Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 4/5, pp. 401–406, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

An improved extraction method for the HPLC determination of morphine and its metabolites in plasma

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Abstract: A new, simple and rapid extraction procedure coupled with a combined coulometric–fluorescence HPLC assay is described for the simultaneous determination of morphine (M) and morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), and normorphine (NM) in plasma. The effect of concentration and pH of selected ion-pairing agents on the extraction of these compounds from plasma by solid-phase extraction was investigated. The extraction procedure was optimized in terms of recovery, reproducibility and lack of interference from endogenous materials. The optimized method uses tetrabutylammonium hydrogen sulphate (TBAHS) at pH 10 followed by separation on a single C_{18} solid-phase extraction cartridge. For routine analysis the procedure provides high and reproducible recoveries over a concentation range of 1.0–1000 ng ml^{-1} for morphine, M6G and normorphine and 20–1000 ng ml^{-1} for M3G. The method was used successfully to analyse plasma samples from a pharmacokinetic study in which sheep had received an intravenous dose of 0.015 mg kg^{-1} of M6G.

Keywords: *High-performance liquid chromatography; morphine; morphine-6-glucuronide; morphine-3-glucuronide; solid-phase extraction; plasma.*

Introduction

Opiates and their derivatives are still the most potent analgesics available today, and have been used since early Egyptian times. More recently, the increasing use of morphine in a wide range of patients for the treatment of acute and chronic pain, has led to considerable interest in developing methods for its determination in biological fluids.

Morphine (M) is extensively metabolized in the liver, mainly through conjugation [1]. The major metabolite is morphine-3-glucuronide (M3G), and the significant minor metabolites are morphine-6-glucuronide (M6G), morphine-3-sulphate and normorphine (NM) [1–3]. M6G and NM are of special interest clinically, as they have been shown to be pharmacologically active [4–6].

In order to study the pharmacokinetics of morphine and its metabolites there is a need to develop a very sensitive and specific assay for their determination. Radioimmunoassay, though able to determine picogram quantities of morphine, is not specific enough for the determination of morphine and all of its metabolites simultaneously [7]. GC and GC–MS techniques have also been used because of their sensitivity, but the sample preparation is complicated and time-consuming as well as the technique being relatively expensive [8, 9]. Most of the currently used assay methods for morphine and its metabolites are based on the HPLC method of Svensson [10, 11], using UV detection for M3G followed by electrochemical detection for morphine, M6G and normorphine. Various modifications and improvements to simplify the method or increase its sensitivity have been published but few authors have made a detailed investigation of the sample preparation procedures involved [11–19]. Yet, the limit of reliable detection of compounds in biological fluids by HPLC, especially in the pico- or nanogram range, also greatly depends on the selectivity of the chosen extraction procedure in removing unwanted endogenous materials from the sample matrix whilst maintaining a high and reproducible recovery of the compounds of interest.

This communication describes the development of a new solid-phase extraction procedure for a sensitive, reliable and rapid assay, to determine morphine, M3G, M6G and normorphine in plasma, using both fluorescence and coulometric detection. The method was used to measure M6G and morphine in sheep plasma after intravenous administration of M6G.

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Experimental

Chemicals

Morphine hydrochloride was purchased from May & Baker (UK). M3G, M6G, NM, and nalorphine were purchased from Sigma (Poole, UK). Octanesulphonic acid (OSA), sodium dodecylsulphate (SDS), and acetonitrile were of HPLC grade purchased from Fisons (Loughborough, UK). Tetrabutylammonium hydrogensulphate (TBAHS), and ammonium sulphate were of analytical grade, purchased from Fluka (Glossop, UK). Potassium dihydrogenphosphate and sodium hydrogéncarbonate were HPLC grade, whilst all other chemicals were of analytical grade purchased from BDH Ltd (Poole, UK).

Chromatography

The HPLC system consists of: RR/066L solvent pump (HPLC Technology Ltd, Macclesfield, UK); Gilson 401 dilutor and Gilson 231 sample injector (Gilson Medical Electronics, Villiers le Bel, France); Pellicular ODS (60–80 μm) guard column; Spherisorb S5 ODS 2 analytical column (150 × 4.6 mm i.d., 5 μm; Phase Separations, Queensferry, UK); Merck–Hitachi F1000 fluorescence spectrophotometer with excitation wavelength 280 nm, emission wavelength 335 nm; ESA Coulochem II electrochemical detector, with a 5020 guard cell and 5011 analytical cell (ESA, Bedford, MA, USA); and Spectra Physics SP4400 integrator (Spectra Physics, CA, USA). The Coulochem detector potentials were set at +0.80, +0.30 and +0.45 V, for guard cell, cell 1 and cell 2, respectively. The two detectors were connected in series with the electrochemical detector first and the fluorescence detector second. The mobile phase consisted of acetonitrile–potassium dihydrogenphosphate (pH 2.1, 0.01 M) with sodium dodecylsulphate (1 mM), (27:73, v/v). The flow rate was 1.0 ml min^{-1}, and the injection volume was 20 μl.

