ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 179–187

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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LC determination of morphine and morphine glucuronides in human plasma by coulometric and UV detection

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Received 22 September 2000; received in revised form 9 January 2001; accepted 3 February 2001

Abstract

A reversed-phase high-performance liquid chromatographic method with coulometric and UV detection has been developed for the simultaneous determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide. The separation was carried out by using a Supelcosil LC-8 DB reversed-phase column and 0.1 M potassium dihydrogen phosphate (pH 2.5)–acetonitrile–methanol (94:5:1 v/v) containing 4 mM pentanesulfonic acid as the mobile phase. The compounds were determined simultaneously by coulometry for morphine and with UV detection for morphine-3-glucuronide and morphine-6-glucuronide. Morphine, morphine glucuronides and the internal standard were extracted from human plasma using Bond-Elut C18 (1 ml) solid-phase extraction cartridges. In the case of coulometric detection, the detection limit was 0.5 ng/ml for morphine; in the case of UV detection the detection limit was 10 ng/ml for morphine-3-glucuronide and for morphine-6-glucuronide, too.

Keywords: Morphine; Morphine-3-glucuronide; Morphine-6-glucuronide; HPLC; Simultaneous UV and ECD detection; Solid-phase extraction

1. Introduction

Morphine (M) is a highly effective and preferred drug for the treatment of moderate to severe pain. The metabolism of M in human subjects is primarily through glucuronidation by microsomal UDP-glucuronyltransferase to morphine-3-glucuronide (M3G) and, to a lesser extent to morphine-6-glucuronide (M6G). M6G is a potent μ receptor agonist with an analgesic activity greater than that of the M. M3G has no opioid action but it seems to be a functional antagonist of M and M6G and it may have a role in the side-effects of M [1].

Several methods were described for the simultaneous determination of M and its major metabolites. The most common analytical techniques currently used are high-performance liquid chromatography (HPLC) with fluorescence [2,3], UV [4–6], electrochemical [7,8] and mass-spectrometry (MS) detection [9–11], or the combination of

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0731-7085/01/$ - see front matter
PII: S0731-7085(01)00393-4

these detectors [12–15]. The HPLC tandem MS assays may give reasonable results, but are extremely expensive and consequently are not commonly available for pharmacokinetic analysis.

The aim of our study was to develop a HPLC method for the analysis of M, M3G and M6G in human plasma for a bioequivalence investigation of an orally administered retard preparation containing morphine sulphate. According to the results of the previously published studies, the measured plasma concentration values of M, M3G and M6G were about 1–20, 50–200, and 15–150 ng/ml, respectively after oral administration of 30 mg morphine sulphate. The solid-phase extraction followed by reversed phase HPLC with simultaneous UV and coulometric detection described in our paper ensure the sensitive and reliable determination of morphine and its glucuronides and is less expensive than the LC/MS-LC/MSMS methods.

2. Experimental

2.1. Materials

M, M3G, M6G and *N*-ethyl-normorphine as internal standard (IS) were synthetised and provided by ICN Hungary (Tiszavasvári, Hungary).

Methanol, acetonitrile, dichlormethane (LiChrosolv chromatography grade) and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, sodium hydroxide and ammonium sulphate were obtained from Reanal (Budapest, Hungary). Pentanesulfonic acid sodium salt was from Sigma (St. Louis, USA)

Bond-Elut C18 cartridge of 1 ml/100 mg was the product of Varian (Harbor City, CA, USA).

2.2. Chromatographic conditions

The HPLC system consisted of Shimadzu (Kyoto, Japan) equipments (LC-10AD pump, SIL-10A autosampler, SCL-10A system controller and a CTO-10AC column thermostat). For the detection, a Coulochem II electrochemical detector — equipped with a 5020 model guard and 5011 model analytical cell (ESA Inc, Bedford, MA, USA) — coupled with a Shimadzu 10A UV were used. The potentials for the guard cell and the first and second electrode were 500, 400 and 700 mV, respectively, the UV detector wavelength was set at 210 nm. Fig. 1 shows the schematic flow diagram of the HPLC system.

