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Determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in human serum by solid-phase extraction and liquid chromatography–mass spectrometry with electrospray ionisation

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Abstract

A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of morphine and two of its metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), in serum is described. The compounds are extracted from serum using Sep-Pak light C_{18} solid-phase extraction cartridges, separated on an ODS C_{18} analytical column (100×4.6 mm I.D.) and detected by electrospray ionisation mass spectrometry. The separation was achieved by running a linear gradient from 4 to 70% acetonitrile with formic acid added as modifier. The flow-rate in the column was 1.0 ml/min. After the column, the eluate was subjected to a 1:50 split, with 20 μl/min delivered to the mass spectrometer and 980 μl/min delivered to waste. The compounds were detected in the mass spectrometer by selected-ion monitoring for m/z 286.2 for morphine and 462.2 for M3G and M6G. The spray voltage was 2.4 kV and the sampling cone was set at 40 V. The compounds have been quantified in serum over a concentration range of 2.9–60 nmol/l (0.84–17 ng/ml) for morphine, 11–1080 nmol/l (5.0–500 ng/ml) for M3G and 4.3–220 nmol/l (2.0–100 ng/ml) for M6G using external standardisation. Intra-assay and inter-assay precision were in the range of 2.4–9.0% for all compounds. The major advantage with the present LC–MS method was the shorter analysis time, 10 min per sample compared to 45 min per sample with our previous LC method with dual detectors. The LC–MS method has proved to have both the selectivity and sensitivity needed for pharmacokinetic studies.

Keywords: Liquid chromatography–mass spectrometry; Morphine; Morphine glucuronide; Glucuronide

1. Introduction

Morphine is a potent opioid analgesic with main clinical use for short-term treatment of postsurgical and traumatic pain. The drug is also used for long-term treatment of moderate to severe pain in cancer patients. Morphine is also a common drug of abuse. The main morphine metabolite in humans is its 3-glucuronide (M3G), minor metabolites are the 6-glucuronide (M6G), morphine-3-sulphate and normorphine [1–3].

When developing analytical methods for morphine and its metabolites, it is important to consider the purpose the method should be used for. The demands

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in term of cost, speed, reliability, selectivity and sensitivity differ widely between methods intended for various purposes; e.g., methods for rapid screening for drugs of abuse, methods to be used as evidence in courts of law or methods for research on morphine metabolism. The analytical method presented here is intended for acquiring pharmacokinetic data in clinical trials of new morphine formulations and administration methods. The expected serum concentrations of morphine range from 3 to 70 nmol/l (0.84–20 ng/ml) when measured during 24 h after intake of an oral dose of 20 mg [4]. About the same serum concentrations of morphine were obtained after administration of a single intravenous dose of 5 mg [4]. The number of samples in a clinical trial is normally between 700 and 1500, and since time is an important factor in drug development, the speed of analyses might become critical.

Immunological methods, such as radioimmunoassays (RIA) have been employed for determination of morphine [5], but problems with cross-reactivity towards metabolites restrict the usefulness for pharmacokinetic studies [6]. Gas chromatography (GC) with mass spectrometric (MS) detection [7] or electron capture (ECD) detection [8] can provide the sensitivity and selectivity needed, but require derivatisation, with extensive manual sample preparation procedures as result. Furthermore, no GC derivatisation scheme has been devised for the metabolites of morphine, why samples usually are hydrolysed prior to extraction, thereby loosing important information.

Liquid chromatography with electrochemical detection (LC–ED) has found widespread use for determination of morphine, M3G and M6G, often preceded by solid-phase extraction from plasma or serum [9–12]. A major concern with LC–ED methods is the presence of late eluting interference peaks, restricting sample throughput to about one sample per hour [13]. For further references and discussions on other detection methods, the reader is referred to some of the reviews on the subject [14–16].

