Journal of Chromatography, 565 (1991) 457–464
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5749

Short Communication

High-performance liquid chromatographic determination of morphine and its 3- and 6-glucuronide metabolites: improvements to the method and application to stability studies

R. W. MILNE* and R. L. NATION

School of Pharmacy, South Australian Institute of Technology, North Tce., Adelaide 5000 (Australia)

G. D. REYNOLDS

School of Chemical Technology, South Australian Institute of Technology, Adelaide 5000 (Australia)

and

A. A. SOMOGYI and J. T. VAN CRUGTEN

Department of Clinical and Experimental Pharmacology, University of Adelaide, Adelaide 5000 (Australia)

(First received June 12th, 1990; revised manuscript received November 6th, 1990)

ABSTRACT

Improvements to previously reported methods for the determination of morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in human plasma are described. The improved methods involve the use of a solid-phase extraction cartridge and a chromatographic system which uses paired-ion reversed-phase high-performance liquid chromatography with a radially compressed column. Only one cartridge is used to prepare each sample for chromatography and each cartridge may be used for at least fourteen 1-ml plasma samples. The recovery is greater than 85%. The improvements to the method of sample pretreatment and in the chromatographic conditions have allowed determination of morphine, M3G and M6G in human plasma down to 13.3 nmol/l (coefficient of variation = 9.3%), 108 nmol/l (6.6%) and 41 nmol/l (6.7%), respectively, using ultraviolet detection alone. It was shown that all three compounds were stable in plasma for up to 101 weeks when stored at −20°C.

INTRODUCTION

In man, morphine is metabolized primarily to morphine-3-glucuronide (M3G), and to a lesser extent to morphine-6-glucuronide (M6G), morphine-3-ethereal-sulphate and normorphine [1,2]. Evidence from animal [3–6] and human [7,8] studies indicating that M6G may contribute to morphine analgesia and side-effects highlights the importance of determining the plasma concentrations

0378-4347/91/$03.50

of M6G and M3G, in addition to morphine, when conducting pharmacokinetic and pharmacodynamic studies on morphine [7,9].

Svensson *et al.* [10] reported a high-performance liquid chromatographic (HPLC) method, with ultraviolet (UV) detection, for the simultaneous determination of morphine, M3G and M6G in plasma. Improvements to the sensitivity and selectivity for morphine and M6G using coulometric detection, were reported by Svensson [11] and Joel *et al.* [12]. Both latter methods required an additional detector in series (UV or fluorescence, respectively) for monitoring M3G. The original method [10] and its modifications [11,12] used two solid-phase extraction cartridges to isolate morphine, M3G and M6G from plasma. The compounds were collected from the second cartridge in 3 ml of eluate and, to achieve the required sensitivity, up to 1 ml of this eluate was injected into the HPLC system.

This communication describes improvements to the sample preparation and chromatographic conditions while retaining the use of a single and readily available method, namely UV, for the detection and quantification of all three compounds. Using the improved method, the stability of morphine, M3G and M6G in plasma was studied, an important consideration in pharmacokinetic studies.

EXPERIMENTAL

Chemicals

Morphine · HCl (McFarlane Smith, Edinburgh, U.K.) and M3G (Sigma, St. Louis, MO, U.S.A.) were used as supplied. M6G was synthesized by the method of Yoshimura *et al.* [13]. Purity was established using a small sample of M6G provided by the National Institute of Drug Abuse (Bethesda, MD, U.S.A.). The synthesized material was found to contain less than 0.1% morphine. Hydromorphone (the internal standard) was obtained from commercial tablets (Dilaudid®, Knoll, Ludwigshafen, Germany) or from Sigma. 1-Dodecylsulphate sodium salt was obtained from Regis (Morton Grove, IL, U.S.A.). HPLC-grade acetonitrile (UV cut-off 190 nm) and methanol were from Waters Assoc. (Lane Cove, Australia). All other chemicals were of analytical grade and were used as received.

Plasma sample pretreatment

Two methods for the preparation of plasma samples for HPLC are described. Method II is an improvement on method I. For both, each new Sep-Pak C_{18} cartridge (Waters Assoc.) was washed successively with 10 ml of methanol, 5 ml of 25% acetonitrile in 10 mmol/l sodium dihydrogenphosphate (pH 2.1) and 10 ml of water under positive pressure at an approximate flow-rate of 5 ml/min. Prior to re-use, each cartridge was washed with 5 ml water followed by the washing procedure above. During application of the sample, and thereafter until elution of the compounds of interest, solvents were passed through the cartridge under positive pressure at an approximate flow-rate of 2 ml/min.