The separation was accomplished at ambient temperature (air-conditioned room with temperature 22 ± 2°C, the column thermostat was adjusted at 23°C), on a Supelcosil LC8-DB (150×4.6 mm i.d., 5 μm particle size) analytical column equipped with a Supelcosil LC8-DB (20×4.6 mm) guard column (Supelco, Bellefonte, PA, USA). The mobil phase consisted of 0.05 M potassium dihydrogen phosphate buffer (pH 2.5), acetonitrile and methanol (94:5:1 v/v) containing 4 mM pentanesulfonic acid. The flow rate was 1 ml/min. The eluent was filtered through a Supelco Nylon (0.2 μm) membrane (Supelco, Bellefonte, PA, USA) and degassed by ultrasonication.

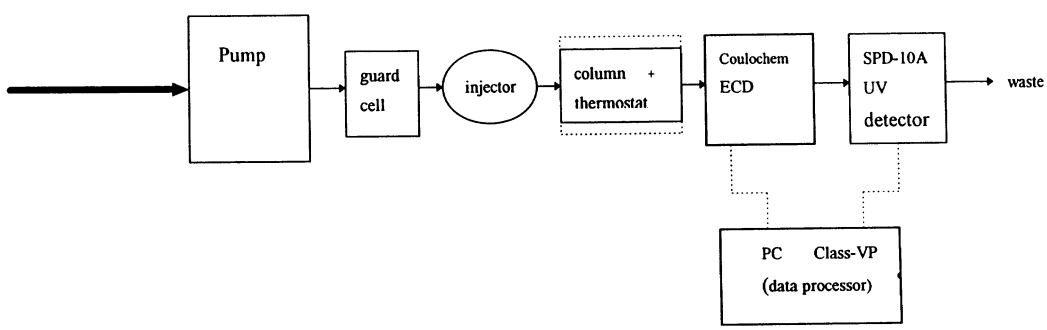

Fig. 1. Schematic flow diagram of the HPLC system.

Chromatograhy was performed using a ClassVP 4.2 software (Shimadzu, Kyoto, Japan).

2.3. Solutions

Stock solutions (1 mg/ml): M and IS were prepared in methanol, M3G and M6G were prepared in distilled water. When stored at −20°C, stock solutions were stable for at least 8 weeks.

Working standard solutions of 100, 10, 1 μg/ml, and 100 ng/ml of the compounds and 10 μg/ml of IS were obtained by diluting the stock solution with distilled water.

The working standard solutions were prepared freshly every week and stored at 4°C.

2.4. Plasma samples

Blank human plasma was prepared from blood of drug-free volunteers obtained by venipuncture from the cubital vein. Heparin sodium (25 000 IU) was used as anticoagulant, 20 μl to 7 ml blood. Whole blood was centrifuged at $1500 \times g$ for 10 min and the resulting plasma was stored at −20°C until processing.

Biological samples were collected from healthy volunteers similarly, at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16 and 24 h after drug administration. During the bioequivalence study retard test and reference preparations — containing 30 mg morphine — were administered orally.

2.5. Quality control (QC) samples

For the method validation, QC samples were prepared from pooled drug-free human plasma, in advance. (QC1: 10 ng/ml M, 300 ng/ml M3G, 100 ng/ml M6G; QC2: 50 ng/ml M, 500 ng/ml M3G, 200 ng/ml M6G; QC3: 100 ng/ml M, 1000 ng/ml M3G, 300 ng/ml M6G). QC samples were stored deep-frozen at −20°C. The IS was added to each QC sample just prior to sample processing.

2.6. Sample processing

To 1 ml of plasma 300 ng of the IS was added, using a 10 μg/ml concentration working solution and then homogenized with a vortex mixer for 5 s. One millilitre of 0.5 M ammonium sulphate (pH 9.3) solution was added to the mixture, and vortexed for 5 s. M, M3G, M6G and IS were extracted from the plasma with solid phase extraction (SPE), using a vacuum manifold (Supelco, Bellefonte, PA, USA). The Bond-Elut C18 cartridges were activated with 2×1 ml of methanol and 2×1 ml distilled water. Homogenized plasma samples were then applied onto the cartridge. The column was not allowed to dry before sample application. However, prior to washing the cartridge, it was dried in on air stream. The column was consecutively washed with 1 ml of 0.005 M, pH 9.3 ammonium sulphate buffer, and 2 ml of dichlormethane. The column was then dried on air stream, and allowed to stand for 15 min at room temperature. The sample was then eluted with 400 μl of (methanol:dichlormethane = 9:1):1N HCl = 98:2 (v/v) solution. The eluates were evaporated to dryness under a stream of nitrogen, and dissolved in 200 μl of the mobile phase. The injected volume was 50 μl.

