ELSEVIER

Journal of Chromatography B, 664 (1995) 329–334

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic–electrospray mass spectrometric determination of morphine and its 3- and 6-glucuronides: application to pharmacokinetic studies

R. Pacifici[a], S. Pichini[a], I. Altieri[a], A. Caronna[b], A.R. Passa[a], P. Zuccaro[a,*]

[a]*Clinical Biochemistry Department, Istituto Superiore di Sanità, V. le Regina Elena 299, 00161 Rome, Italy*
[b]*Santo Spirito Hospital, Rome, Italy*

First received 21 June 1994; revised manuscript received 11 October 1994; accepted 25 October 1994

Abstract

A rapid and selective assay of morphine and its 3- and 6-glucuronides in serum, based on high-performance liquid chromatography–electrospray mass spectrometry has been developed. The analytes and the internal standard, codeine or naltrexone, were subjected to solid-phase extraction, using ethyl solid-phase extraction columns, prior to chromatography. A reversed-phase column and a gradient mobile phase consisting of water and methanol were used. The mass spectrometer was operated in the selected-ion monitoring mode. The following ions were used: *m/z* 286 for morphine, *m/z* 300 for codeine, *m/z* 342 for naltrexone, and *m/z* 462 for morphine 3- and 6-glucuronides. The limit of quantitation observed with this method was 10 ng/ml morphine, 50 ng/ml morphine-6-glucuronide and 100 ng/ml morphine-3-glucuronide. The present method proved useful for the determination of serum levels of the parent drug and its metabolites in pain patients, heroin addicts and in morphine-treated mice.

1. Introduction

The analgesic properties of morphine are used for the treatment of cancer pain [1]. On the other hand, morphine is the major metabolite of heroin (3,6-*o*-diacetylmorphine), the opioid most commonly sold in the illicit drug market [2].

Morphine is metabolized in man to the inactive morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) with an analgesic activity greater than that of the parent compound, and is present in plasma in concentrations that exceed those of morphine itself [3–5]. Conversely, M-6-G is not present in all animal species [6,7] and M-3-G, which is inactive in man, shows antinociceptive effect in rat [8,9].

Radioimmunoassays (RIA) have often been used to measure serum concentrations of morphine, but cross-reactions between M-3-G and M-6-G and the antiserum to morphine rendered the results obtained difficult to interpret [10–12]. M-3-G and M-6-G were quantified in human serum for the first time in 1982 using ion-pair high-performance liquid chromatography [13]. Subsequently, other HPLC techniques were de-

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00496-X

veloped using amperometric, fluorimetric or UV detection [14–18].

High-performance liquid chromatography (HPLC) combined with mass spectrometry has been widely applied in recent years [19]. Indeed, mass spectrometry is still the best detector for HPLC since it offers both universality and selectivity, even if the HPLC–MS coupling still suffers from the lack of sensitivity.

This paper describes an HPLC assay for morphine and its two glucuronides in serum with electrospray-mass spectrometric detection. A solid-phase extraction was performed for serum samples, using codeine and naltrexone as internal standards. Data on serum levels of the drug and its metabolites in two cancer patients, five heroin addicts and in morphine-treated mice are reported.

2. Experimental

2.1. Chemicals and reagents

Morphine-HCl, codeine-HCl, naltrexone-HCl, morphine-3-glucuronide were purchased from Salars (Como, MI, Italy) and morphine-6-glucuronide from Sigma (Poole, UK). Ethyl solid-phase extraction columns (1 ml volume, 100 mg sorbent) were from J.T. Baker (Milan, Italy). All solvents were analytical grade.

2.2. HPLC–MS conditions

HPLC was performed with a Waters 600 MS multisolvent delivery system (Waters Chromatography Division, Rome, Italy) equipped with a U6K universal liquid chromatography injector. Chromatographic separation was achieved at ambient temperature with a reversed-phase Supelcosil ABZ column (25 cm × 4.6 mm I.D., 5 μm particle size; Supelchem, Milano, Italy) and a convex gradient elution consisting of 85% water and 15% methanol at the start changing to 40% water and 60% methanol in 10 min, in which the analysis was completed (gradient curve equation: $y = -0.46\ e^{-x/1.18} + 0.60$, where x is the time in minutes and y is the percentage of methanol). The flow-rate was 0.8 ml/min splitted to 18 μl/min before the electrospray MS interface.

