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Short Communication

High-performance liquid chromatographic determination of morphine, morphine-3-glucuronide, morphine-6-glucuronide and codeine in biological samples using multi-wavelength forward optical detection

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ABSTRACT

An isocratic high-performance liquid chromatographic method has been developed for the determination of morphine, morphine-3-glucuronide, morphine-6-glucuronide and codeine in plasma, urine and cerebrospinal fluid. The use of an efficient solid-phase extraction procedure together with a forward optical scanning detector allows a detection limit of 500 pg/ml. The method was evaluated by examination of biological samples taken from newborn infants following the intravenous administration of morphine sulfate.

INTRODUCTION

Morphine (M) is being increasingly used in the treatment of chronic pain and as a drug of abuse. Recent studies show that not only M but also its metabolites are clinically active [1–4]. Therefore, there is a need to develop a sensitive and specific method for the determination of M and its metabolites in biological samples. Methods to determine the concentration of M or its metabolites in biological samples have used radioimmunoassay (RIA) [5,6], gas chromatography (GC) with electron-capture detection [7] or mass spectrometry [8] or high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [9–12], fluorescence detection [13,14] or UV detection [15,16].

RIA allows picogram amounts of M to be detected [5], but it does not measure M specifically since the antisera commonly used may cross-react with M metabolites like morphine-3-glucuronide (M-3-G), morphine-6-glucuronide (M-6-G), codeine (C) and normorphine [6]. RIA therefore measures M-like immunoreactivity and is therefore not specific. Much better specificity and reliability are

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present in GC, but only mass fragmentography [17] and electron-capture detection [7] achieve the sensitivity limits comparable to RIAs. However, GC methods require several tedious and time-consuming sample preparation steps including derivatization to reduce the high polarity of M making electron capture possible [9]. These techniques are relatively expensive and are not commonly available. The method of detection of M and its metabolites by using HPLC has been introduced during the last ten years and is being adopted in an increasing number of laboratories because of its specificity, reliability, sensitivity and the fact that derivatization is not necessary.

The limit of detection is one of the major concerns when HPLC methods are applied in the biomedical field. The level of detection has been improved considerably by modifying extraction procedures and by the use of advanced detection systems. The use of solid-phase extraction columns as an alternative to liquid–liquid extraction for the isolation of drugs has gained popularity over recent years because of the reported excellent recoveries and ease of operation [18,19]. The use of a bonded-phase cation exchanger with some non-polar character has been found to be suitable for chromatographic methods [20].

Several types of detectors have been tried with HPLC to improve the sensitivity. Electrochemical detectors are by far the most widely used. However, the simultaneous detection of M and its metabolites using an electrochemical detector is not possible because of differing redox potentials of these compounds. Simultaneous determination of M and its metabolites by using ED with an additional screening electrode to determine M-6-G and fluorescence detection to quatitate M-3-G has been described [1,2]. Fluorescence detection has been used following HPLC. Several methods to convert M to a fluorescent product (pseudomorphine) were devised [21,22]. Unfortunately the increased sensitivity achieved by derivatization to a fluorescent product was poor. Some of the methods using dimerization of M to pseudomorphine [23,24] could not detect M-3-G because of a dimerization reaction occurring through the phenolic (3-) position. However, using the native fluorescence of M and other opiate agonists a sensitive method to estimate M and its metabolites has been described [14]. UV detectors [15,16] have been used, but the sensitivity and the limit of detection varies from 5 to 50 ng/ml [15] much above the desired values of less than 1 ng/ml.

We have developed a method combining solid-phase extraction of M and its metabolites from urine, plasma and cerebrospinal fluid (CSF) with isocratic HPLC and multi-wavelength forward optical detection to analyze M, M-3-G, M-6-G and C.

EXPERIMENTAL

Reagents

Methanol and acetonitrile were HPLC grade (Fischer Scientific, Fairlawn, NJ, USA). Potassium phosphate, phosphoric acid and acetic acid were AR grade

(Sigma, St. Louis, MO, USA). M, M-3-G, M-6-G and C were donated by National Institute on Drug Abuse (NIDA, Washington, DC, USA), courtesy of Dr. B. Walsh. Plasma, urine and CSF samples were obtained from the University of Illinois Hospitals (Chicago, IL, USA).