3. Results and discussion

An ion-pair reversed-phase liquid chromatographic method with a simultaneous coulometric and UV detection has been developed for the quantitative determination of morphine and its active metabolites in human plasma.

In the present method, the mobile phase, together with a Supelcosil LC8-DB stationary phase ensured symmetrical, close to optimum peak shape both for the study compounds and the IS.

The described solid phase extraction procedure ensured the cleanest extract and offered a rapid and reliable way to isolate the investigated compounds together with the IS.

During the analysis we used a coupled detector system: we applied coulometric detection for the determination of M; in the case of M3G and M6G we detected the compounds by UV detection.

Under the conditions described above, the total analysis cycle was 25 min per sample.

Fig. 2 demonstrates the chromatogram of a typical blank plasma extract and a blank plasma

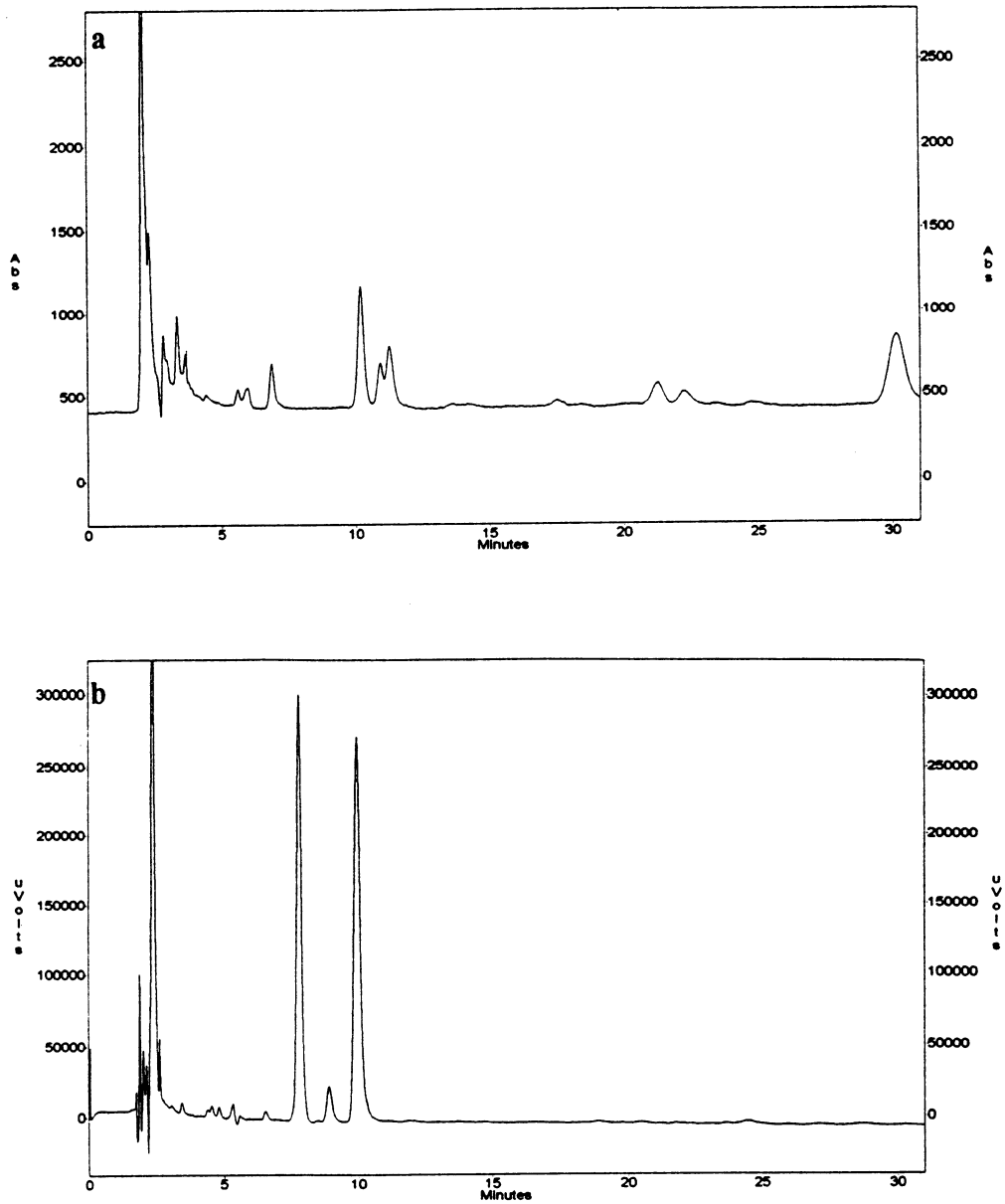

Fig. 2. Chromatogram of a typical blank plasma extract by UV (a) and coulometric (b) detection and a blank plasma spiked with 100 ng M, 1000 ng M3G and 300 ng M6G by UV (c) and coulometric (d) detection.

spiked with 100 ng M, 1000 ng M3G and 300 ng M6G by UV and coulometric detection. Fig. 3 shows a typical chromatogram of a volunteer's plasma sample after oral administration of the drug containing 30 mg morphine by UV and coulometric detection. Endogenous compounds interfering with retention times of the compounds or that of the IS could not be seen in the chromatograms.

3.1. Validation

For the examination of system suitability, five injections were made from the same biological

sample containing 300 ng/ml M3G, 100 ng/ml M6G, 10 ng/ml M and 300 ng/ml IS. Based on the system suitability test, the mean retention times and S.D. for M were 11.19 (0.23), for M3G were 4.95 (0.26), for M6G were 8.29 (0.25) and for the IS 19.59 (0.33) min. On the basis of five replicate determinations, the reproducibility (R.S.D.%) of the retention time was 2.09, 5.15, 2.57%, for the compounds and 1.68% for IS, whereas the reproducibility of the peak area values was 1.11, 2.57, 3.33 and 4.48% for the compounds and IS, respectively.

Validation data indicated that the limit of quantitation (LOQ) was 1 ng/ml (M), 25 ng/ml (M3G) and 15 ng/ml (M6G) and the limit of detection (LOD) was 0.5 ng/ml for M; in the case

