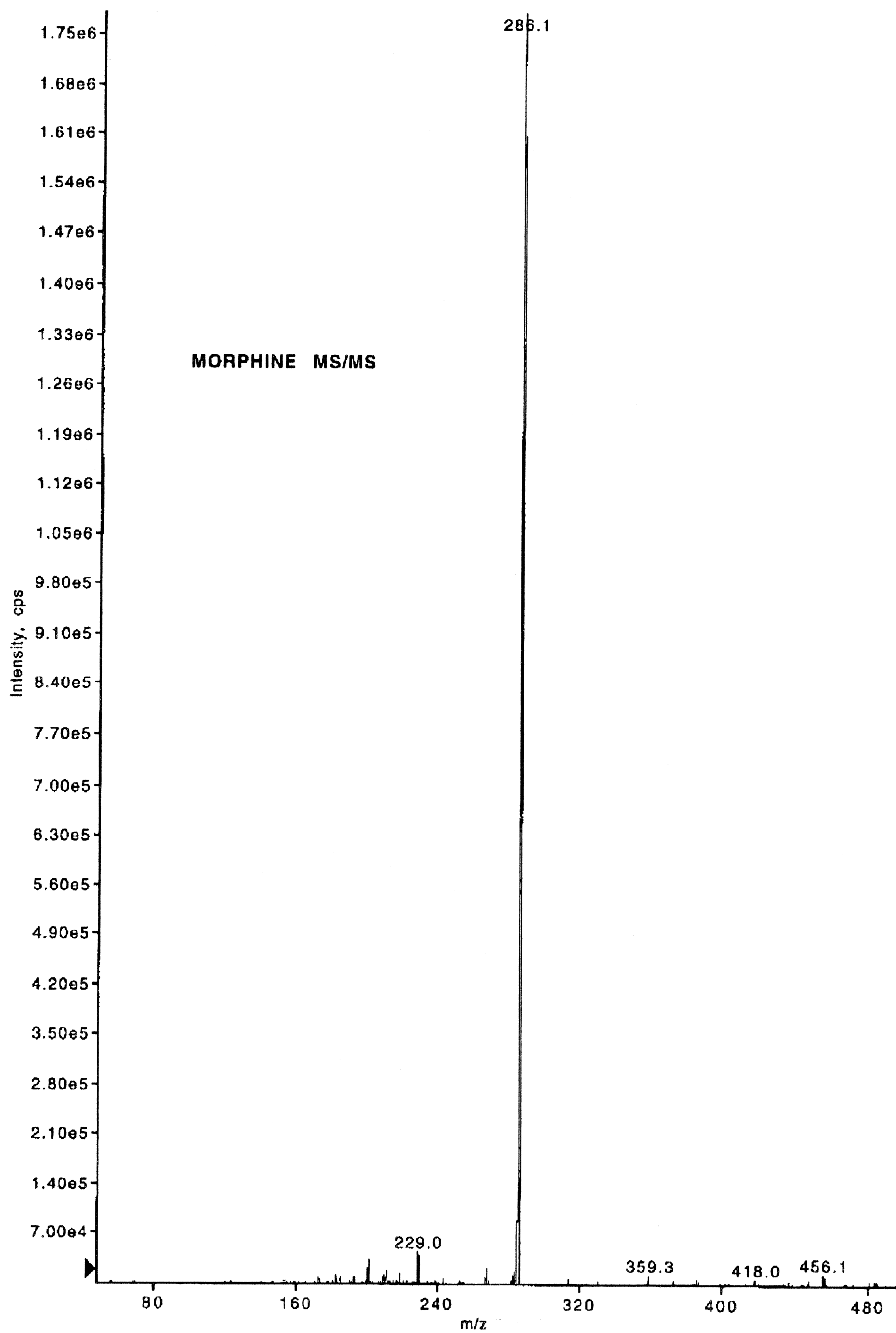

Fig. 3. Mass spectrum of morphine — MS–MS.