The MS system (TRIO 2 with electrospray interface, Fisons Instruments, Altrincham, UK) was operated with a capillary tip voltage of 2.97 kV for the probe, a counter-electrode voltage of 0.39 kV, a sampling-cone voltage of 66 V, −106 V, −17 V, for the three couples of lenses, respectively. These parameters were checked daily and slightly modified if needed, to obtain optimum sensitivity. The source temperature was held at 60°C. The selected-ion monitoring (SIM) mode was used, and the following ions were monitored: m/z 286 for morphine, m/z 300 for codeine, m/z 342 for naltrexone, and m/z 462 for morphine 3- and 6-glucuronides.

2.3. Standards and controls

Solutions of stock reference standards (1 mg/ml, 10 μg/ml and 1 μg/ml) were prepared in methanol and stored below 0°C. Dilutions were freshly made daily for each analysis. Serum standards were prepared daily by adding known amounts of the stock standards to drug-free serum; these standards were used to create HPLC–MS calibration curves as a control and to determine analytical recoveries, intra-day and inter-day variabilities.

2.4. Biological samples collection

Blood samples from cancer patients requiring opioid analgesia were obtained from two male inpatients of Santo Spirito Hospital in Rome. Patient No. 1 was treated orally with controlled-release discoids of morphine sulphate (Oblioser, Recordati, Milano, Italy). A 10-mg dose was administered at 8 a.m., and a 20-mg dose at 8 p.m. Blood samples were collected 1 h after the morning dose and 1 h before the evening dose. Patient No. 2 received a continuous intravenous infusion of morphine (10 mg/24 h) and blood was drawn at 10 a.m., 12 a.m., and 3 p.m. Blood samples from heroin addicts were obtained from subjects who had been enrolled in an outpatient maintenance and detoxification study at Santo

Spirito Hospital. All blood samples were immediately centrifuged at 1000 *g* for 5 min. Serum was collected and stored at −20°C until analysis. Male C57BL/6 inbred mice (Charles River, Calco, Como, Italy), 8–10 weeks of age, weighing 18–20 g, were treated with morphine-HCl dissolved in saline, at doses of 20, 60, and 150 mg/kg, administered subcutaneously (s.c.). In the case of the 20 mg/kg treatment, blood samples were collected at 10, 20, and 120 min and at 6, 10, 12 and 24 h after administration. Ten mice were used for each time tested. For the other doses, blood was collected at 40 min after the treatment. Serum obtained by centrifugation was stored at −20°C until analysis.

2.5. Extraction of biological samples

A 1-ml aliquot of serum, with 100 μl of internal standard (1 μg/ml aqueous solution) added, was extracted using ethyl solid-phase extraction columns. The columns were conditioned with 2 column volumes of methanol, one column volume of water, followed by two column volumes of 0.01 *M* ammonium hydrogen carbonate buffer (pH 9.3). Serum samples were loaded onto column, washed with one column volume of 0.01 *M* ammonium hydrogen carbonate buffer, and eluted with one column volume of methanol. The eluate was evaporated to dryness under nitrogen, redissolved in 100 μl of mobile phase and a 20-μl aliquot was injected onto the HPLC column. The extraction procedure reported above was compared with a solid-phase extraction procedure reported in the literature for strong cation-exchange (SCX) cartridges [17].

3. Results and discussion

Solid-phase extraction with C_2 columns was used in preference to procedures described in the literature. In the case of extractions performed with Sep-Pak C_{18} cartridges [13,14,16], the eluate consisted of non-volatile salts of buffers, which could not be injected onto the mass spectrometer. Using strong cation-exchange cartridges [17], the recovery of M-3-G was always under 40%. C_2 solid-phase extraction gave the best results. Over the concentration ranges tested in the analysis, recoveries were above 95% for all the analytes, except for M-3-G, the recovery of which was 70%.