Extraction procedure

Extractions were performed using a C_{18} Bond Elut (1 ml, 100 mg) solid-phase extraction cartridge (Varian, CA, USA), with a Vac Elut extraction apparatus which enables 10 samples to be processed at a time. The cartridge was first pre-wetted with methanol (1 ml), followed by water (1 ml). The sample was then applied to the cartridge, and washed with 4 × 1 ml of water. Morphine and its metabolites were then eluted with 0.5 ml of HPLC mobile phase, of which 20 μl was injected directly onto the HPLC column.

Effect of pH on compound retention by extraction column

Spiked plasma samples were buffered to different pH values prior to extraction, by adding 0.5 ml of water, pH 8 or pH 9 phosphate buffer (0.2 M), followed by vortex mixing. The samples were then extracted as described above.

Optimization of extraction procedure

To investigate the effect of different ion-pairing reagents, spiked plasma samples were buffered with 350 μl carbonate buffer (pH 9.0, 0.2 M) and 150 μl ion-pairing reagent (10 mM) was added followed by vortex mixing. The samples were extracted as described above. To optimize the washing procedure, the extractions were repeated using only TBAHS. The eluate was collected from the Bond Elut at each stage of the extraction, i.e. during application of the sample, from each successive 1 ml wash, and during the final elution with mobile phase. The morphine, M3G, M6G, and normorphine concentration in each fraction was then determined. Further optimization of the buffer pH was carried out by comparing the effects of using a pH 9 and pH 10 carbonate buffer (0.2 M) using 20 mM TBAHS as the ion-pairing reagent and washing with only 2 × 1 ml carbonate buffer (pH 9.0, 5 mM).

Final extraction procedure

To 0.5 ml of sample was added internal standard (nalorphine; 20 μl of a 5 μg ml^{-1} solution), 350 μl of carbonate buffer (pH 10.0, 0.2 M), and 100 μl TBAHS (20 mM), followed by vortex mixing after each addition. The cartridge was pre-wetted with 1 ml methanol followed by 1 ml carbonate buffer (pH 9.0, 5 mM), the sample was applied to the cartridge and washed with 2 × 1 ml of carbonate buffer (pH 9.0, 5 mM) and the analytes eluted with 1 ml of mobile phase. This extraction procedure was used to assay the samples from the pharmacokinetic study.

Recovery

Plasma samples (0.5 ml) were spiked with known amounts of morphine, M6G, M3G and normorphine extracted using the final ex-

traction procedure and peak areas determined by HPLC analysis. The recovery for each compound was calculated by comparison of these peak areas with the peak areas obtained from unextracted aqueous standards of concentrations equivalent to 100% recovery of the compounds.

Results and Discussion

Effect of sample pH on retention

The pH at which the sample is applied to the solid-phase extraction cartridge affects the retention of morphine and its metabolites. Increasing the pH from 7 to 9 improves the recovery of morphine and normorphine from about 25 to 100% (Table 1a), whilst the increase is much smaller for M3G and M6G. By increasing the pH, the ionization of the basic nitrogen group (pK_a 8.0) is suppressed, thus enabling morphine and normorphine to interact more with the C_{18} groups on the silica. The pK_a of the glucuronide group (for both M3G and M6G) is 3.2, thus the glucuronides remain completely ionized at high pH values, and so their retention is not increased. Due to the pK_a values of the compounds being so far apart, it is not possible to extract fully all four compounds at a particular pH, so an ion-pairing agent has to be employed.