Method I. A mixture of plasma (0.5–1.0 ml) and hydromorphone solution (0.1 ml, 15.5 μmol/l), buffered with 3 ml of 0.5 mol/l ammonium sulphate (adjusted to pH 9.3 with ammonia, 28%, w/w), was applied to the Sep-Pak cartridge. The cartridge was rinsed successively with 20 ml of 5 mmol/l ammonium sulphate (pH 9.3), 0.5 ml of water and 0.5 ml of 25% acetonitrile in 10 mmol/l sodium dihydrogenphosphate buffer (pH 2.1). Morphine, M3G, M6G and hydromorphone were eluted with a further 0.5 ml of the acetonitrile–phosphate buffer mixture, of which 0.2 ml was injected onto the HPLC column.

Method II. Plasma (0.5–1.0 ml) was mixed with 0.1 ml of hydromorphone solution (15.5 μmol/l), 3 ml of 0.5 mol/l sodium hydrogencarbonate–sodium carbonate buffer (pH 9.3) and applied to the Sep-Pak cartridge. The cartridge was rinsed successively with 20 ml of 5 mmol/l sodium hydrogencarbonate–sodium carbonate buffer (pH 9.3), 0.5 ml of water and 0.35 ml of 25% acetonitrile in 10 mmol/l sodium dihydrogenphosphate buffer (pH 2.1). Morphine, M3G, M6G and hydromorphone were eluted with a further 0.8 ml of the acetonitrile–phosphate buffer mixture, of which 0.25 ml was injected onto the HPLC column.

HPLC instrumentation and conditions

Chromatography was performed using a Model 6000A pump, a Wisp 710A autosampler, a guard column (23.2 mm × 3.6 mm I.D.) packed with Bondapak C_{18}/Corasil, an RCM-100 radial compression module containing a Nova-Pak C_{18} 4-μm, Radial-Pak cartridge (10 cm × 5 mm I.D.) and a Model 481 UV detector set at 210 nm (all Waters Assoc.). Peak areas were determined using a Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan) or a Baseline 810 chromatography workstation (Waters Assoc.). Mobile phase, consisting of 26.5% acetonitrile and 0.8 mmol/l 1-dodecylsulphate sodium in 10 mmol/l sodium dihydrogenphosphate (pH 2.1), was pumped at a flow-rate of 0.8 ml/min.

Stability studies

The stability of morphine, M3G and M6G in human plasma was determined after each had been added to separate drug-free plasma samples at concentrations of 133, 10 800 and 2040 nmol/l, respectively, and stored at −20°C. The concentration of morphine in the stored samples was determined at 0, 1, 2, 5, 12 and 36 weeks, using method I, and at 101 weeks using method II, by reference to a morphine calibration curve constructed from freshly prepared standard solutions of morphine. Limited amounts of authentic M3G and M6G precluded the preparation of fresh standards at each time point.

RESULTS

Previous workers used 10% acetonitrile in 10 mmol/l phosphate buffer to elute morphine, M3G and M6G from the Sep-Pak cartridge [10–12]. To improve sensitivity, up to 1 ml of the eluate was injected onto the HPLC system [12]. In our

laboratory, collection of the compounds of interest from the Sep-Pak cartridge was achieved in a reduced volume using 25% acetonitrile in phosphate buffer as eluent. The volume was established by treating a plasma sample containing morphine (2660 nmol/l), M3G (2170 nmol/l), M6G (2040 nmol/l) and hydromorphone (3110 nmol/l), as described above, and collecting approximately 0.1-ml fractions of the acetonitrile–phosphate buffer mixture. For methods I and II, the recoveries of morphine, M3G, M6G and hydromorphone, determined after HPLC of each fraction, are shown in Fig. 1. It is clear that all four compounds were collected from the Sep-Pak in 0.5 ml of eluate using method I or 0.8 ml of eluate with method II. During the use of either method, the recovery for each compound was calculated by comparison of the peak area obtained by direct injection of an aqueous solution to that obtained after injection of the eluate prepared from each of the calibration standards. For method I the mean (± S.D.) recoveries for morphine, M3G, M6G and hydromorphone were 90 ± 5, 85 ± 4, 89 ± 6 and 86 ± 6%, respectively. Each cartridge may be used for at least five 1-ml plasma samples without significant losses in recovery or qualitative changes in the HPLC profiles. For method II the mean (± S.D.) recoveries for morphine, M3G, M6G and hydromorphone were 88 ± 8, 99 ± 6, 85 ± 9 and 93 ± 3%, respectively. With method II, when one Sep-Pak cartridge was used to treat fourteen replicate plasma samples containing morphine (266 nmol/l), M3G (2170 nmol/l), M6G (509 nmol/l) and hydromorphone (1040 nmol/l), the mean (±