We have analysed morphine, M3G and M6G with liquid chromatography–mass spectrometry (LC–MS) with atmospheric pressure ionisation [17] with the method presented here. The pharmacokinetic data from the study will be presented in another publication. During validation of the method, an internal standard, $^{2}H_{3}$-morphine was used. The validation was evaluated using both peak-area ratios of morphine, M3G and M6G to the internal standard and peak-area measurements with external standards. Direct peak-area measurements with external standards produced better accuracy and precision and those data are presented below. Our purpose was to obtain at least the same sensitivity as with our previous LC method with ultraviolet and electrochemical detection.

2. Experimental

2.1. Chemicals and reagents

Morphine sulphate, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) were obtained from Sigma (St. Louis, MO, USA). The concentrations in the text given as ng/ml refer to concentration of the compounds as base. All chemicals were of analytical reagent grade and were used without further purification.

2.2. Solid-phase extraction

Serum, 1 ml, was mixed with 1 ml of 0.5 mol/l potassium carbonate (pH 9.3). The mixture was loaded on a Sep-Pak light C_{18} cartridge (Waters, Millipore, Milford, MA, USA) which was prewashed with 3 ml methanol followed by 3 ml of water. The cartridge was washed with 5 ml 5 mmol/l potassium carbonate (pH 9.3) and 0.25 ml water. Air was passed through the cartridge for about 30 s to dry the cartridge followed by a second washing step with 0.2 ml of 16% acetonitrile in 30 mmol/l potassium phosphate (pH 2). Finally the analytes were eluted with 0.6 ml of 16% acetonitrile in 30 mmol/l potassium phosphate (pH 2). The flow-rate was 1.5 ml/min. The eluate was diluted with an equal volume of water and 100 μl of the final solution was injected onto the LC–MS system.

2.3. Liquid chromatography

Mobile phases were: (A) 3 mmol/l formic acid (E. Merck, PA grade) in water (Elga Maxima, Bucks,

UK); and (B) 3 mmol/l formic acid in acetonitrile (E. Merck, LiChrosolve gradient grade) delivered at a flow of 1 ml/min by a Beckman 126 solvent delivery module (Beckman Instruments, CA, USA). A linear gradient from 4% A to 70% A in 3.5 min was used. Samples were injected with a Gilson ASPEC sample preparation robot equipped with a 200-μl sample loop in a Rheodyne 7010 valve and Gilson type 21 sample racks (Gilson Instruments, Villiers, France). A 100×4.6 mm I.D. YMC ODS-AL column (YMC, NC, USA) preceded by an external 0.5-μm sintered frit filter was housed in a column heater (Microlab, Aarhus, Denmark) operated at +40°C. The eluate from the column was subjected to a 1:50 split, with 20 μl/min delivered to the mass spectrometer and 980 μl/min delivered to waste. The selectivity obtained on the column for the compounds was tested with varying amounts of formic acid in the mobile phase.

2.4. Mass spectrometry

The mass spectrometer was a Fisons Instruments VG Platform, equipped with pneumatically-assisted electrospray and an RF-ion bridge in the second vacuum stage. Selected-ion monitoring was performed for m/z 286.2 for morphine and 462.2 for M6G and M3G. The spray voltage was 2.4 kV and the sampling cone was set at 40 V. Optimisation of the interface variables, such as gas flows, voltages and probe position was done manually during direct infusion of a 10 μmol/l solution of the target analytes dissolved in 20% acetonitrile in water with 3 mmol/l formic acid, at a flow-rate of 20 μl/min. The response in the MS for morphine was tested by flow-injection of 150 μl samples of morphine dissolved in water containing different concentrations of formic acid, acetic acid or TFA and 10% acetonitrile.

2.5. Validation

The method was validated by analysis of human serum quality control samples prepared at three concentrations spanning the calibration range. Five samples of each quality control pool and calibration samples were analysed on six different days. Precision and accuracy were determined. Precision was expressed as the percent coefficient of variation of each pool (C.V.%). Accuracy was measured according to the following equation: Percent difference from theoretical value=$[(X/C_T)-1]\times100$, where X is the mean determined concentration of a quality control pool and C_T is the theoretical concentration. The statistics were calculated by ANOVA in SAS 6.08 for Windows.

