Journal of Chromatography, 615 (1993) 164–168
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6772

Short Communication

High-performance liquid chromatographic determination of morphine and its metabolites in plasma using diode-array detection

D. Wielbo, R. Bhat, G. Chari and D. Vidyasagar

Department of Pediatrics, University of Illinois at Chicago, Chicago, IL 60612 (USA)

I. R. Tebbett* and A. Gulati

Department of Pharmacodynamics, University of Illinois at Chicago, Chicago, IL 60612 (USA)

(First received October 20th, 1992; revised manuscript received February 1st, 1993)

ABSTRACT

An isocratic high-performance liquid chromatographic method has been developed for the determination of morphine, codeine, normorphine, morphine 3-glucuronide and morphine 6-glucuronide in plasma using a diol column and diode-array detection. Samples were extracted using solid-phase extraction with recoveries in excess of 90%. The limit of determination was 1 ng/ml for morphine, codeine and morphine 3-glucuronide, and 10 ng/ml for normorphine and morphine 6-glucuronide. Inter- and intra-day precision were better than 10%.

INTRODUCTION

The extensive use of morphine as an analgesic together with the widespread abuse of opiates has necessitated the development of rapid and sensitive techniques for the detection of these compounds in biological samples. Although immunoassay systems offer the sensitivity required for the detection of opiates, these rarely differentiate between morphine metabolites and the parent compound [1]. The ability to identify and quantify morphine metabolites has assumed increasing importance with the realization that many of these are pharmacologically active [2]. While GC–MS offers both the sensitivity and specificity required for the examination of morphine and metabolites, the technique requires an often time-consuming derivatization step [3]. Many workers have therefore concentrated on HPLC as a method for the detection of these compounds and papers have been published in which electrochemical [4–6], fluorescence [7] and UV absorbance de-

* Corresponding author. Present address: Department of Pharmaceutics, University of Florida, Box J-484, Gainesville, FL 32610, USA.

0378-4347/93/$06.00

tectors [8–11] have been described. A combination of UV and fluorescence detectors has been described for the determination of morphine and metabolites [12]. However, only UV detection allows the simultaneous determination of morphine and its metabolites without the need for derivatization. Unfortunately this detection system is thought to lack the sensitivity necessary for the detection of physiological concentrations of opiates in biological samples. This is due in part to the fact that on a reversed-phase system, the polar morphine glucuronides elute very close to the solvent front and are therefore prone to being hidden by co-eluting endogenous materials. We recently published such an article describing the use of reversed-phase HPLC with a very sensitive forward optics detection system for the analysis of these compounds [13]. This, however, was not ideal, since computer data manipulation was required in order to obtain the necessary sensitivity.

We have therefore developed an alternative HPLC method for the examination of morphine and its metabolites using a diol column. Morphine and its metabolites were eluted several minutes after the solvent front on this essentially normal-phase column and were free from interference from endogenous materials. The limit of determination was therefore improved.

EXPERIMENTAL

Materials

Methanol and acetonitrile were HPLC grade (Fisher Scientific, Fairlawn, NJ, USA). Potassium phosphate and phosphoric acid were analytical grade and were obtained from Sigma (St. Louis, MO, USA) as were morphine, codeine, normorphine and morphine 3- and 6-glucuronides. Serum was obtained from catheterized piglets and kept at −20°C until required for analysis.

Extraction

Solid-phase extraction was performed using 3-ml Clean Screen columns (Worldwide Monitoring, Horsham, PA, USA) containing 40-μm bonded silica particles. The cartridges were positioned on a 24-station Vac-Elut (Varian, Harbor City, CA, USA) manifold, and vacuum pressure was set to approximately 2.7 kPa. The columns were conditioned with methanol (3 ml), water (3 ml) and phosphate buffer at pH 3 (1 ml). A 1-ml sample of plasma was mixed with 2 ml of 10 m*M* phosphoric acid and applied to the extraction column. The cartridge was air-dried for 30 s and then washed with phosphate buffer (pH 3) (1 ml), followed by methanol (1 ml). The column was again air-dried for 30 s, before elution of the retained compounds with 3 ml of 2% ammoniacal methanol. The solvent was evaporated to dryness under nitrogen at 45°C and the residue reconstituted in 50 μl of HPLC mobile phase. Extraction recovery and reproducibility of this procedure were determined by the comparison of HPLC peak areas of standard solutions with those obtained after extraction of spiked plasma.