Effects of ion-pairing reagents

Different ion-pairing reagents were tested in order to increase the retention of the glucuronides, without altering the retention of morphine and normorphine (Table 2). The use of acidic ion-pairing reagents (pentane sulphonic acid (PSA), OSA and SDS) showed no significant increase in the retention of the glucuronides, but increased the retention of morphine and normorphine. PSA has previously been shown to increase the recovery of M6G [16], but in theory an acidic ion pairing reagent should not improve the extraction of acidic glucuronides. This discrepancy was probably partly due to cartridges with more packing material being used. Tetramethylammonium chloride (TMAC; 10 mM) and TBAHS (10 mM) were shown to increase the retention of both the glucuronides, but TBAHS exerted a much greater effect. A more concentrated solution of TBAHS (25 mM) was tested, but although the retention of the glucuronides was significantly increased, the retention of normorphine was greatly reduced. On the basis of these results TBAHS (20 mM) was chosen as the ion-pairing reagent and the method was further developed to improve the recovery of normorphine.

Optimization of washing procedure

The washing step was examined to assess the

Table 1
The effect of pH on the retention of morphine and its metabolites on C_{18} solid-phase extraction cartridges

	Percentage recovery of compound				
	(a) Using buffer only			(b) Using TBAHS as an ion-pairing reagent (20 mM)	
Compound	pH 7	pH 8	pH 9	pH 9	pH 10
M3G	2.9 (±1.8)	2.3 (±1.2)	9.6 (±3.6)	35.5 (±4.7)	59.6 (±4.3)
M6G	6.0 (±4.1)	2.9 (±1.7)	12.2 (±2.2)	62.5 (±3.8)	78.0 (±3.9)
NM	26.4 (±3.7)	27.8 (±3.3)	103 (±3.6)	33.8 (±9.1)	93.7 (±5.4)
M	24.1 (±7.8)	35.1 (±4.6)	110 (±7.5)	85.6 (±5.2)	95.4 (±3.6)

Results are expressed as mean (±standard deviation) for four replicates.

Table 2
The effect of different ion-pairing reagents on extraction recovery

	Percentage recovery of compound from plasma (n = 4)			
Ion-pairing reagent	M3G	M6G	NM	M
PSA	—	6.6 (±3.1)	81 (±4.5)	90 (±5.0)
OSA	—	—	102 (±5.0)	104 (±5.5)
SDS	—	—	97 (±3.5)	91 (±12.3)
TMAC	3.0 (±0.5)	4.3 (±0.9)	99 (±3.0)	102 (±2.0)
TBAHS (10 mM)	30.0 (±4.5)	66.1 (±7.6)	60 (±8.1)	114 (±5.4)
TBAHS (25 mM)	82.8 (±2.8)	107 (±5.3)	22 (±6.9)	119 (±5.6)

Results are expressed as mean (±standard deviation) for four replicates.

loss of analytes at each stage. The greatest loss occurred during the initial application of the sample (Table 3). A significant further amount of M3G and M6G was lost during the first 1 ml of washing, whilst for normorphine a loss occurred during the final 1 ml of washing (morphine retention was unaffected throughout the extraction procedure). On investigation with blank plasma, it was found that a 2 × 1 ml wash was sufficient to remove the majority of the interfering endogenous material, thus for subsequent extractions a 2 × 1 ml wash was used. The loss on initial application of the sample was reduced considerably to less than 5% by pre-wetting the cartridges with 1 ml of carbonate buffer (pH 9.0, 5 mM) instead of water.

Optimization of pH during extraction

Increasing the concentration of the TBAHS solution from 10 to 20 mM was found to decrease the pH of the buffer to below pH 9.0 which considerably reduced the initial retention of normorphine ($pK_a \approx 11.5$). By increasing the pH of the initial buffer used for sample preparation to pH 10.0 (and washing with a dilute pH 9.0 carbonate buffer) the recovery of all the compounds was significantly increased, and that of normorphine improved to 93.7% (Table 1b).

Comparison with existing extraction method

Two identical sets of spiked plasma samples were extracted; the first using the established extraction procedure developed by Svensson *et al.* [10], and the second using the method developed. The Svensson method (and subsequent modifications), involves an extraction using ammonium sulphate, and multiple washing through two solid phase cartridges. The new method shows improved reproducibility, increased recoveries of the M3G and M6G, and comparable recoveries for normorphine and morphine (Table 4). Preliminary studies have shown that the new method also extracts morphine-3-sulphate from plasma with a recovery in excess of 90%. Also it has the advantages of being faster and more economic, as only one cartridge is used per extraction.