2.6. Quantitation

Each calibration curve consisted of 6–7 calibration points, 2.9–60 nmol/l (0.84–17 ng/ml) for morphine, 11–1080 nmol/l (5.0–500 ng/ml) for M3G and 4.3–220 nmol/l (2.0–100 ng/ml) for M6G). Calculations were made using external standardisation and peak-area measurements. Linear least-square regression was used to fit straight lines to the data. The lines were forced through the origin. The limit of quantitation was determined by running nine samples of a low concentration of each compound through the assay. The detection limits of the compounds were also determined by direct injection of standards into the LC column. The detection limit was defined as the amount injected giving a signal three times the noise.

3. Results and discussion

3.1. Solid-phase extraction

The design of a sample preparation method is heavily dependent on the context in which it is intended to be used. Since we intend to analyse series containing 600–1500 samples from clinical trials, speed, simplicity and possibility of automation are critical issues. Solid-phase extraction can provide the speed and simplicity necessary to make this kind of sample series possible. It is, however, important to avoid solvent evaporation and reconstitution if possible, since this step takes a long time and is difficult to automate. Method development becomes significantly more complex when several analytes with different properties have to be recovered simultaneously [18,19]. A close look at the structures of morphine, M3G and M6G reveals some interesting properties (Fig. 1). Morphine has a tertiary amine with pK_a of 7.9 and a phenolic hydroxyl group at the

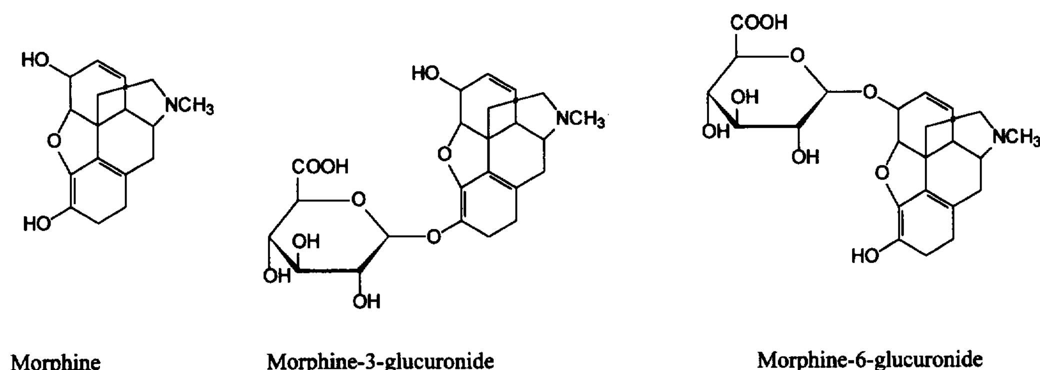

Fig. 1. Structures of morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G).

3′ carbon with estimated pK_a of 9–10. Both glucuronides have a carboxylic group with pK_a in the range 3–4, but M3G has lost the phenolic hydroxyl group. The present extraction method employs a fine balance between pH and polarity manipulations and secondary interactions to achieve retention and elution of the compounds.

3.2. Liquid chromatography

One of the primary means to affect retention in LC is by mobile-phase manipulations. Morphine and its glucuronides have traditionally been separated on reversed-phase columns with ion-pair additives in the mobile phase. All LC–MS interfacing techniques, except, possibly, dynamic fast atom bombardment (FAB), require volatile mobile phases in order to avoid fouling of interface components, thus traditional ion-pair additives are precluded in LC–MS. The main route to affect retention when using LC with MS detection is by varying the percentage of organic modifier in the mobile phase. If organic modifier manipulations cannot provide the retention or selectivity needed, the remaining route is by stationary-phase selection.