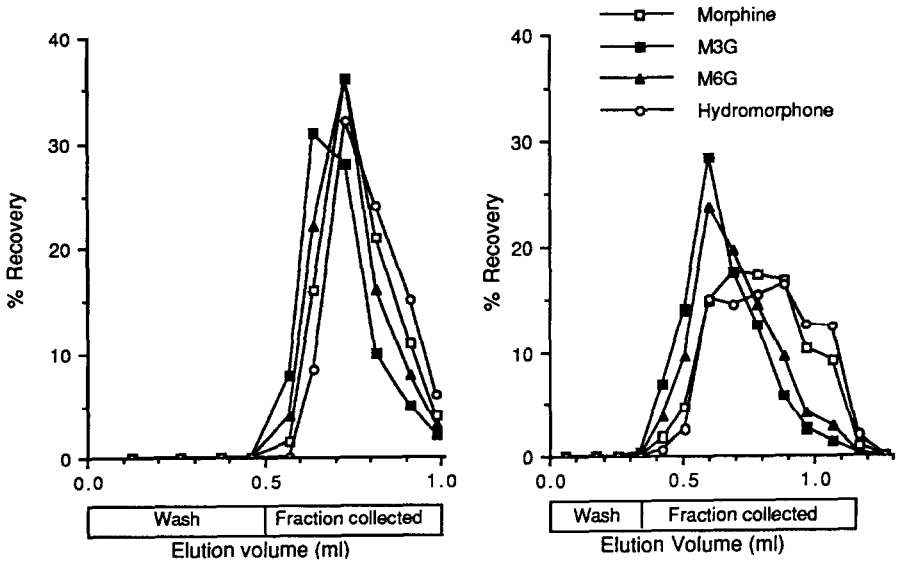

Fig. 1. Percentage recovery of morphine (2660 nmol/l), M3G (2170 nmol/l), M6G (2040 nmol/l) and hydromorphone (3110 nmol/l), as a function of the volume of 25% acetonitrile in 10 mmol/l sodium dihydrogenphosphate buffer (pH 2.1) as eluate, from a plasma sample treated with a Sep-Pak cartridge using method I (left) and method II (right).

S.D.) recoveries were 86 ± 4, 98 ± 3, 89 ± 4 and 91 ± 5%, respectively, with no observable trend. There were no qualitative differences in the HPLC profiles for the fourteen samples.

Using the sample preparation of method II and chromatographic conditions described in this report, a typical chromatogram obtained from a plasma sample containing morphine (133 nmol/l), M3G (1083 nmol/l) and M6G (204 nmol/l) is shown in Fig. 2. M3G, M6G, morphine and hydromorphone were eluted after approximately 9, 12, 23 and 32 min, respectively. There was no interference from endogenous compounds present in drug-free plasma (Fig. 2) and, there were only minor differences in the appearance of the chromatograms resulting from the different sample preparations of method I and II. There were no important differences in the appearance of the chromatograms between those resulting from the use of two Sep-Pak cartridges, as in the original method of Svensson *et al.* [10], and from one cartridge as in our modified method.

Standard curves for morphine (13.3–532 nmol/l), M3G (108–4330 nmol/l) and M6G (20.4–1020 nmol/l) were linear with correlation coefficients greater than 0.990. Using 1 ml of plasma, the coefficients of variation ($n = 5$) were 6.2% (26.6 nmol/l) and 2.3% (266 nmol/l) for morphine, 4.5% (108 nmol/l) and 4.4% (4330