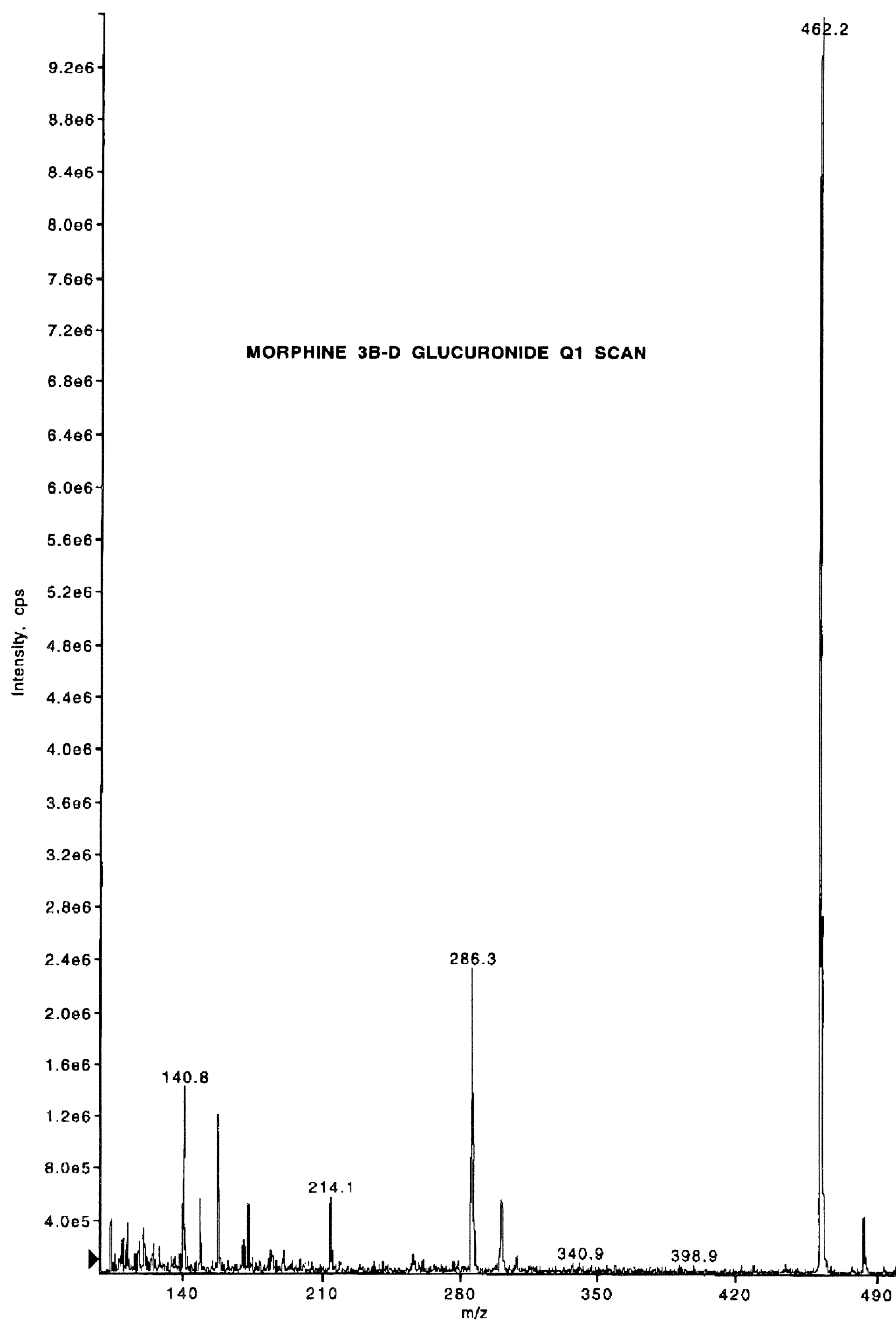

Fig. 4. Mass spectrum of morphine 3-β-D-glucuronide — Q1.