Extraction

Solid-phase extraction was performed using 1-ml Bond Elut strong cation-exchange (SCX) cartridges (Analytichem, Harbor City, CA, USA) containing 40-μm silica particles. The cartridges were positioned on a Vac Elut processing station (Analytichem). The cartridges were conditioned with methanol (2 ml), water (1 ml) and 10 m*M* phosphoric acid (0.5 ml at pH 3.4). A 0.4-ml urine, plasma or CSF sample was mixed with 0.5 ml of 10 m*M* phosphoric acid and applied to the SCX cartridges. The cartridge was air-dried for about 30 s and then washed firstly with 10 m*M* phosphoric acid (1 ml), 0.1 *M* acetic acid (0.5 ml) followed by methanol (1 ml). The column was again air-dried for 30 s. M and its metabolites were eluted with 3% ammoniacal methanol (2 ml) and collected in the tubes. The solvent was evaporated to dryness under nitrogen at 45°C and reconstituted in 150 μl of the mobile phase.

Chromatographic conditions

HPLC was performed at ambient temperature using a 600E multi-solvent delivery system (Waters Assoc., Milford, MA, USA). The mobile phase was composed of 10% acetonitrile in 0.05 *M* potassium phosphate buffer pH 4.68. The flow-rate of the mobile phase was 1.0 ml/min. Aliquots (50 μl) of the reconstituted samples were injected into a Hibar LiChrospher 100 CM-8/III, 250 mm × 4.6 mm I.D. column (E. Merck, Rahway, NJ, USA). The HPLC analysis was performed under isocratic conditions. The injector was a 50-μl loop injector (Rheodyne, Cotati, CA, USA). The steel column was protected by a 0.2-μm filter (Water Assoc.). Detection was performed using a UV–VIS Spectra Focus forward optical detector and Spectra Focus software (Spectra Physics, San Jose, CA, USA). The detector was set at three wavelengths, 230, 255 and 280 nm, to scan at high speed. Data manipulation was achieved with an IBM PS/2 computer. Full spectra were also monitored from 190 to 310 nm for each peak. The data were further analyzed using derivative spectroscopy to determine absorbance maxima and inflection points. The data for each chromatogram were saved to do three-dimensional plots and spectral analysis. The data were used to distinguish the peaks of M and its metabolites from other peaks. A re-equilibrium time of 5 min was given between injections. Each run was monitored for 15 min.

Peak areas were used to calculate the concentrations of M, M-3-G, M-6-G and C, based on the standard curves prepared from biological samples spiked with M and its metabolites.

RESULTS AND DISCUSSION

Extraction

The solid-phase extraction using Bond Elut SCX cartridges provided excellent recoveries for M and its metabolites. Similar results were obtained by Venn and Michalkiewicz [14], using Bond Elut C_2 columns and fluoresence detection. The advantage of a cation-exchange extraction procedure was that cleaner extracts were obtained in a relatively small volume. The final eluent (3% ammonical methanol) disrupted both ion-paired and non-polar interactions.

Recovery

The recovery was determined for each compound by comparing the differences in area under the peak between plasma extracted and unextracted standards and were determined over a concentration range of 10–50 ng/ml. The recoveries of M, M-3-G, M-6-G and C from plasma were 92, 91, 87 and 87%, respectively. The recoveries of M and its metabolites from urine were similar to that of plasma.

Chromatographic conditions

Chromatographic separation of M and its metabolites was achieved with a mobile phase consisting of 10% acetonitrile in 0.05 *M* potassium phosphate buffer pH 4.68 with a flow-rate of 1.0 ml/min. The retention times of M-3-G, M-6-G, M and C were 3.82, 4.35, 4.99 and 9.28 min, respectively. It was observed that a slight change in mobile phase condition had a significant effect on the retention times. A chromatogram showing full UV spectra of M and its metabolites is shown in Fig. 1. The best separation and detection of M and its metabolites was obtained at 280 nm wavelength. A chromatogram for standard concentrations of M and its metabolites extracted from plasma is shown in Fig. 2.

The urine, plasma and CSF samples from newborn infants administered a single intravenous dose of 0.1 mg/kg M sulfate were analysed. The study protocol was approved by the Institutional Review Board. The urine, plasma and CSF samples were collected 24 h after the administration of M. The chromatogram of extracted blank plasma is shown in Fig. 3. The chromatogram of samples ob-

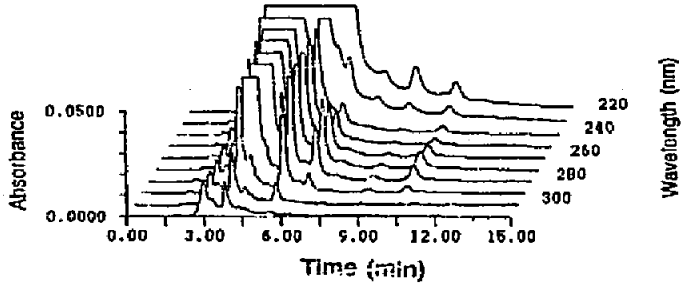

Fig. 1. Chromatogram showing full UV spectra of extracted plasma spiked with morphine (5 μg/ml), morphine-3-glucuronide (5 μg/ml), morphine-6-glucuronide (5 μg/ml) and codeine (5 μg/ml). The retention times of M-3-G, M-6-G, M and C were 3.82, 4.35, 4.99 and 9.28 min, respectively.