Fig. 1 shows SIM chromatograms of serum samples obtained from a drug addict (A), and from morphine-treated mice (B). Morphine and both the glucuronides were present in human serum, while in murine serum only morphine and its 3-glucuronide were found. There were no interfering peaks in blank human and murine samples.

Calibration curves of the peak height (counts × 10^{-3}) versus the amount of the analytes (μg/ml) were prepared and checked daily from sera spiked with five different concentrations of the analytes and carried through the entire procedure. The calibration curves were linear over the ranges 10–1000 ng/ml for morphine, codeine, and naltrexone; 50–1000 ng/ml for M-6-G; and 100–1000 ng/ml for M-3-G. The quantitation limit (signal-to-noise ratio of 3) and the linearity of the method are shown in Table 1.

The analytical imprecision was determined from analyses of five spiked serum samples performed for up to six days. The within-day coefficients of variation for 10 ng/ml morphine, 50 ng/ml M-6-G and 100 ng/ml M-3-G were 6.5, 6.9, and 7.2%, respectively. The between-day coefficients of variation for the same amounts of morphine, M-6-G and M-3-G were 6.9, 7.1 and 7.4%, respectively.

The procedure as described was used to determine the concentration of morphine and its glucuronides in serum from two cancer patients receiving different drug treatments (Table 2). In the case of a patient receiving morphine sulphate by the oral route, 2 ml of serum were used for the determination in order to measure the low concentrations expected for the drug and its metabolites, due to the controlled-release formulation.

The concentration of morphine and its glucuronides was determined also in the serum from five drug addicts (Table 3). Only in one subject, both the glucuronides could be determined; in