Morphine and its glucuronides tend to show very weak retention on most reversed-phase columns when using acidic mobile phases. mobile-phase pHs in the interval 6–8 give good retention but very poor ionisation efficiencies in electrospray. After trying several columns from various manufacturers, we found that the YMC ODS-AL material provided some retention of our target analytes. The YMC ODS-AL material is not end-capped, and since it is intentionally designed to provide a high degree of residual silanol activity, the retention in this case probably is heavily dependent on secondary interactions between the analytes and the stationary phase. Representative chromatograms are shown in Fig. 2a.

It was discovered that retention of all analytes, but especially morphine, was dependent on the formic acid concentration, as shown in Fig. 2b. Since sensitivity is an issue, it was desirable to inject as much as possible of the eluate from the solid-phase extraction. This is beneficial only if sufficient retention – and thus enrichment – of the analytes can be achieved. From an enrichment point of view, it would be desirable with as low a formic acid concentration as possible, but a too low concentration would result in insufficient control of separation pH, and thus a less robust method. Our experience is that 2–3 mmol/l formic acid is a good compromise between retention, robustness and sensitivity (see next section)

3.3. Mass spectrometry

The response obtained in the mass spectrometer depends on the compound, how easy it is ionised, the amount of organic solvent, nature and concentration of buffer used in the mobile phase. For morphine, both TFA and ammonium acetate buffers, commonly used buffers in connection with electrospray ionisation, reduced the signal while a low concentration of formic acid could be used to obtain a useful signal in the MS. Fig. 3a shows how the signal (as peak height

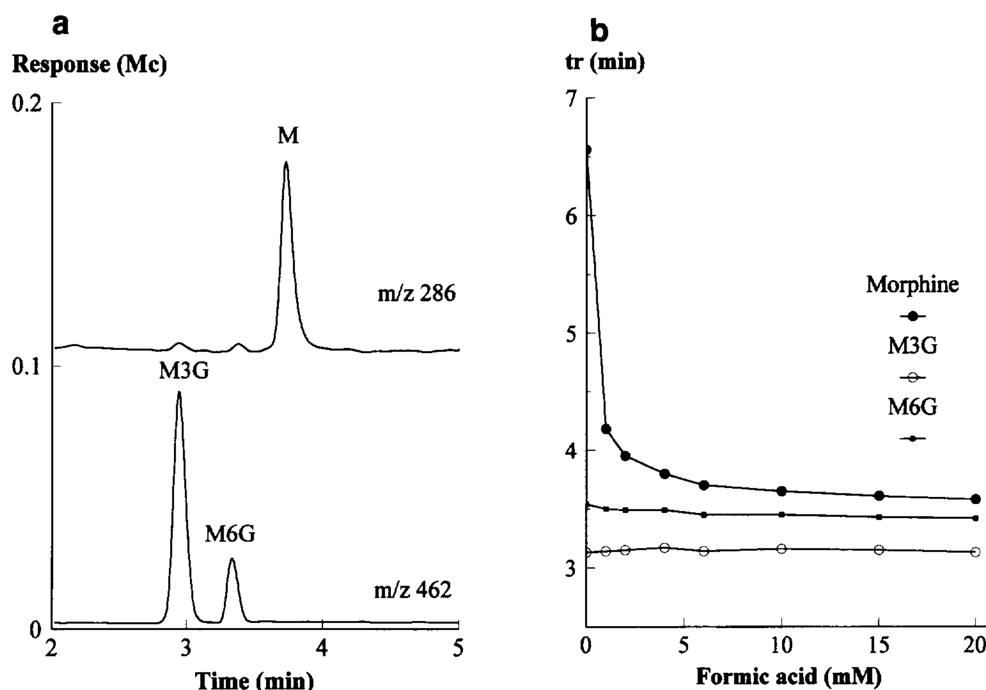

Fig. 2. (a) Chromatogram obtained by injecting 50 μl of a standard containing 32, 40 and 90 nmol/l (9.1, 18 and 42 ng/ml) of morphine, M6G and M3G, respectively. Indications of fragmentation can be observed in the 286 trace at the location of M3G and M6G. (Mc on the y-axis refers to megacounts). (b) Retention as function of formic acid concentration. Morphine is affected significantly more than the glucuronides. (●) Morphine, (○) M3G, (■) M6G.