Chromatographic conditions

A Waters 600E multi-solvent HPLC pump was used to deliver solvent at a flow-rate of 1 ml/min. The mobile phase consisted of 0.05 *M* sodium dihydrogenphosphate buffer–acetonitrile (20:80, v/v). The effect of pH on the separation of morphine and metabolites was investigated by the addition of orthophosphoric acid to this mobile phase to a final pH of 3. Separation was achieved on a 20 cm × 4.5 mm I.D., 5-μm LiChrosphere diol column (Merck, Rahway, NJ, USA) fitted with a Rheodyne injection system (Cotati, CA, USA) incorporating a 20-μl loop. Eluting compounds were detected with a Waters 991 photodiode-array detector (Millford, MA, USA) with integration at 230 nm. Data manipulation was achieved with a NEC 386 computer.

Following the initial development of the chromatographic system and extraction procedure, calibration curves were prepared in drug-free plasma. Each calibrator was divided into individual aliquots and stored frozen at −20°C until needed. Standard curve calibrators and control samples were prepared for each compound of interest (drug and/or metabolites) in the biological matrix at concentrations of 0, 1, 5, 10, 25, 50 and

100 ng/ml, with codeine as internal standard (50 ng/ml). Each point on the calibration curve was taken as the average of two determinations. The limit of detection was determined from twice the signal-to-noise ratio as documented by replicate analysis of blank (drug-free) samples and samples containing drug at minimal detectable concentration in plasma.

Precision

Intra-day and inter-day precision were determined on three control samples (low, medium and high concentration range). Replicate (n = 7) analysis of control samples was performed on the same day and over a period of two weeks. Precision is expressed as the relative standard deviation of the intra-day and inter-day replicate analysis for each control sample.

RESULTS AND DISCUSSION

Optimal separation of morphine and its metabolites was achieved with a mobile phase consisting of 0.05 M sodium dihydrogenphosphate buffer–acetonitrile (20:80, v/v) adjusted to pH 3.0 with orthophosphoric acid. A typical chromatogram illustrating the separation of these compounds is shown in Fig. 1. While the diol column is primarily a weakly polar stationary phase, it does have some cation-exchange properties. This