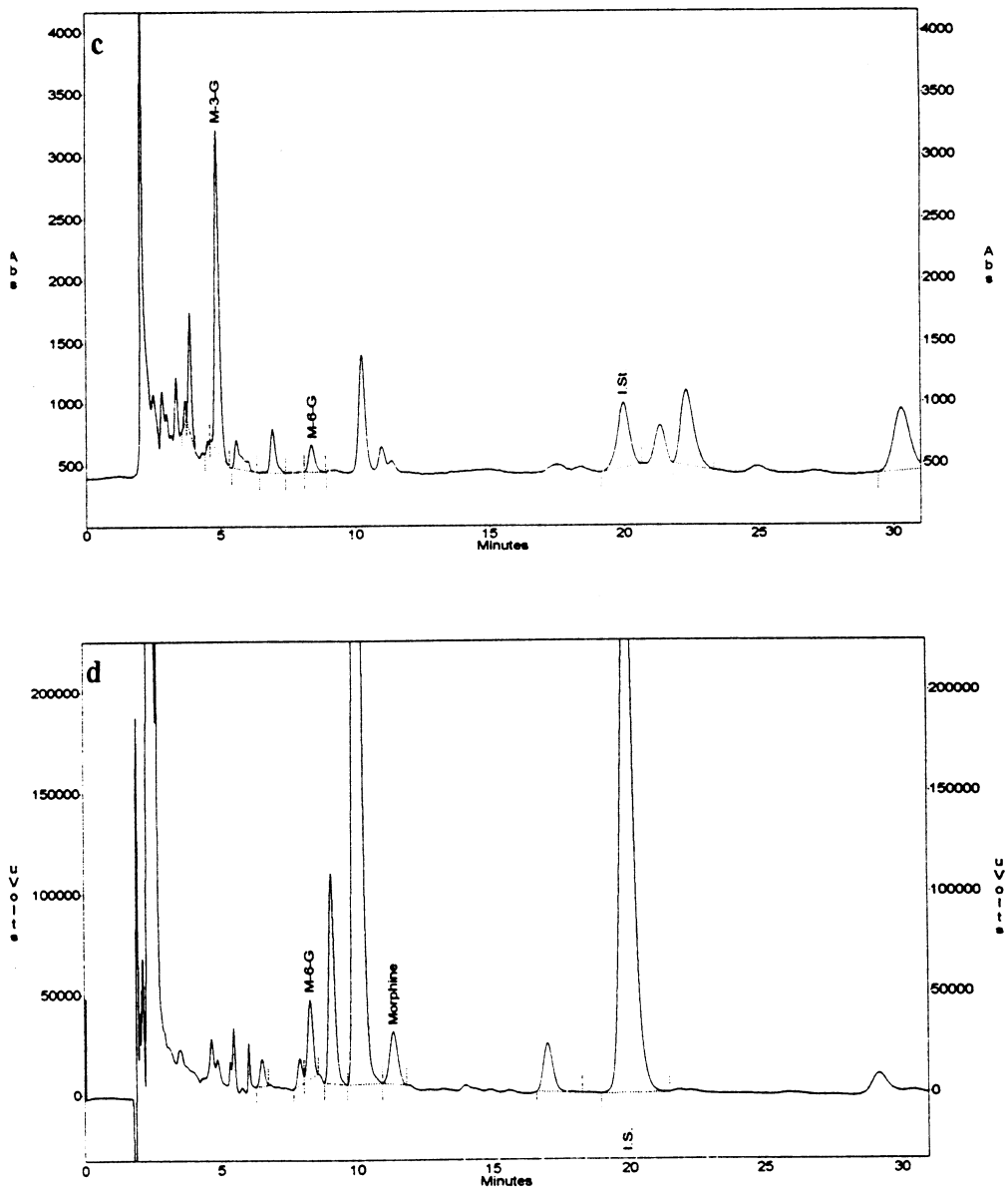

Fig. 2. (*Continued*)