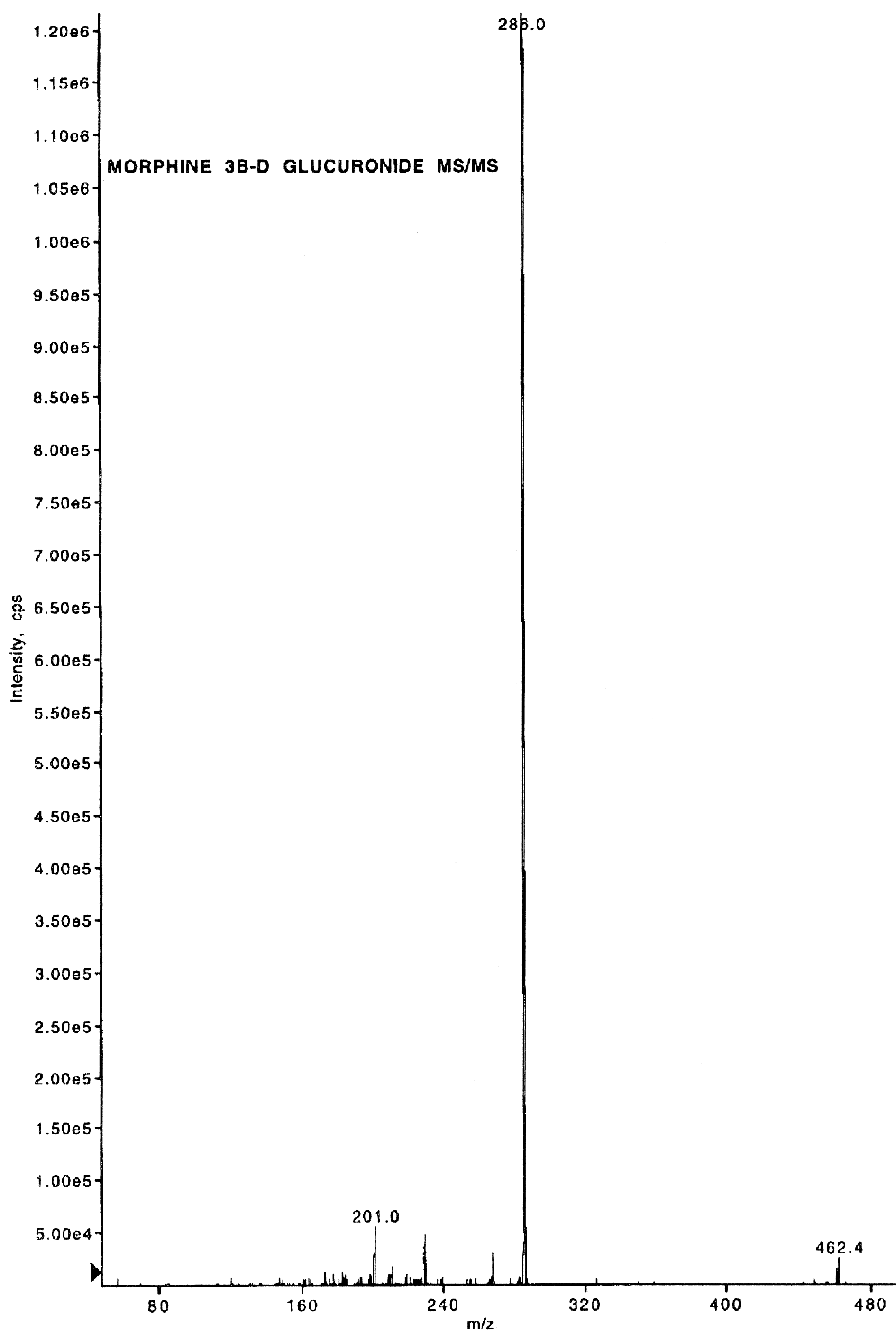

Fig. 5. Mass spectrum of morphine 3-β-D-glucuronide — MS–MS.

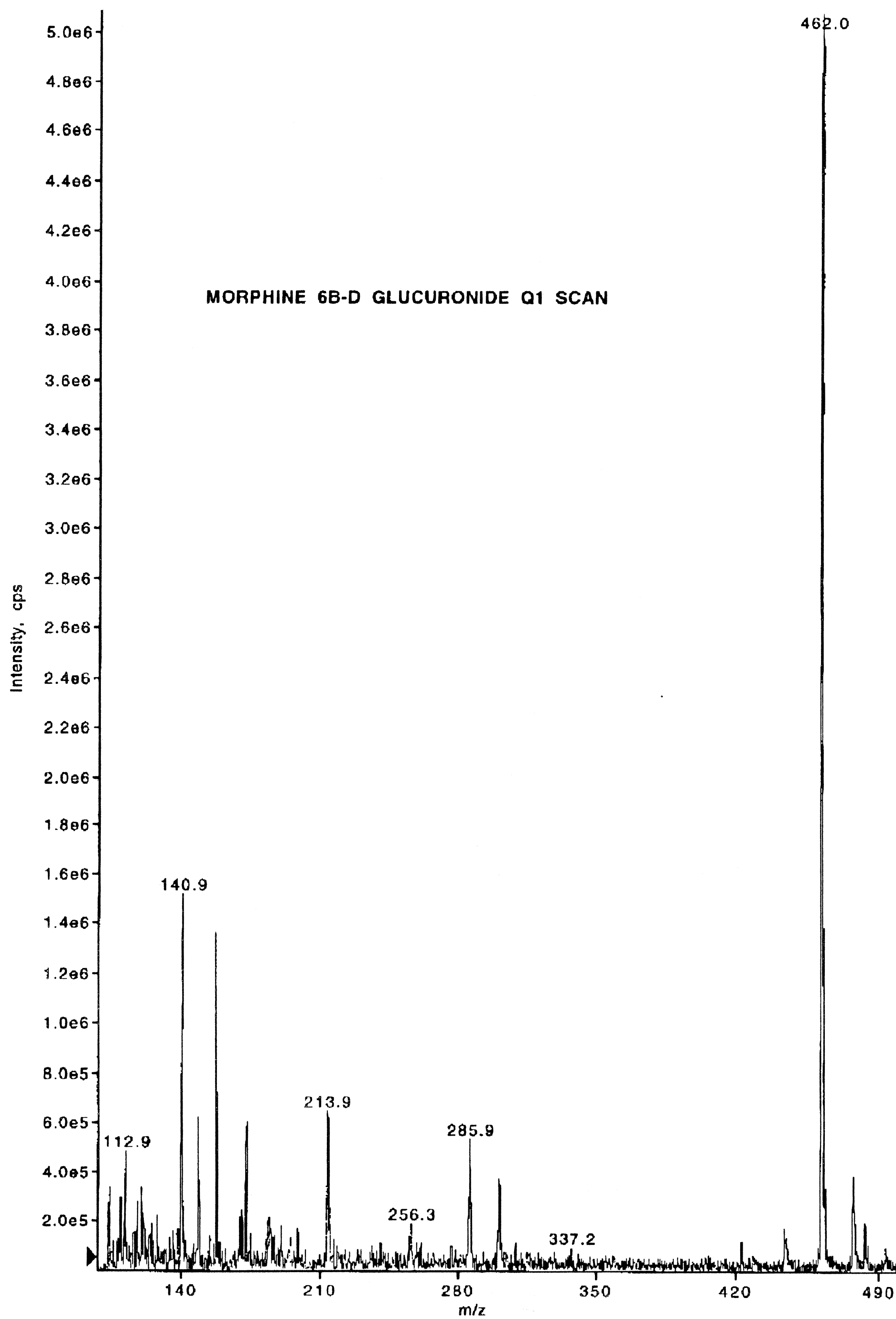

Fig. 6. Mass spectrum of morphine 6-β-D-glucuronide — Q1 scan.