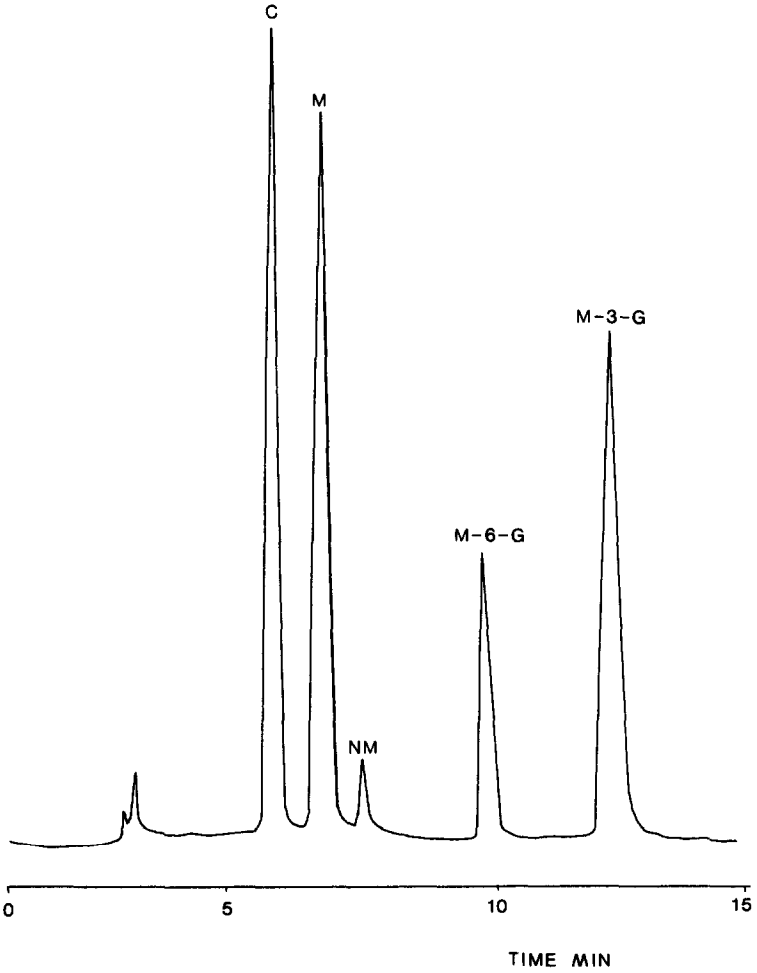

Fig. 1. Chromatogram showing the separation of a standard solution (100 ng/ml) of codeine (C), morphine (M), normorphine (NM), morphine 6-glucuronide (M-6-G) and morphine 3-glucuronide (M-3-G).

is illustrated by the effect of pH on the retention times of codeine with the above mobile phase (Fig. 2). The same response of retention time to pH was observed with the other opiates in this study.

Calibration graphs were linear over the concentration range 1–100 ng/ml, and regression equations showed correlation coefficients of greater than 0.998. Intra-day assay precision for 10, 50 and 100 ng/ml plasma concentrations of morphine, morphine 3-glucuronide, morphine 6-glucuronide and codeine was better than 7% for each compound. Similarly, the inter-day assay precision for 10 ng/ml serum concentrations for the same compounds was less than 10%. Accuracy was determined as being better than 5% for each compound. Limit of determination (signal-to-noise ratio >2) was determined as 1 ng/ml for morphine, codeine and morphine 3-glucuronide, and 10 ng/ml for normorphine and morphine 6-glucuronide.

The extraction procedure described gave clean extracts of the compounds of interest with recoveries in excess of 90%. The elution solvent methylene chloride–isopropanol–ammonium hydroxide described by Worldwide Monitoring for the extraction of opiates [14] gives good recoveries of codeine, morphine and normorphine. However, the use of ammoniacal methanol is required for the elution of the more polar glucuronide metabolites.

The analytical procedure was validated by the examination of plasma samples taken from piglets after the administration of a single intravenous dose of 0.5 mg/kg morphine. A chromatogram typical of these analyses is represented in Fig. 3.

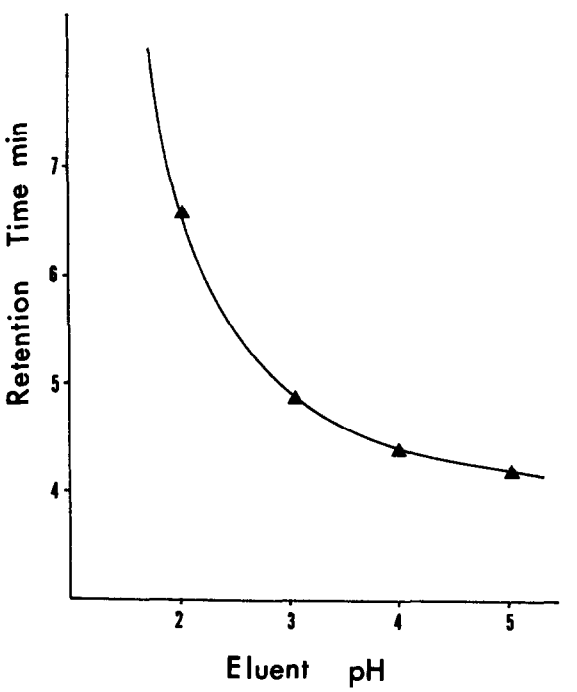

Fig. 2. Effect of mobile phase pH on the retention time of codeine.