in flow-injection experiments) depends on formic-, acetic- and trifluoroacetic acid concentrations for morphine. Since the glucuronides are structural isomers, and give the same mass, it was necessary to physically separate them on the column. Furthermore, since both glucuronides showed a slight tendency to fragmentation, with the main fragment being morphine, it was necessary to physically separate at least M3G from morphine. Fig. 3b shows spectra for morphine, M3G and M6G (traces a, b, and c, respectively).

3.4. Stability

The instrumentation proved to be stable and batches of 60 samples have been repetitively analysed. The liquid chromatographic column shows a tendency to lose separation power over time and was changed after the injection of 200–400 samples. The precolumn 0.5-μm frit filter had to be changed after every sample batch (60 samples) due to pressure build-up, the cause of which could be either elution of small amounts of plasma protein or particulate matter from the SPE column.

3.5. Validation

The obtained precision and accuracy for each quality control pool are given in Table 1. The concentration range for respective compounds was chosen to reflect the concentration obtained in clinical situations after giving morphine to patients. The obtained precision is better than that of our previous LC method.

3.6. Quantitation

The limits of quantitation were set to 2.9, 11 and 4.3 nmol/l (0.84, 5.0 and 2.0 ng/ml) of morphine, M3G and M6G, respectively, where the C.V. was below 15% (Table 2). A chromatogram obtained from a serum spiked with these concentrations of the