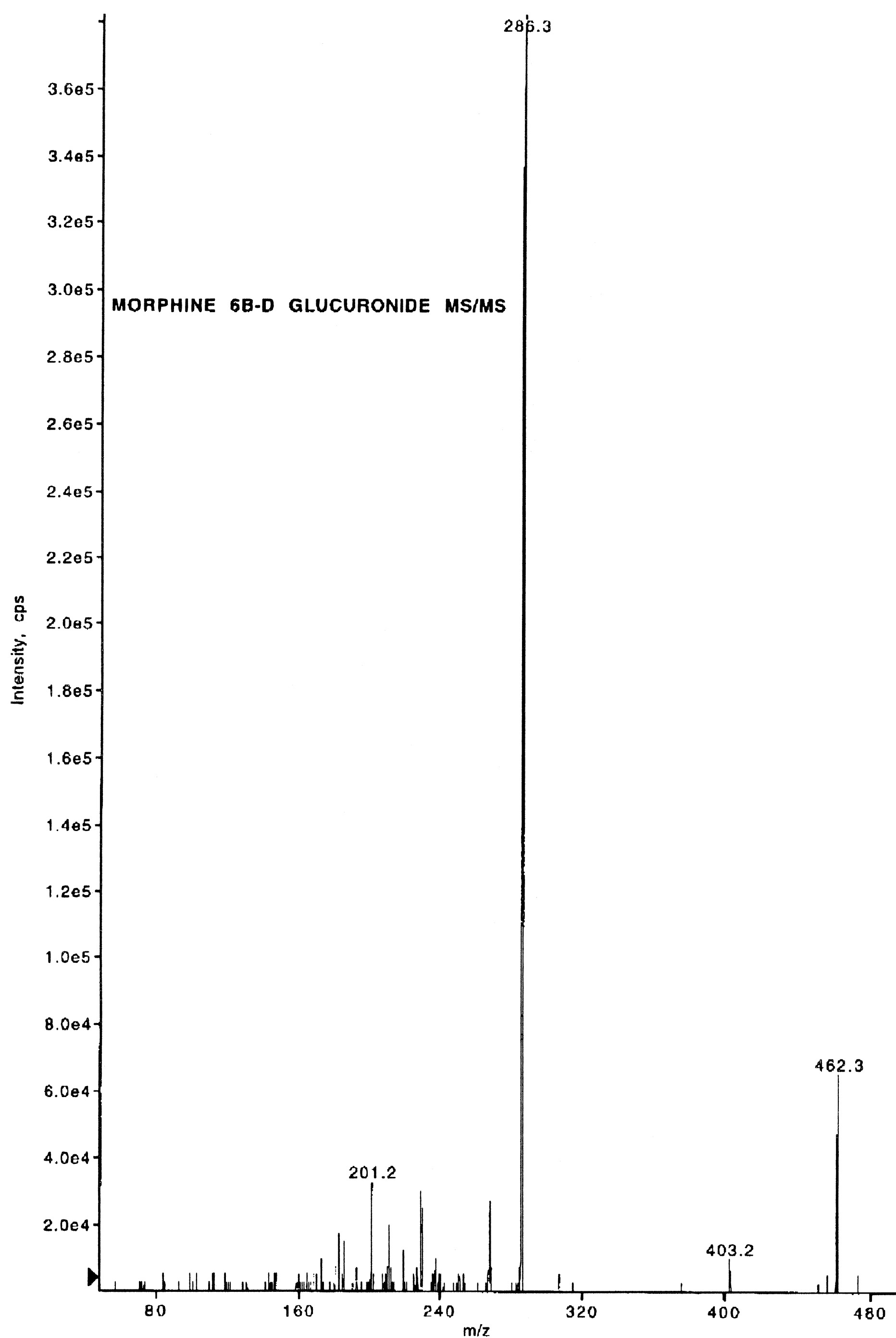

Fig. 7. Mass spectrum of morphine 6-β-D-glucuronide — MS–MS.