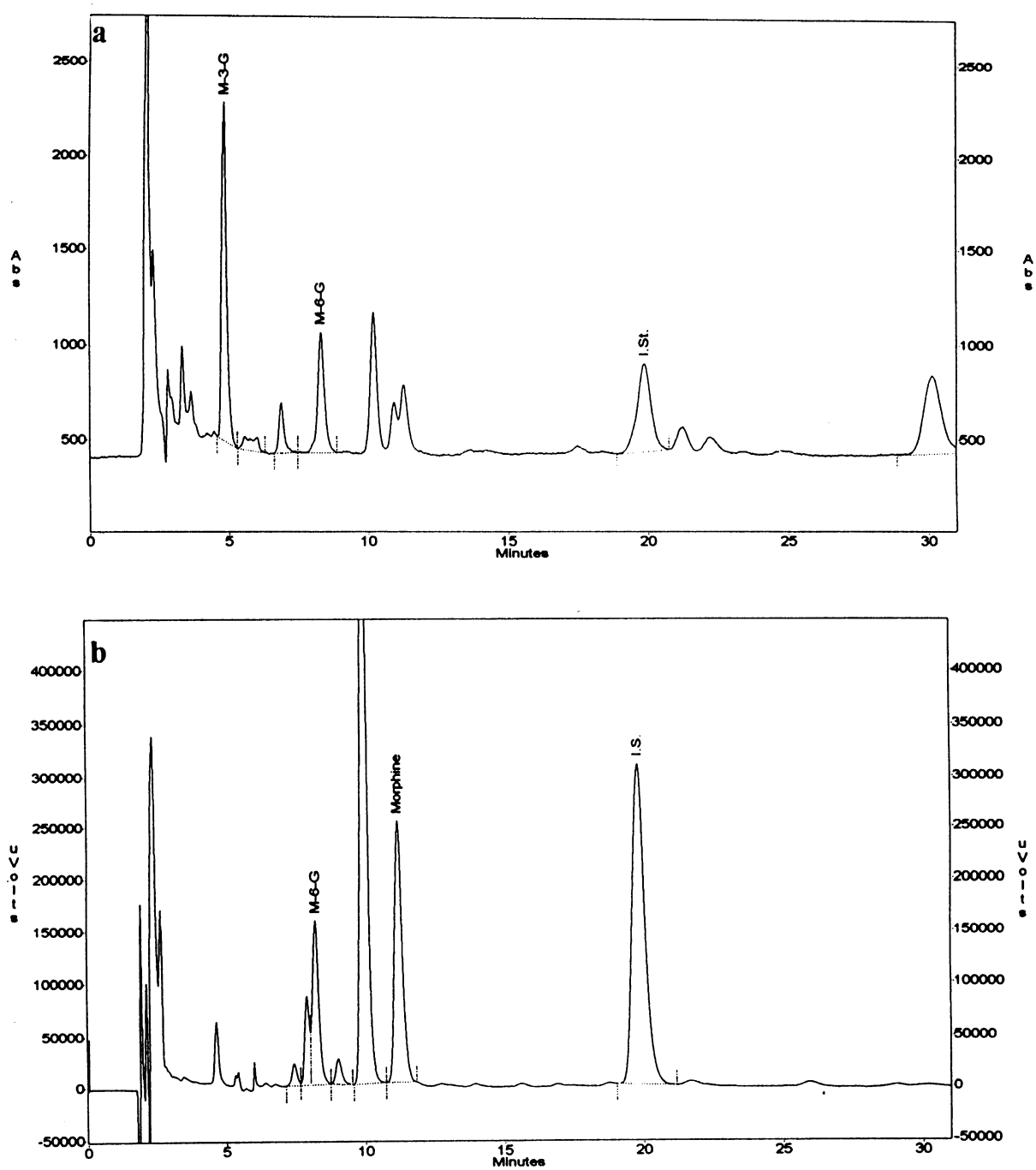

Fig. 3. Typical chromatogram of a volunteer's plasma sample after oral administration of a retard morphine formulation containing 30 mg morphine sulphate by UV (a) and coulometric (b) detection.

Table 1
Between-day precision and accuracy of the method

Nominal concentration (ng/ml)	Measured concentration (ng/ml) mean ± S.D.	R.S.D. (%)	Accuracy (%)	*n*
Morphine				
10	8.510 ± 0.605	7.38	14.90	5
50	46.041 ± 4.120	8.94	7.92	5
100	91.074 ± 5.459	5.99	8.93	5
M-3-G				
300	283.37 ± 17.77	6.27	5.55	5
500	486.49 ± 2.984	6.13	2.80	5
1000	1000.69 ± 39.43	3.94	0.07	5
M-6-G				
100	102.406 ± 12.52	12.22	2.41	5
200	197.96 ± 5.68	2.87	1.02	5
300	313.61 ± 22.988	7.33	4.53	5

of UV detection the detection limit was 10 ng/ml for M3G and for M6G, too.

To evaluate the linearity of the method, standard curves were prepared by spiking drug-free pooled human plasma with different amounts of M (1–30 ng/ml), M3G (50–2000 ng/ml) and M6G (15–1000 ng/ml).

To each sample, 300 ng IS was also added. The extraction and liquid chromatography were carried out as described above.

Calibration curves were constructed from the peak height ratios of M, M3G, M6G to IS versus the respective concentrations, calculated by the $1/y^2$ weighting method using the Class-VP 4.2 software. The *F* test for linearity and linear regression analysis were chosen for testing linearity.