Validation

The method was validated using sheep plasma. Eight point calibration curves (using coulometric detection) for M6G, normorphine and morphine (1.0–1000 ng ml^{-1}), and using fluorescence detection for M3G (20–1000 ng ml^{-1}), were found to be linear with correlation coefficients of 0.998 or greater. The lower limit of quantitation (20 μl injection volume; signal to noise ratio of 3:1) was 1.0 ng ml^{-1} for M6G, normorphine and morphine with electrochem-

Table 3
Percentage recovery of compounds during the different stages of extraction

Stage	M3G	M6G	NM	M
Application	39.0 (±2.3)	10.2 (±5.7)	26.0 (±7.6)	—
1st ml	14.3 (±2.1)	18.4 (±3.8)	2.6 (±0.7)	—
2nd ml	4.4 (±0.3)	2.8 (±0.4)	—	—
3rd ml	1.5 (±0.2)	3.3 (±0.3)	4.6 (±0.4)	—
4th ml	1.1 (±0.1)	1.5 (±0.1)	15.0 (±1.4)	—
Elution	29.3 (±2.8)	61.7 (±5.5)	47.1 (±7.0)	91.5 (±4.6)
Total recovered	89.3 (±3.9)	97.3 (±5.7)	95.3 (±6.7)	91.5 (±4.6)

Results are expressed as mean (±standard deviation) for six replicates.

Table 4
Comparison of methods

	Svensson method		New method	
Compound	Mean % recovery	% RSD	Mean % recovery	% RSD
M3G*	69 (±15.9)	23.0	90 (±1.9)	2.1
M6G	76 (±12.2)	16.0	82 (±5.5)	6.7
NM	96 (±113.3)	13.9	89 (±10.5)	11.8
M	99 (±6.0)	6.0	100 (±3.5)	3.5

Results are expressed as mean (±standard deviation) for 24 replicates.
*($n = 6$, instead of $n = 24$).

ical detection; and 20 ng ml^{-1} for M3G, and 50 ng ml^{-1} for M6G, normorphine and morphine using fluorescence detection. These were appropriate limits of detection as shown by a recent pharmacokinetic study [6], in which the concentrations were found to range from 1 to 79 ng ml^{-1} for morphine, 1 to 37 ng ml^{-1} for M6G and 20 to 188 ng ml^{-1} for M3G, after I.V. administration of morphine.

The intra- and inter-day reproducibilities (expressed as a relative standard deviation of at least six replicate samples) at analyte concentrations of 5.0, 50 and 500 ng ml^{-1} are shown in Table 5.

Figures 1 and 2, show typical chromatograms from extracted plasma samples. When using coulometric detection (Fig. 2), M6G, normorphine, morphine and the internal standard nalorphine are eluted after 4.1, 5.8, 6.5 and 11.5 min, respectively. There was no interference from the endogenous compounds present in the drug-free plasma, whether using fluorescence or coulometric detection.

The method was then used to assay sheep plasma samples from a pharmacokinetic study, in which sheep were dosed intravenously with M6G (0.015 mg kg^{-1}). Samples were taken at intervals, up to 360 min after dosing, and M6G concentrations were determined. No detectable amount of morphine or normorphine was observed in any of the samples. Figure 3 shows the plasma M6G concentration versus time profile for one animal. The pharmacokinetic parameters of M6G were as follows: the mean terminal half-life was 51 min, the mean clearance was 5.6 ml min^{-1} kg^{-1}, and the mean apparent volume of distribution was 0.41 l^{-1} kg^{-1}.

Table 5
Intra- and inter-day reproducibilities for the analysis of morphine, M3G, M6G and normorphine

Compound	Spiked conc. (ng ml^{-1})	Intra-day variation (n = 6)		Inter-day* variation (n = 18)	
		SD	% RSD	SD	% RSD
M3G	100	6.32	6.8	7.62	8.2
M6G	5	0.26	5.3	0.36	7.9
	50	1.58	3.3	3.74	7.3
	500	23.41	5.0	39.75	8.5
NM	5	0.37	6.2	0.56	10.6
	50	1.37	3.0	3.03	6.2
	500	24.21	4.9	38.56	8.3
M	5	0.25	3.6	0.43	9.2
	50	2.55	4.9	2.66	6.1
	500	28.74	6.3	40.02	8.1

*Determined over 3 separate days.