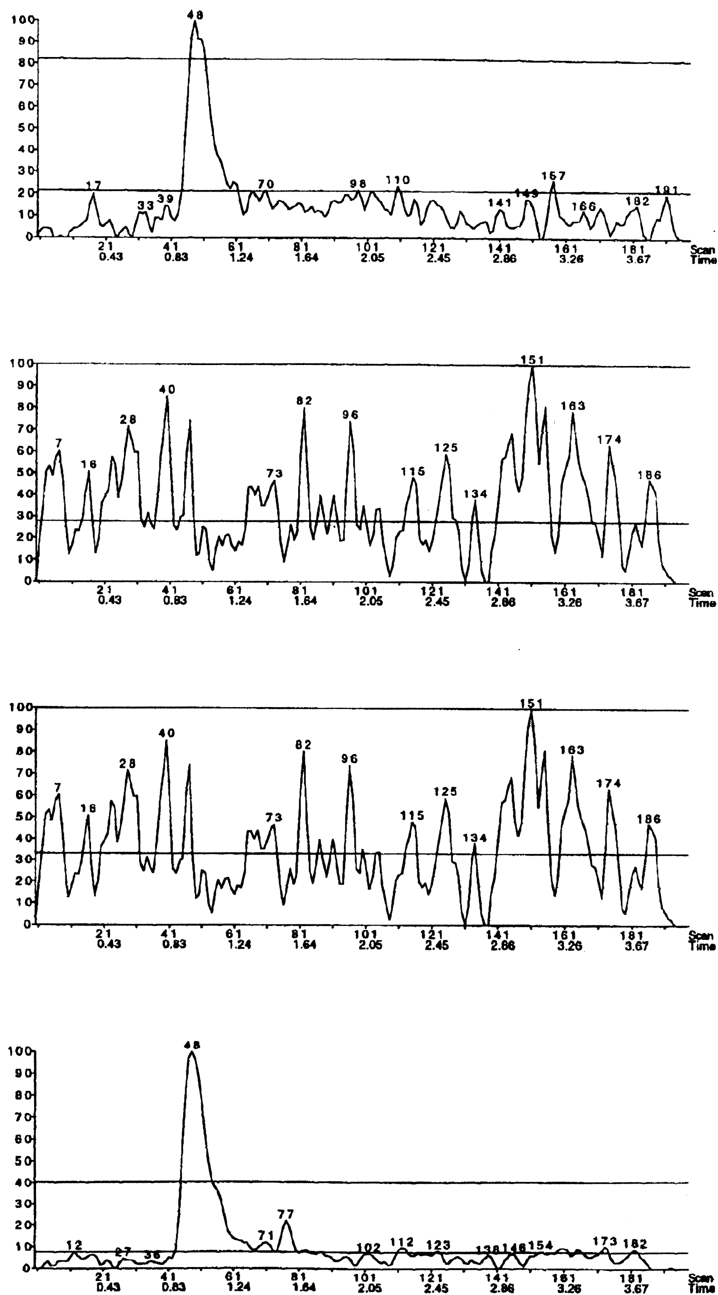

Fig. 8. Chromatograms of blank human serum. Traces: top, morphine; second, morphine-3-glucuronide; third, morphine-6-glucuronide; bottom, deuterated morphine.

Table 2
Mass selected parameters

	Glucuronides	Morphine
Focusing ion voltage (V)	400	400
Orifice voltage (V)	25	25
Curtain gas flow-rate (l/min)	0.95	0.95
Nebulizer gas (l/min)	1.53	1.53
Collision energy (eV)	46.5	24.5
Parent ion kinetic energy (eV)	1	1
Product ion kinetic energy (eV)	3	5

volatile acid to facilitate ionisation was needed. According to the publication of Tyrefors et al. [6] the results showed that both trifluoroacetic acid and ammonium acetate buffers commonly used in connection with electrospray ionisation reduced the signal. Optimized conditions were found with 217 m*M* formic acid instead of 2–3 m*M* as published by Tyrefors et al. [6].

3.3. MS–MS conditions

Morphine-3-glucuronide and morphine-6-glucuronide gave as mother ion m/z 462.0 in positive mode ionisation. The selected parameters to optimise the collision to the m/z 286 ion corresponding to the product ion of morphine were set up as described in Table 2. Unfortunately all the tested conditions of collision were too strong for morphine which appeared as a compound providing numerous product ions after collision. None of them being candidate for the required sensitivity, it was decided to keep the same product ion as the mother ion. This methodology even though proceeding by MS in tandem for morphine-3-glucuronide and morphine-6-glucuronide, operates in the selected ion monitoring (SIM) mode for morphine and its internal standard, deuterated morphine.

Retention times of the four compounds were approximately as follows: morphine-3-β-D-glucuronide, 1.44 min; morphine-6-β-D-glucuronide, 2.07 min; morphine-d_3 internal standard, 2.31 min; morphine, 2.35 min.

3.4. Assay performance

3.4.1. Calibration

Calibration curves were carried out using seven standard points as follows:

- 1.00, 2.00, 5.00, 7.50, 10.0, 15.0 and 20.0 ng/ml for morphine,
- 5.00, 10.0, 25.0, 50.0, 100, 250 and 500 ng/ml for morphine 3-β-D-glucuronide,

Table 3
Standard calibration curves of morphine in human serum

	r[a]	a[b]	b[c]	1.00	2.00	5.00	7.50	10.0	15.0	20.0
n	5	5	5	5	5	5	5	5	5	5
Mean	0.9994	−0.002	0.1037	1.06	2.00	4.84	7.27	9.92	14.9	20.5
SD	0.0001		0.0025	0.027	0.060	0.094	0.063	0.193	0.17	0.19
RSD (%)	0.01		2.4	2.5	3.0	1.9	0.9	1.9	1.1	0.9
Accuracy (%)				106.0	100.0	96.8	96.9	99.2	99.3	102.5

[a] r=Correlation coefficient.
[b] a=Slope.
[c] b=Intercept.