Calibration curves showed good linearity (M3G, $Y = 0.06022 + 0.003876x$; M6G, $Y = 0.104009 + 0.0048075x$; M, $Y = 0.004848 + 0.008162x$), the correlation coefficients were always greater than 0.995 for all compounds.

The within and between-day precision and accuracy of the method were determined using QC samples at three different concentration levels. Five replicate determinations were made at each concentration level.

As indicated by the results, the R.S.D values always remained below ± 15% and the accuracy of the determination did not deviate from 100% by more than ± 15% (between-day, − 12.58 and + 3.45%, within-day, − 14.9 and + 4.53%) (Table 1 Table 2).

Absolute recovery was measured by direct comparison of peak heights of non-extracted, mobil phase-dissolved standards versus plasma extracts, containing the same concentration of M, M3G and M6G. The IS was added to the sample after extraction when the dry residue was dissolved in mobile phase.

The concentration dependence of recovery was negligible, the average extraction efficiencies of M, M3G and M6G were 95.4, 96.1 and 96.8%, respectively.

Stability tests proved that M, M3G, M6G and the IS remained stable for at least 4 weeks in stock solution. Morphine and its metabolites did not show significant decomposition in human plasma during 2 and 4 weeks of storage at − 20°C. The accuracy of the compounds in the plasma after storage was between − 8.5 and + 6.7%.