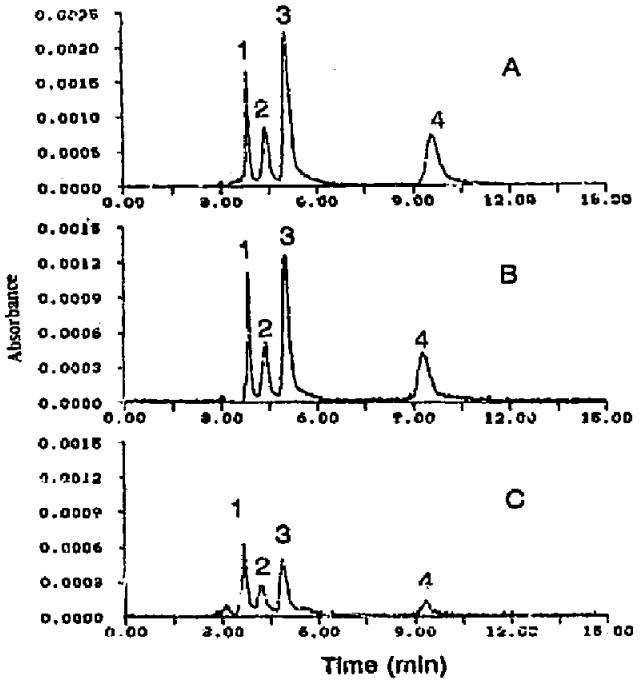

Fig. 2. Chromatograms showing extracted plasma spiked with (C) 5 ng/ml, (B) 10 ng/ml and (A) 20 ng/ml morphine-3-glucuronide (1), morphine-6-glucuronide (2), morphine (3) and codeine (4) (50-μl injection).

tained from one infant is shown in Fig. 4. It can be seen that the peaks of M-3-G are clearly identifiable in urine, plasma and CSF samples, while the peaks of M could be identified in CSF and plasma. There was one peak corresponding to M-6-G which was smaller than M-3-G in the plasma sample only. There were no peaks corresponding to C suggesting that the major metabolite in this infant is M-3-G. In another chromatogram of extracted urine, plasma and CSF samples obtained from another infant (Fig. 5), M-6-G was clearly identifiable in all the samples and an M-3-G peak could be identified in plasma and urine samples. There were no peaks corresponding to M and C. Although M-3-G is the major metabolite in mammals, we found more M-6-G than M-3-G in this infant. This could be due to either prematurity or some pathological process and needs fur-

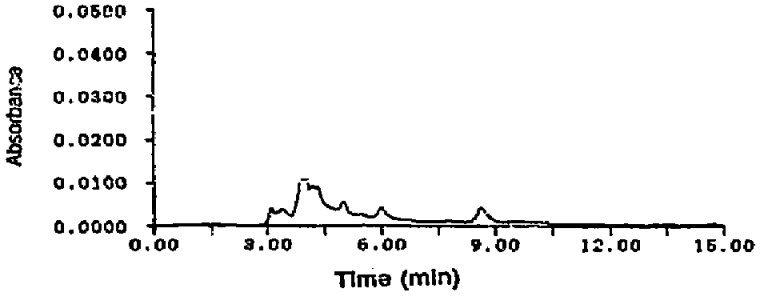

Fig. 3. Chromatogram showing extracted blank plasma.