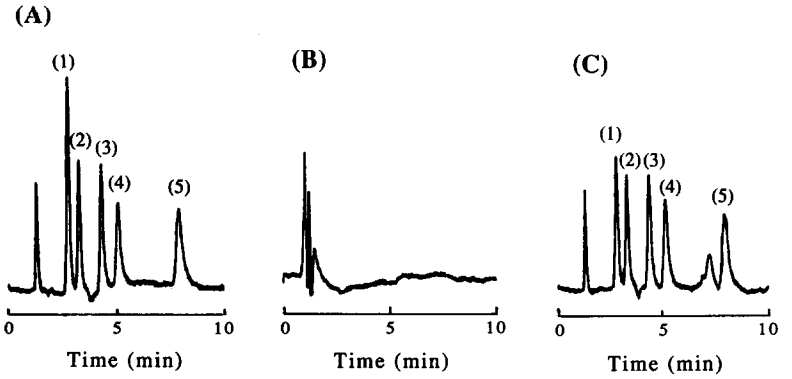

Figure 1
Chromatograms (using fluorescence detection). A slightly modified mobile phase was used, with a 72:28 ratio of aqueous to organic (c.f. 73:27), which altered the retention times to: 2.8, 3.3, 4.3, 5.1 and 7.9 min for M3G, M6G, normorphine, morphine and nalorphine, respectively. (A) Aqueous standard (200 ng ml^{-1} of M3G, M6G, normorphine, morphine and nalorphine). (B) Extracted blank plasma. (C) Extracted plasma spiked with 200 ng ml^{-1} of M3G, M6G, normorphine, morphine and nalorphine. 1, M3G; 2, M6G; 3, normorphine; 4, morphine; 5, nalorphine.

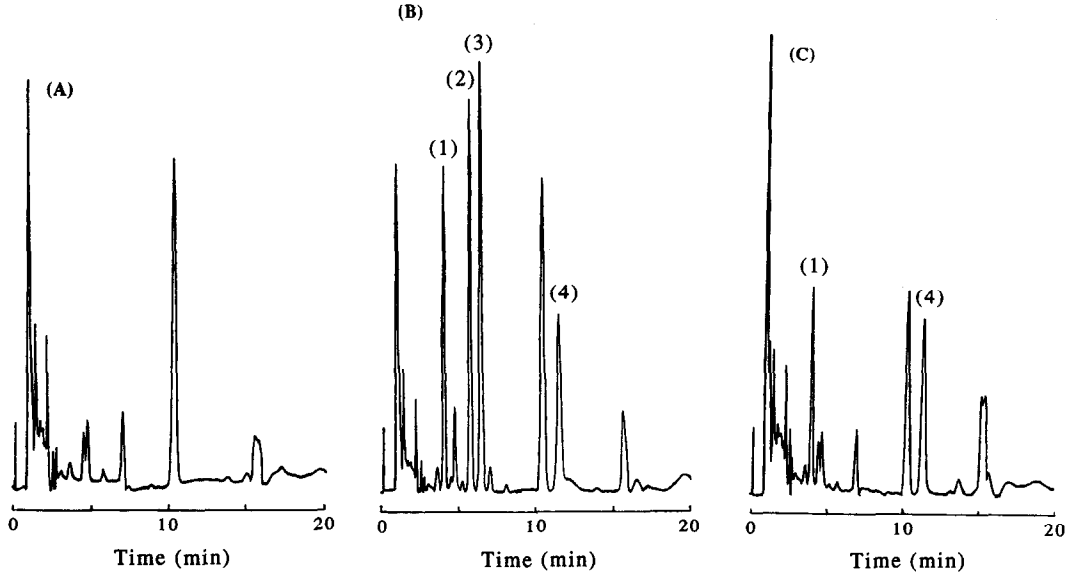

Figure 2
Typical chromatograms using coulometric detection. (A) Blank plasma, (B) spiked plasma 236 ng ml^{-1} M6G, 200 ng ml^{-1} normorphine and morphine and 100 ng ml^{-1} nalorphine, and (C) plasma sample 15 min after dosing sheep with 0.15 mg kg^{-1} of M6G (121 ng ml^{-1} of M6G). 1, M6G; 2, normorphine; 3, morphine, 4, nalorphine.

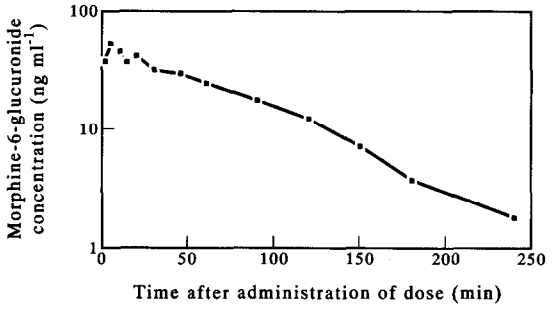

Figure 3
Morphine-6-glucuronide plasma concentration versus time after intravenous administration of M6G to a sheep.

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[Received for review 21 May 1992; revised manuscript received 30 July 1992]