Table 4
Standard calibration curves of morphine 3-β-D-glucuronide in human serum

	r	a	b	5.00	10.0	25.0	50.0	100	250	500
n	5	5	5	5	5	5	5	5	5	5
Mean	0.9992	0.0029	0.0126	4.72	10.2	26.4	48.3	103	254	495
SD	0.0011		0.0036	0.631	0.76	1.45	1.99	4.4	11.3	13.2
RSD (%)	0.1		28.6	13.4	7.5	5.5	4.1	4.3	4.4	2.7
Accuracy (%)				94.4	102.0	105.6	96.6	103.0	101.6	99.0

Table 5
Standard calibration curves of morphine 6-β-D-glucuronide in human serum

	r	a	b	2.00	5.00	10.0	25.0	50.0	75.0	100
n	5	5	5	5	5	5	5	5	5	5
Mean	0.9984	0.0011	0.0100	2.06	5.04	9.93	24.5	49.9	75.5	101
SD	0.0012		0.0016	0.233	0.408	0.326	2.93	1.46	3.50	4.8
RSD (%)	0.1		16.0	11.3	8.1	3.3	12.0	2.9	4.6	4.8
Accuracy (%)				103.0	100.8	99.3	98.0	99.8	100.7	101.0

- and 2.00, 5.00, 10.0, 25.0, 50.0, 75.0 and 100 ng/ml for morphine 6-β-D-glucuronide.

Theses ranges suit the serum concentrations observed in the present study and those published by Osborne et al. [3] after different routes of administration comprising oral, buccal, sublingual and intravenous. For higher levels dilution can be carried out as described below.

Different stock solutions of morphine, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide were used for the preparation of QC and calibration samples.

Results are presented in Tables 3–5.

3.4.2. Interday reproducibility

Five different runs were carried out over 5 days, results are presented in Tables 6–8.

Table 6
Interday reproducibility for morphine (n=10)

Nominal conc. in QC sample (ng/ml)	Accuracy[a]	Precision[b] (%)
1.00	102.0	5.7
2.00	98.5	3.0
10.0	101.0	2.7
20.0	102.5	2.2

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

Table 7
Interday reproducibility for morphine 3-β-D-glucuronide (n=10)

Nominal conc. in QC sample (ng/ml)	Accuracy[a]	Precision[b] (%)
5.00	99.2	15.2
10.00	97.9	9.3
250	103.2	5.5
500	102.2	6.1

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

3.4.3. Dilution test

QC samples were prepared at the following nominal concentrations: morphine, 30 ng/ml; morphine 3-β-D-glucuronide, 600 ng/ml and morphine 6-β-D-glucuronide, 150 ng/ml.

Samples were 2–5-fold diluted in blank human serum (see Tables 9–11).

Table 8
Interday reproducibility for morphine 6-β-D-glucuronide (n=10)

Nominal conc. in QC sample (ng/ml)	Accuracy[a]	Precision[b] (%)
2.00	96.0	12.6
4.00	98.0	6.0
50.0	109.8	5.6
100	104.0	5.5

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

Table 9
Dilution test for morphine (n=6)

Dilution	Accuracy[a]	Precision[b] (%)
1/2	94.7	1.1
1/5	97.7	4.9

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

Table 10
Dilution test for morphine 3-β-D-glucuronide (n=6)

Dilution	Accuracy[a]	Precision[b] (%)
1/2	101.5	3.4
1/5	97.8	2.3

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

Table 11
Dilution test for morphine 6-β-D-glucuronide ($n=6$)

Dilution	Accuracy[a]	Precision[b] (%)
1/2	99.3	4.8
1/5	102.0	5.3

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

Table 13
Stability for morphine 3-β-D-glucuronide ($n=6$)

	Accuracy[a]	Precision[b] (%)
t_0	100.8	2.8
After 3 cycles	92.0	7.2

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

Tables 6–8 display the interday precision of four quality control levels for each analyte. All the results being included (morphine, morphine-3-glucuronide and morphine-6-glucuronide), after five different runs, mean recovery was included in the range 97.9–109.8% and the precision 2.2–15.2%.

The dilution test was achieved on QC samples prepared at the following nominal concentrations: morphine, 30 ng/ml; morphine 3-β-D-glucuronide, 600 ng/ml and morphine 6-β-D-glucuronide, 150 ng/ml. QC samples were two- and fivefold diluted in human serum. All the results being concerned (morphine, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide), mean recovery was included in the range 94.7–102.0% and the precision was included in the range 1.1–5.3%. Results are presented in Tables 9–11.

Since the limit of quantitation (LOQ) is the lowest concentration of quality control being supported by precision and accuracy in the ± 20% limits, LOQ was found as 1.00 ng/ml for morphine, 5.00 ng/ml for morphine3-β-D-glucuronide and 2.00 ng/ml for morphine 6-β-D-glucuronide.

3.4.4. Stability

Stability of the three components was tested after three freeze/thaw cycles to face reassays if necessary. All results being included (morphine, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide) mean recovery was included in the range 97.0–104.0% and precision was included in the range 2.7 – 7.2%.

Results are presented in Tables 12–14.