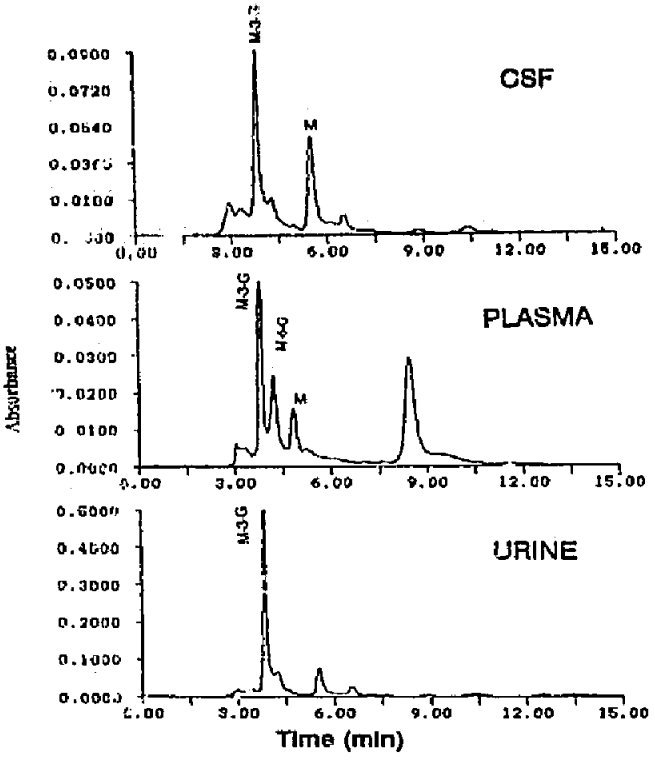

Fig. 4. Chromatograms of CSF, plasma and urine samples of an infant obtained at 24 h after the administration of morphine. Extraction was done and morphine and its metabolites were eluted from SCX cartridges. This chromatogram shows that the major metabolite is M-3-G.

ther evaluation. It is clear that with the technique developed, M and its metabolites can be detected in urine, plasma and CSF samples with very low detection limits.

Limit of detection

The detection limit was determined as the concentration of sample which corresponds to the voltage deflection of a peak that was twice that mean of the baseline noise. When extracted from urine, plasma and CSF (0.4 ml) using a Bond Elut SCX cartridge the minimum quantifiable concentration of M and its metabolites was 500 pg/ml. The sensitivity of the assay did not differ significantly for the four compounds. The forward optical detector was found to be more sensitive than a regular UV detector.

Precision

In order to determine the reproducibility, experiments were performed for ten consecutive days and it was found that the retention times for M and its metabolites were fairly close.

The calibration was performed using seven concentrations (10–50 ng/ml) of M, M-3-G, M-6-G and C in plasma prepared in duplicate. These samples were

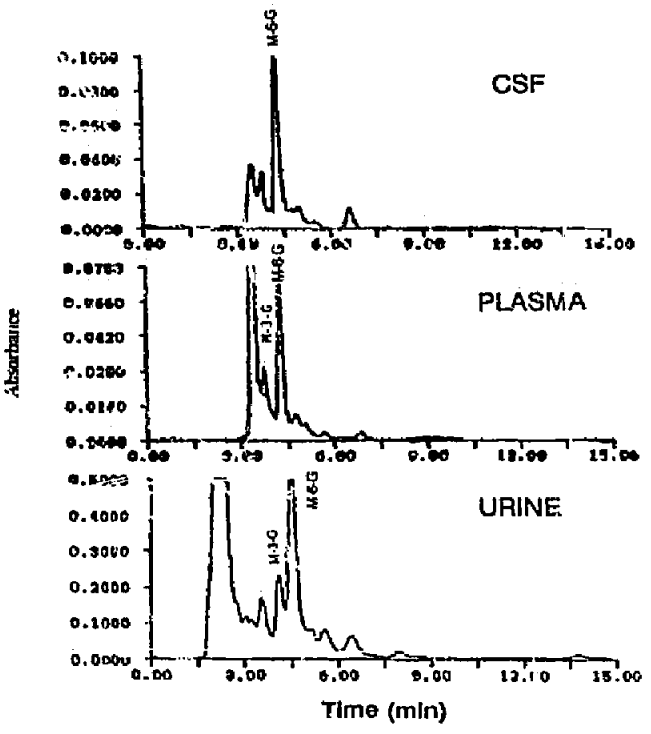

Fig. 5. Chromatograms of CSF, plasma and urine samples of another infant obtained at 24 h after the administration of morphine. Extraction was done and morphine and its metabolites were eluted from SCX cartridges.

extracted and subjected to HPLC analysis. The mean coefficient of variation (C.V.) of extraction was 11.3%. Lower concentrations of M and its metabolites had higher coefficients of variation. Linear correlations between the area under the peak and the original concentrations were performed by regression analysis. It was found that each compound had a strong linear correlation between area under the peak and concentration ($P < 0.0001$) and the correlation coefficients were more than 0.998. The intra-assay precision for 10 ng/ml plasma concentrations of M, M-3-G, M-6-G and C were 7.3, 7.1, 6.9 and 6.6%, respectively. The inter-assay precision for 10 ng/ml plasma concentrations of M, M-3-G, M-6-G and C were 7.6, 7.2, 6.3 and 6.9%, respectively. Precision studies were also done at various concentrations and were found to be less than 11%.

CONCLUSIONS

The results of the present study indicate that cation-exchange solid-phase extraction followed by HPLC with multi-wavelength forward optical detection is effective in the detection of M and its metabolites in urine, plasma and CSF samples at physiological concentrations. The method described is rapid, reliable, accurate, sensitive and reproducible. The method can be readily used to estimate the levels of M and its metabolites in biological fluids of patients being treated with M or to screen subjects for M abuse.

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