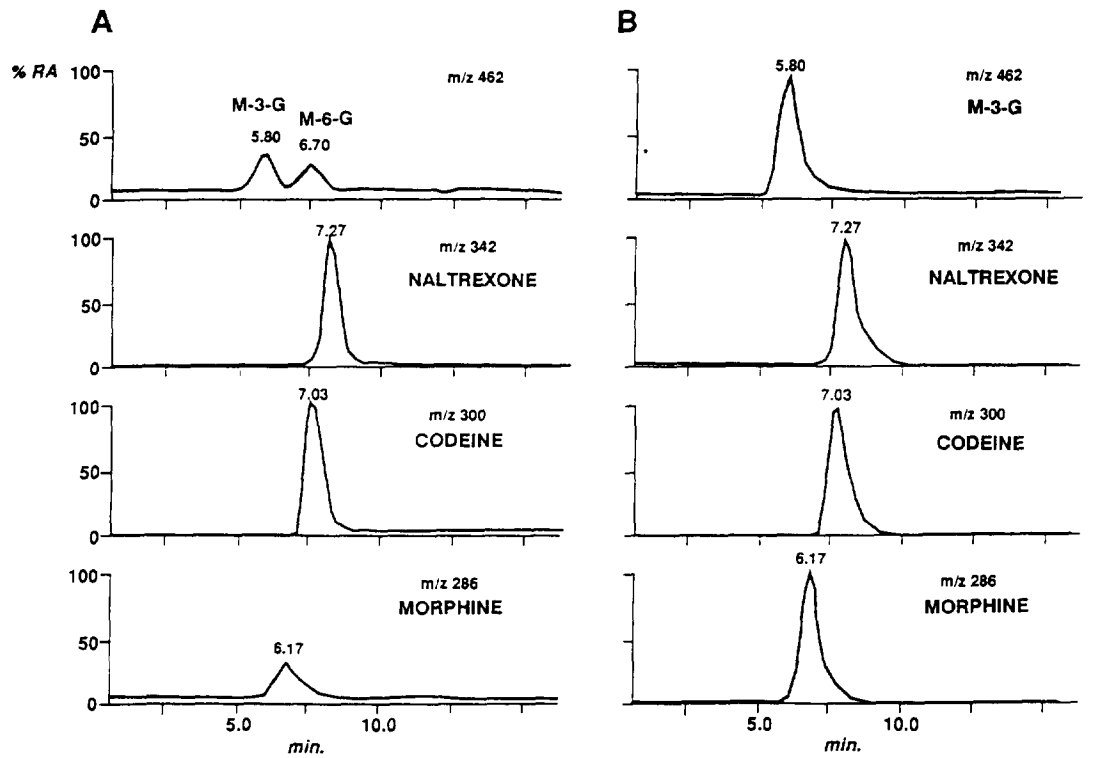

Fig. 1. SIM chromatograms of extract of (A) serum sample from a drug addict containing morphine (38 ng/ml), codeine as an internal standard (1 μg/ml), naltrexone as an internal standard (1 μg/ml), M-3-G (305 ng/ml), M-6-G (69 ng/ml); (B) serum sample from morphine-treated mice containing morphine (110 ng/ml), codeine (I.S., 1 μg/ml), naltrexone (I.S., 1 μg/ml), and M-3-G (2 μg/ml).

the other samples only morphine and its 3-glucuronide, or morphine alone could be detected. This difference in drug metabolism may be due to inter-individual variability or to different amounts of heroin injected and time elapsed after the last injection. An other application of the present method was the determination of morphine and its metabolites in the serum of C57BL/6 mice. Several studies have shown the

Table 1
Quantitation limit and linearity

Compound	Retention time (min)	Quantitation limit (ng/ml)	Linearity[a]
Morphine	6.17	10	$y = 1.3x - 0.7$
M-3-G	5.80	100	$y = 0.08x + 0.2$
M-6-G	6.70	50	$y = 0.15x + 0.09$
Codeine[b]	7.03	10	
Naltrexone[b]	7.27	10	

[a] y = peak height (counts × 10^{-3}); x = amount of the analytes (μg/ml).
[b] Internal standard.

Table 2
Serum concentrations of morphine, M-3-G and M-6-G in cancer patients receiving morphine by oral route (patient No. 1) or continuous intravenous infusion (patient No. 2)

Patient	Sample	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)
1[a]	9 a.m.	14	81	35
1	7 p.m.	5	108	20
2	10 a.m.	31	730	120
2	12 a.m.	51	597	298
2	3 p.m.	35	840	593

[a] A 2-ml serum sample was used.

Table 3
Concentrations in serum samples of drug addicts

Subject	Morphine (ng/ml)	M-3-G (ng/ml)	M-6-G (ng/ml)
1	38	305	69
2	17	231	N.D.
3	18	264	N.D.
4	50	N.D.	N.D.
5	30	N.D.	N.D.

N.D. = not detected.

formation of M-3-G and M-6-G in various animal species such as rhesus monkeys [20], guinea pigs, rabbits [6,7], while M-6-G did not appear in rats and mice [7]. Only morphine and its 3-glucuronide were found in serum of mice treated with a 20 mg/kg dose of morphine (Fig. 2). This result was confirmed by comparing mice treated with 20, 60 and 150 mg/kg morphine (Table 4), even though the presence of amounts of morphine-6-glucuronide, too small to be determined, cannot be excluded. These results fairly agree with the observations of Kuo et al. [7]. Indeed, M-6-G was not detected in 24-h urine of mice treated with 10 mg/kg morphine, although in-vitro studies demonstrated the capability of mice liver toward the glucuronidation of 6-hydroxyl group of morphine.

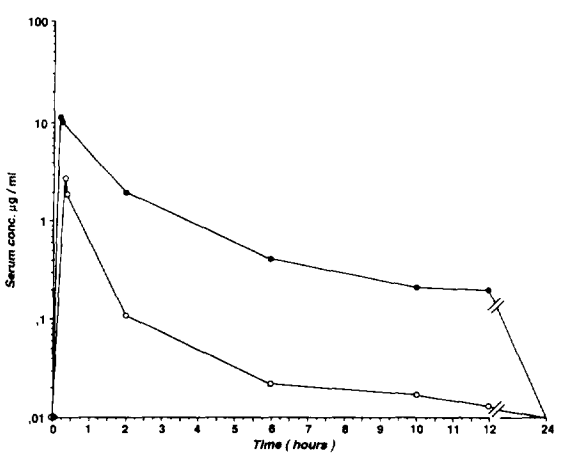

Fig. 2. Profile of serum concentrations of morphine (○) and M-3-G (●) after 20 mg/kg administration of morphine hydrochloride to mice.