4. Conclusion

This methodology led to a robust and routine assay method and showed the following advantages, compared to the previous LC–MS methods already published:

- shorter analysis time, 5 min versus 25 min [10],
- isocratic HPLC conditions for robustness versus gradient conditions [6],
- MS in tandem for higher specificity for morphine-3-glucuronide and morphine-6-glucuronide [5,6,10].

Five weeks were required for set up of this method while 5 days for validation and 7 days for 550 assays and reassays for pharmacokinetic reasons.

This method was applied to a routine analysis of a pharmacokinetic interaction study after administration of 7.5 mg of morphine as intravenous infusion over 2 h to 18 healthy volunteers.

Table 12
Stability for morphine ($n=6$)

	Accuracy[a]	Precision[b] (%)
t_0	104.0	3.0
After 3 cycles	100.0	3.7

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

Table 14
Stability for morphine 6-β-D-glucuronide ($n=6$)

	Accuracy[a]	Precision[b] (%)
t_0	97.0	2.7
After 3 cycles	99.4	5.1

[a] Accuracy=% of nominal concentration.
[b] Precision=100×standard deviation/mean.

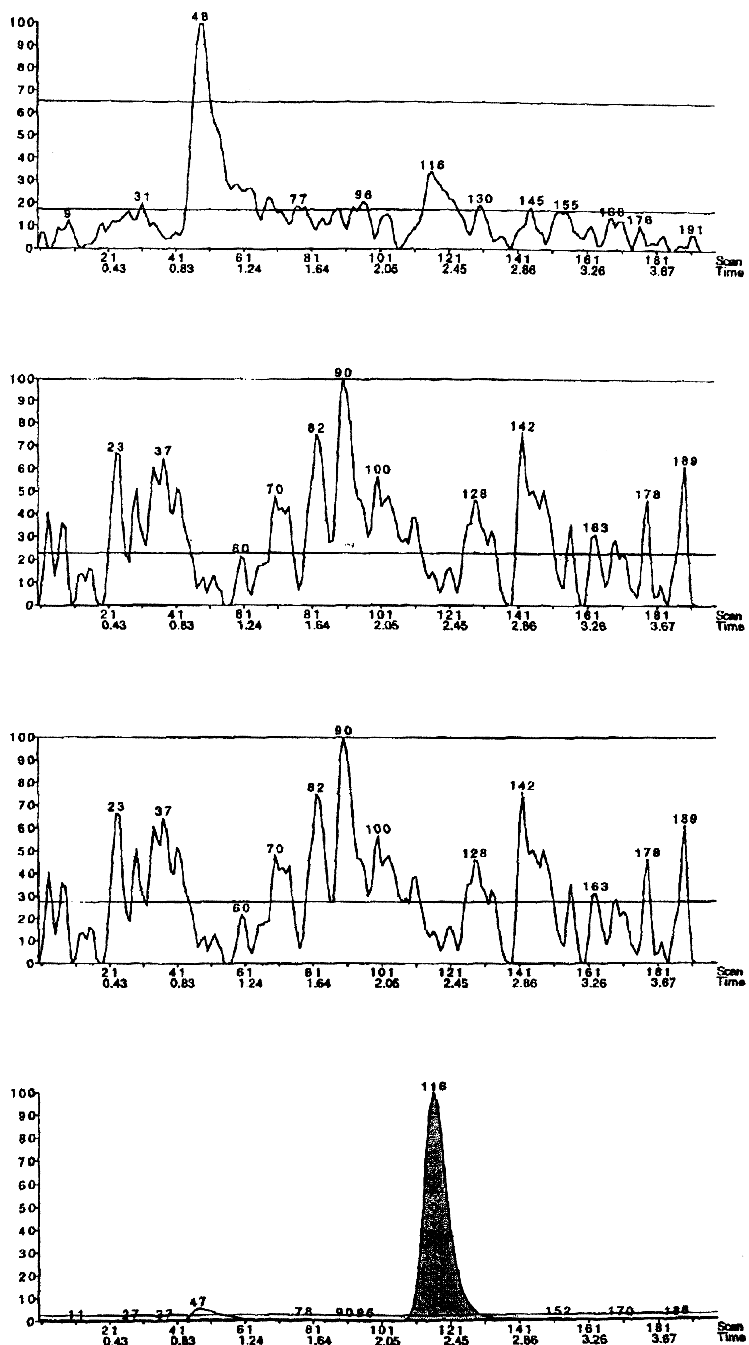

Fig. 9. Chromatograms of blank human serum spiked with 10 ng/ml of deuterated morphine. Traces: top, morphine; second, morphine-3-glucuronide; third, morphine-6-glucuronide; bottom, deuterated morphine.