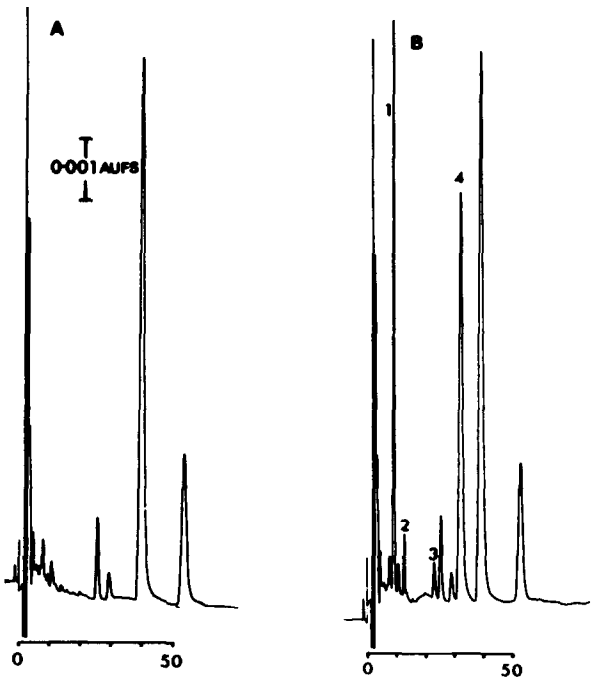

Fig. 2. Chromatograms of (A) blank plasma (0.5 ml) and (B) plasma (0.5 ml) containing 133 nmol/l morphine (3), 1083 nmol/l M3G (1) and 204 nmol/l M6G (2) to which 1.55 nmol of hydromorphone (4) was added as internal standard. Both samples were prepared for chromatography using method II.

nmol/l) for M3G, and 7.4% (41 nmol/l) and 2.9% (1020 nmol/l) for M6G using method I; for method II the coefficients of variation ($n = 5$) were 9.3% (13.3 nmol/l) and 2.4% (266 nmol/l) for morphine, 6.6% (108 nmol/l) and 4.0% (4330 nmol/l) for M3G, and 6.7% (41 nmol/l) and 5.9% (1020 nmol/l) for M6G. For the reproducibility studies, each group of five replicates was treated with the same Sep-Pak cartridge.

Morphine, M3G and M6G were found to be stable in human plasma stored at −20°C for up to 101 weeks. Over this period, the morphine concentration in plasma samples spiked with morphine was within 10% of the nominal value. In plasma samples containing M3G (10 800 nmol/l) or M6G (2040 nmol/l) the morphine concentration was less than 13.3 nmol/l.

DISCUSSION

We report here on improvements to the original and modified HPLC methods for the determination of morphine, M3G and M6G in plasma [10–12]. These methods required two Sep-Pak cartridges for sample clean-up and the injection of a relatively large volume (up to 1 ml) of a weaker eluate (3 ml of 10% acetonitrile–phosphate buffer) into the HPLC system. Paired-ion chromatography on a stainless-steel column packed with an octadecylsilane stationary phase was used to separate morphine, M3G and M6G. A chromatographic peak which may interfere with morphine quantification, especially with UV detection, is apparent from the reports of Svensson *et al.* [10] and Svensson [11]. To overcome this problem, the methods of Svensson [11] and Joel *et al.* [12] employed coulometric detection while maintaining the original chromatographic conditions [10]. However, both methods still required UV [11] or fluorescence [12] monitoring for the detection and quantification of M3G.

The modified sample preparation and chromatographic conditions described in the present report have allowed baseline separation of morphine from any interfering peaks and, with UV detection alone, permitted the determination of morphine concentrations in plasma down to 13.3 nmol/l with a precision of 9.3%. A similar precision for morphine was achieved by Svensson *et al.* [10], with UV detection, and Joel *et al.* [12], but at plasma concentrations of 77 and 70 nmol/l, respectively. The modified method of Svensson [11] allowed the determination of plasma morphine concentrations of 5 nmol/l with a precision of 5.9%. The coefficient of variation for M3G at 108 nmol/l in the present report compares favourably with the values of 1.3% (433 nmol/l) and "less than 6%" (433 nmol/l) achieved by Svensson *et al.* [10] and Joel *et al.* [12], respectively. The precision achieved for M6G at 41 nmol/l in the present report compares well with the "less than 6%" obtained by Joel *et al.* [12] at 43 nmol/l.

The use of 1-ml injection volumes of 10% acetonitrile-phosphate buffer, as described previously [12], resulted in poor peak shape for M3G and M6G. The peak-height/peak-area ratio of 0.76 min^{-1} and 0.91 min^{-1} for M3G and M6G,

respectively, after a 1-ml injection in 10% acetonitrile–phosphate buffer was increased to 1.59 and 1.54 min^{-1} after injection of the same amount of each compound in 0.25 ml of 25% acetonitrile–phosphate buffer. The peak-height/peak-area ratios for morphine and hydromorphone remained unchanged at 0.90 and 0.70 min^{-1}, respectively. The injection of a reduced volume onto the HPLC column gave improved peak shape for the glucuronide metabolites and better resolution from endogenous compounds. Increasing the concentration of acetonitrile in the eluent allowed quantitative recovery of morphine, M3G and M6G from the Sep-Pak cartridge in a smaller eluate volume than was used previously [10–12] and allowed a greater fraction of the eluate to be injected onto the HPLC column.