Our method has been used in bioequivalence studies on morphine kinetics. Fig. 4 shows the pharmacokinetic curves of morphine and its glucuronide metabolites in a representative volunteer after oral administration of a retard morphine formulation containing 30 mg morphine sulphate.

4. Conclusion

We have developed a sensitive, rapid and reliable method for the analysis of morphine and its glucuronide metabolites in human plasma in the course of a bioequivalence study.

Table 2
Within-day precision and accuracy of the method

Nominal concentration (ng/ml)	Measured concentration (ng/ml) mean ± S.D.	R.S.D. (%)	Accuracy (%)	*n*
Morphine				
10	8.742 ± 1.238	14.16	12.58	5
50	51.724 ± 1.211	2.34	3.45	5
100	102.683 ± 3.087	3.00	2.68	5
M-3-G				
300	285.487 ± 8.59	3.01	4.84	5
500	503.69 ± 19.422	3.85	0.73	5
1000	1015.37 ± 43.365	4.27	1.54	5
M-6-G				
100	98.196 ± 3.884	3.95	1.81	5
200	198.27 ± 5.35	2.70	0.87	5
300	302.326 ± 8.599	2.84	0.77	5

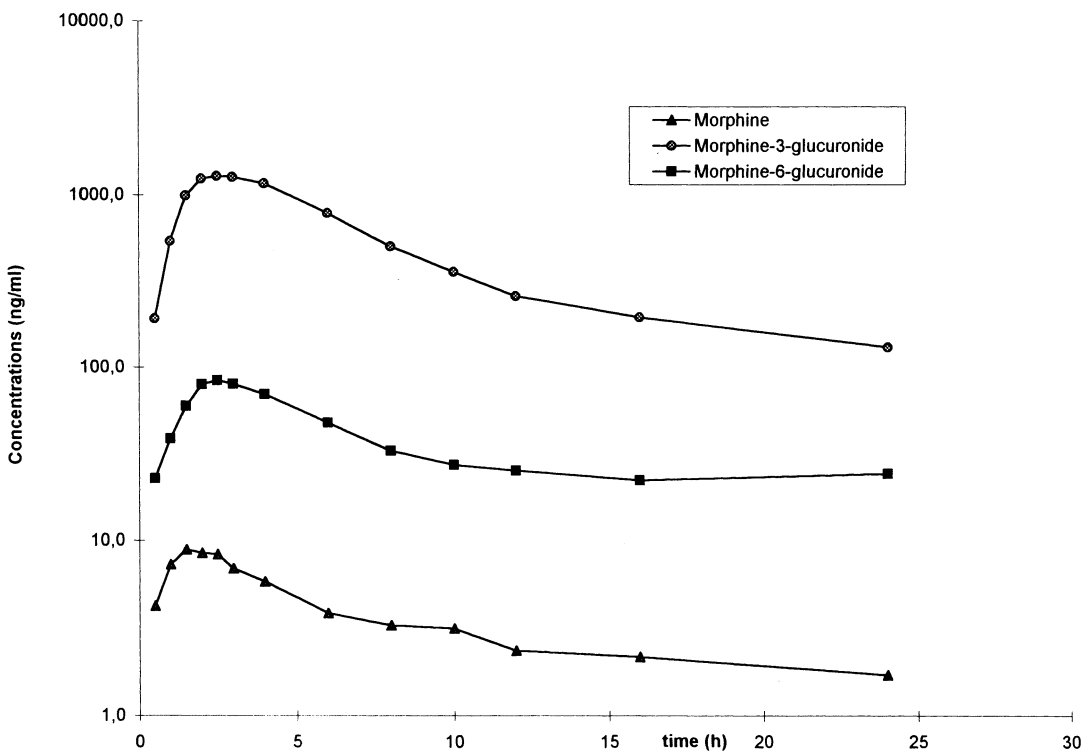

Fig. 4. Pharmacokinetic curves of morphine and its glucuronide metabolites in a representative volunteer after oral administration of a retard morphine formulation containing 30 mg morphine sulphate.

The assay proved to be useful in the analysis of more than 1200 plasma samples.

Acknowledgements

We are grateful to S. Hosztafi for the synthesis of morphine metabolites and the IS.

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