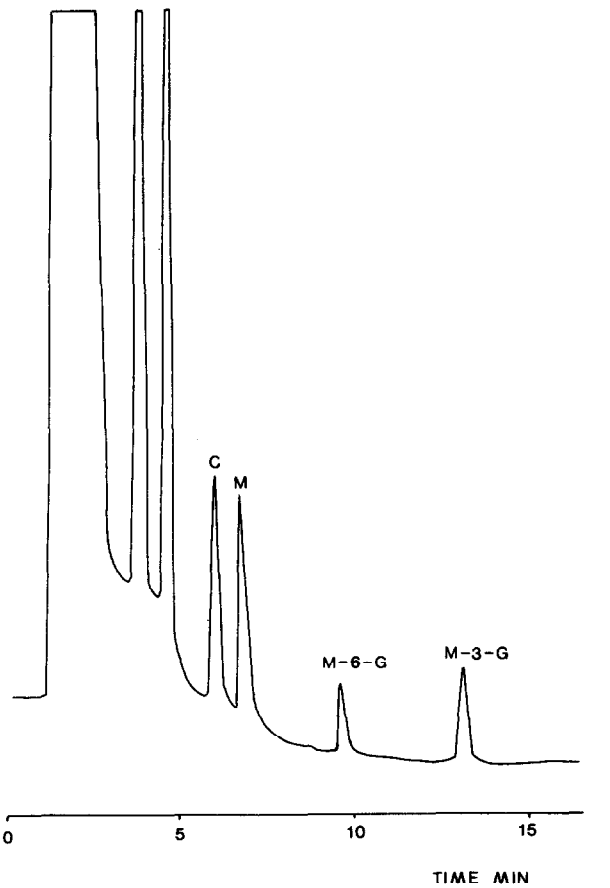

Fig. 3. Chromatogram of a plasma extract showing the presence of approximately 50 ng/ml morphine (M), 100 ng/ml morphine 6-glucuronide (M-6-G), 20 ng/ml morphine 3-glucuronide (M-3-G) and 50 ng/ml internal standartd, codeine (C).

CONCLUSIONS

The HPLC method described above represents a rapid and sensitive method for the determination of morphine and its glucuronide metabolites in biological samples. The use of a diol column offers an advantage over previously reported reversed-phase HPLC methods, since it allows a much greater separation of the opiate metabolites from any endogenous compounds eluting at or near the solvent front. Sensitivity is therefore improved without the need for sophisticated data manipulation involving chromatogram subtractions.

ACKNOWLEDGEMENT

This work was funded in part by a grant from the National Institute on Drug Abuse, Grant No. DA06794.

REFERENCES

1 C. W. Hand, R. A. Moore, H. J. McQuay, M. C. Allen and J. W. Sear, *Ann. Clin. Biochem.*, 24 (1987) 153.
2 D. Paul, K. M. Standifer, C. E. Inturriis and G. W. Pasternak, *J. Pharmacol. Exp. Ther.*, 251 (1989) 477.
3 R. A. Tasker and K. Nakatsu, *Analyst*, 111 (1986) 563.
4 F. Tagliaro, G. Carli, R. Dorizzi and M. Marigo, *J. Chromatogr.*, 507 (1990) 253.
5 J. L. Mason, S. P. Ashmore and A. R. Aitkenhead, *J. Chromatogr.*, 570 (1990) 191.
6 K. P. W. G. M. Verwey-Van Wissen, P. M. Koopman-Kimenai and T. B. Vree, *J. Chromatogr.*, 570 (1991) 320.
7 R. F. Venn and A. Michalkiewicz, *J. Chromatogr.*, 525 (1990) 379.
8 P. A. Glare, T. D. Walsh and C. E. Pippenger, *Ther. Drug Monit.*, 13 (1991) 226.
9 B. L. Posey and S. N. Kimble, *J. Anal. Toxicol.*, 8 (1984) 68.
10 Y. Kumagai, T. Ishida and S. Toki, *J. Chromatogr.*, 421 (1987) 155.
11 R. W. Milne, R. L. Nation, G. D. Reynolds, A. A. Somogyi and J. T. Van Crugten, *J. Chromatogr.*, 565 (1991) 457.
12 S. P. Joel, R. J. Osborne and M. L. Slevin, *J. Chromatogr.*, 430 (1988) 394.
13 G. Chari, A. Gulati, R. Bhat and I. R. Tebbett, *J. Chromatogr.*, 571 (1991) 263.
14 *Clean Screen Applications Manual*, Worldwide Monitoring, Horsham, PA, 1991, p. 22.