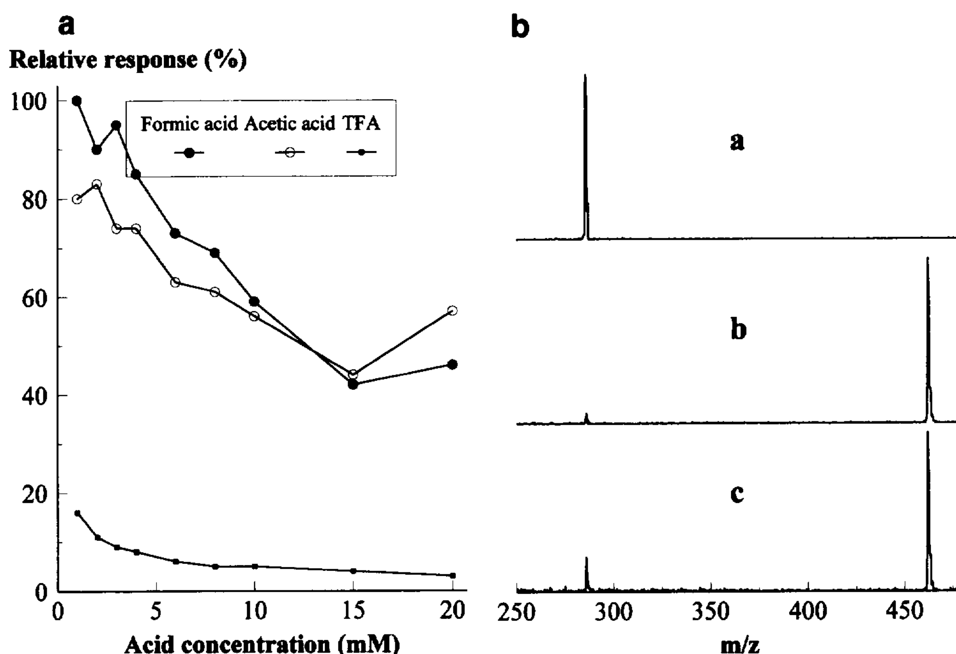

Fig. 3. (a) Morphine signal intensity as function of type and concentration of mobile-phase modifier. (●) Formic acid, (○) acetic acid, (■) trifluoroacetic acid. Experiments were carried out in flow-injection mode, without column attached. (b) Spectra of morphine, M3G and M6G (traces a, b and c, respectively). Sample concentration was approximately 10 μmol/l. Cone voltage, 40 V; spray voltage, 2.4 kV. Note the slight fragmentation of the two glucuronides.

compounds is shown in Fig. 4. The limit of quantitation may be reduced by optimising the LC–MS system. It may also be possible to inject a larger amount of extract; only 50 μl of the 600 μl collected after the Sep-Pak cartridge was used. The detection limit of the compounds obtained by injection of pure standards is also given in Table 2 for comparison. The technique might also be used to analyse blood samples from abusers whose blood might be expected to contain higher concentrations of morphine as the linear range for morphine dissolved in water and injected into the LC–MS system was high; 0.7–7000 nmol/l (0.2–2000 ng/ml) when 100-μl injections were used.

Table 1
Intra- and inter-assay precision and accuracy

Compound	Concentration (nmol/l)	Mean accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)	*n*
Morphine	4.4	3.1	7.7	9.0	30
	15	1.9	4.1	4.8	30
	30	0.59	3.4	5.3	30
M3G	16	8.4	4.5	5.7	30
	217	4.3	2.8	4.2	30
	433	5.1	2.9	5.7	30
M6G	11	3.7	8.6	8.5	30
	33	4.0	4.0	4.6	30
	108	3.0	2.4	3.9	30

Table 2
Limits of quantitation and detection

Compound	Limit of quantitation					Limit of detection[a]
	Concentration (nmol/l)	Intra-assay precision C.V. (%)	Mean accuracy (%)	*n*	Injection (pg)	Injection (pg)
Morphine	2.9	3.8	9.6	9	70	20
M3G	11	7.1	12	9	167	20
M6G	4.3	8.3	2.6	9	417	20

[a] Detection limit (signal-to-noise ratio=3) determined by SIM on standards eluted through the column.

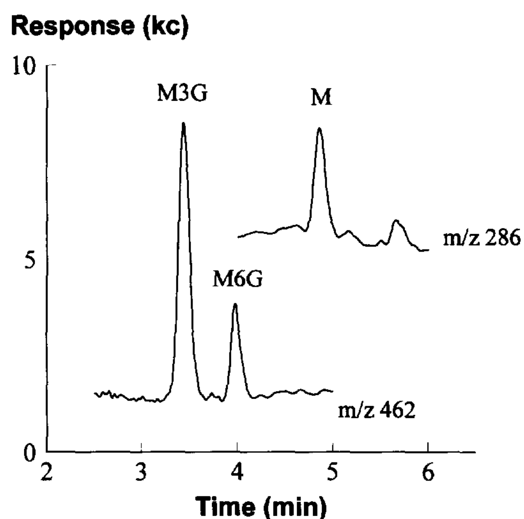

Fig. 4. Chromatogram obtained by injecting 50 μl of an extract of 1 ml serum containing 2.9, 4.3 and 11 nmol/l (0.84, 2.0 and 11 ng/ml) of morphine, M6G and M3G, respectively. Another column was used than in Fig. 3a, and retention differs slightly between the two figures. (kc on *y*-axis refers to kilocounts).

4. Conclusions

The LC–MS method can be used in pharmacokinetic studies. The sensitivity was at present roughly the same as for our previous LC method with dual detectors. The significant advantages with the LC–MS method were the shorter analysis time, 10 min per sample compared to 45 min per sample with the LC method, and the increased impression of reliability with fewer interference peaks. The method is also a very illustrative example of the need for both effective separation and selective detection.

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