A problem encountered with the use of method I for plasma sample preparation was that, after fifteen to twenty injections of eluate into the HPLC, the back-pressure generated at the guard column was approximately doubled and there was a decrease in chromatographic efficiency. The problem was reduced considerably by substituting ammonium sulphate buffer with the sodium hydrogencarbonate–carbonate buffer of method II, so that up to fifty injections of Sep-Pak eluate could be performed before replacement of the guard column became necessary. The reasons for the change in elution profile from the Sep-Pak cartridge (Fig. 1), and the considerable reduction in back-pressure generated at the HPLC guard column when hydrogencarbonate–carbonate buffer was substituted for ammonium sulphate buffer, remain unknown. Method II is the preferred method and is currently being used in this laboratory.

Joel *et al.* [12] criticized the method of Svensson *et al.* [10] as being time-consuming when performed manually. By using one Sep-Pak cartridge only, as in the present report, the sample processing time is reduced considerably without compromising the qualitative appearance of the chromatograms. The re-usability of the Sep-Pak cartridges has not been addressed in previous reports [10–12]. A considerable cost-saving may be achieved by re-using each cartridge for up to five 1-ml plasma samples for method I, or fourteen samples for method II, without significant loss in recovery or qualitative changes in the chromatograms.

While it is common practice for plasma samples to be stored for extended periods prior to analysis, there appears to be no information on the stability of morphine and its glucuronide conjugates during storage. Assuming that any instability of the glucuronides during storage would be due to hydrolysis, yielding free morphine, then for plasma samples from patients receiving morphine, especially those with renal failure where the plasma concentrations of the glucuronides are considerably higher than those of morphine (refs. 7 and 9, and unpublished observations), only a small degree of hydrolysis may produce erroneously high morphine concentrations. The plasma concentrations of M3G and M6G chosen for the stability study approximated those which are achieved in renal failure patients [7,9]. The study indicated that plasma samples containing morphine, M3G and M6G may be stored at $-20°C$ for at least 101 weeks without

any significant change in morphine concentration or hydrolysis of the glucuronides to morphine.

In conclusion, this report describes improvements to the HPLC method for the simultaneous determination of morphine, M3G and M6G in plasma [10–12]. Together with changes to the method of sample pretreatment, which allow greater economy in the use of Sep-Pak cartridges and a smaller elution volume, chromatographic modifications permit the reliable determination of lower concentrations of morphine than was previously possible using UV detection methods alone. The improved method was used to determine the stability of morphine, M3G and M6G, during storage in human plasma. Method II, the preferred method, is currently being used to study the disposition of morphine and its glucuronide metabolites in patients and animals.

ACKNOWLEDGEMENTS

The authors wish to thank the Anticancer Foundation of South Australia, the South Australian Institute of Technology and the Royal Adelaide Hospital for financial assistance, and the National Institute of Drug Abuse for kindly supplying a sample of M6G.

REFERENCES

1 U. Boerner, S. Abbott and R. L. Roe, *Drug Metab. Rev.*, 4 (1975) 39.
2 S. Y. Yeh, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 32 (1973) 763.
3 K. Shimomura, O. Kamata, S. Ueki, S. Ida, K. Oguri, H. Yoshimura and H. Tsukamoto, *Tohoku J. Exp. Med.*, 105 (1971) 45.
4 G. W. Pasternak, R. J. Bodnar, J. A. Clark and C. E. Inturrisi, *Life Sci.*, 41 (1987) 2845.
5 F. V. Abbott and R. M. Palmour, *Life Sci.*, 43 (1988) 1685.
6 D. Paul, K. M. Standifer, C. E. Inturrisi and G. W. Pasternak, *J. Pharmacol. Exp. Ther.*, 251 (1989) 477.
7 R. J. Osborne, S. P. Joel and M. L. Slevin, *Br. Med. J.*, 292 (1986) 1548.
8 R. J. Osborne, S. Joel, D. Trew and M. L. Slevin, *Lancet*, i (1988) 828.
9 J. Sawe and I. Odar-Cederlof, *Eur. J. Clin. Pharmacol.*, 32 (1987) 377.
10 J.-O, Svensson, A. Rane, J. Sawe and F. Sjoqvist, *J. Chromatogr.*, 230 (1982) 427.
11 J.-O. Svensson, *J. Chromatogr.*, 375 (1986) 174.
12 S. P. Joel, R. J. Osborne and M. L. Slevin, *J. Chromatogr.*, 430 (1988) 394.
13 H. Yoshimura, K. Oguri and H. Tsukamoto, *Tetrahedron Lett.*, 4 (1968) 483.