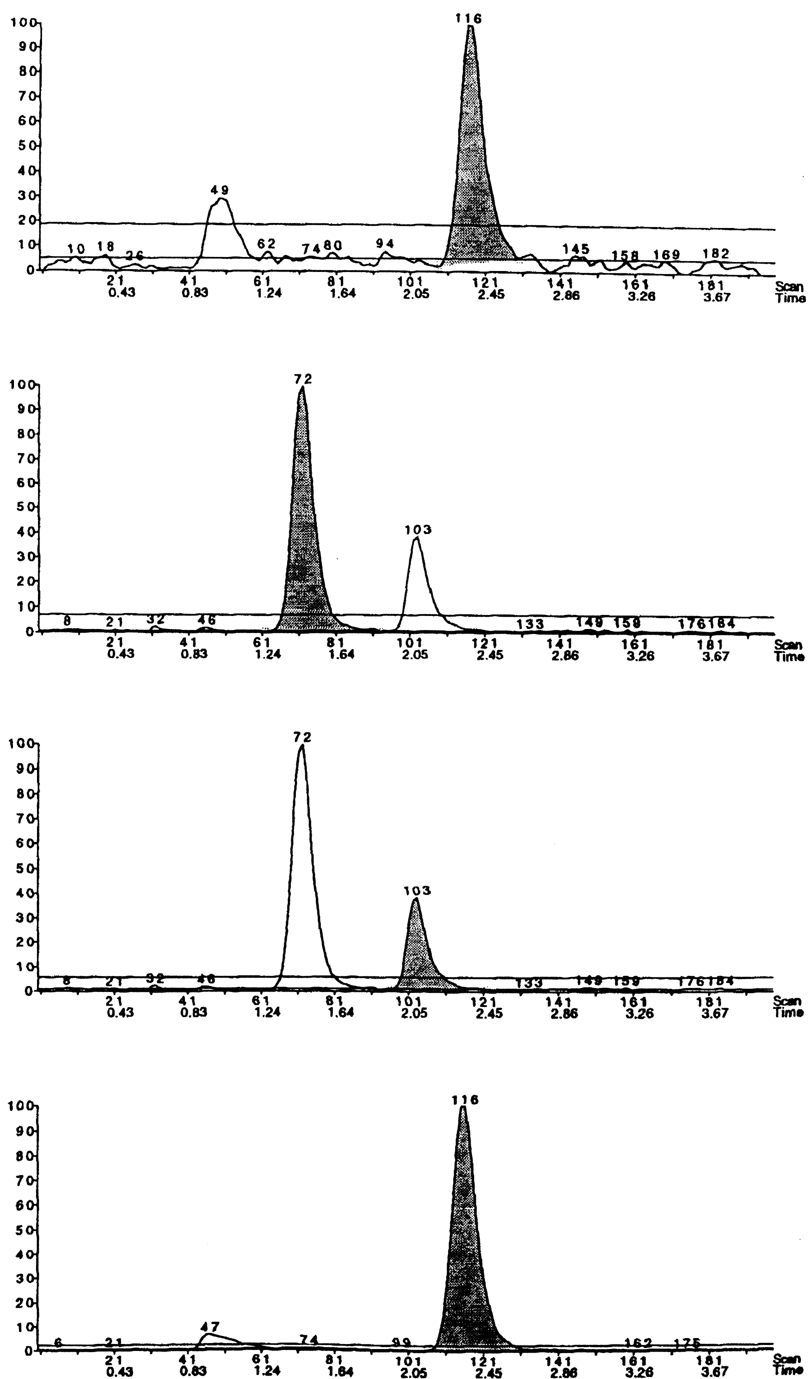

Fig. 10. Chromatograms of human serum at the LOQ; 1 ng/ml for morphine, 5 ng/ml for morphine-3-glucuronide, 2 ng/ml for morphine-6-glucuronide, 10 ng/ml for deuterated morphine.

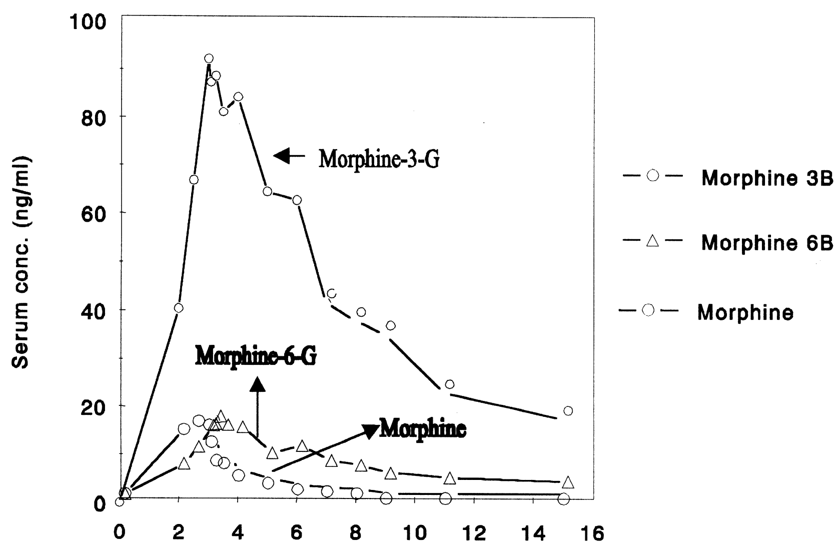

Fig. 11. Morphine, morphine 3-β-D- glucuronide and morphine 6-β-D-glucuronide serum concentrations obtained for one subject.

Typical chromatograms obtained during the study are displayed in Figs. 8–10. A plot of morphine, morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide concentrations in serum versus time after treatment from a single subject is shown in Fig. 11.

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