Table 4
Serum concentration of morphine and its metabolites 40 minutes after morphine administration in C57BL/6 MICE

Drug treatment	Morphine (μg/ml)	M-3-G (μg/ml)	M-6-G (μg/ml)
20 mg/kg	1.6	9.7	N.D.
60 mg/kg	4.3	32.2	N.D.
150 mg/kg	10.5	118.0	N.D.

N.D. = not detected.

In conclusion, this rapid, selective and reliable method can be useful for pharmacodynamic and pharmacokinetic studies, in which data on the concentrations of morphine and each of its metabolites in biological fluids can be of interest.

Acknowledgements

The authors thank Mrs. Simonetta Di Carlo and Mrs. Antonella Bacosi for their technical assistance and Dr. Carla Balestreri for the preparation of the manuscript.

References

[1] World Health Organization, *Cancer Pain Relief Program*, WHO, Geneva, 1986.
[2] E.L. Way, J.W. Kemp, J.M. Young and D.R. Grassetti, *J. Pharmacol. Exp. Ther.*, 129 (1960) 144.
[3] S.Y. Yeh, C.W. Gorodetzky and H.A. Krebs, *J. Pharm. Sci.*, 66 (1977) 1288.
[4] R. Osborne, P. Thompson, S. Joel, D. Trew, N. Patel and M. Slevin, *Br. J. Clin. Pharmacol.*, 34 (1992) 130.
[5] N. Babul and A.C. Darke, *Clin. Pharmacol. Ther.*, 54 (1993) 286.
[6] K. Oguri, N. Hanioka and H. Yoshimura, *Xenobiotica*, 20 (1990) 683.
[7] C.K. Kuo, N. Hanioka, Y. Hoshikawa, K. Oguri and H. Yoshimura, *J. Pharmacobio-Dyn.*, 14 (1991) 187.
[8] Q. Gong, T. Hedner, J. Hedner, R. Bjorkman and G. Nordberg, *Eur. J. Pharmacol.*, 193 (1991) 47.
[9] M. Ekblom, M. Gardmark and M. Hammarlund-Udenaes, *Biopharm. Drug Dispos.*, 14 (1993) 1.
[10] S. Spector, *J. Pharmacol. Exp. Ther.*, 178 (1971) 253.
[11] C.W. Hand, R.A. Moore, H.J. McQuay, M.C. Allen and J.W. Sear, *Ann. Clin. Biochem.*, 24 (1987) 153.

[12] S.P. Joel, R.J. Osborne, N.S. Nixon and M.L. Slevin, *Lancet*, i (1985) 1099.
[13] J.-O. Svensson, A. Rane, J. Sawe and F. Sjoqvist, *J. Chromatogr.*, 230 (1982) 427.
[14] S.P. Joel, R.J. Osborne and M.L. Slevin, *J. Chromatogr.*, 430 (1988) 394.
[15] M. Konishi and H. Hashimoto, *J. Pharm. Sci.*, 79 (1990) 379.
[16] R.W. Milne, L. Nation, G.D. Reynolds, A.A. Somogy and J.T. Van Crugten, *J. Chromatogr.*, 565 (1991) 457.
[17] G. Chari, A. Gulati, R Bhat and I.R. Tebbett, *J. Chromatogr.*, 571 (1991) 263.
[18] R. Harley, M. Green, M. Quinn and M.I. Levene, *Biomed. Chromatogr.*, 7 (1993) 34.
[19] J.F. Garcia and D. Barceló, *J. High Resol. Chromatogr.*, 16 (1993) 633.
[20] A. Rane, J. Sawe, B. Lindberg, J. Svensson, M. Garle, R. Erwald and H. Jorulf, *J. Pharmacol. Exp. Ther.*, 229 (1984) 571.