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DETERMINATION OF MORPHINE IN CEREBROSPINAL FLUID AND PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION*

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SUMMARY

Two methods for the extraction of morphine from cerebrospinal fluid or plasma with quantitation by high-performance liquid chromatography with electrochemical detection were compared for accuracy, precision and ease of preparation. One procedure was a standard extraction procedure and the other utilized a commercially available liquid—liquid extraction column. Both methods produced linear calibration curves over the concentration range of 1—200 ng/ml with coefficients of correlation of 0.999. Since the electrochemical detector is capable of detecting 20 pg of morphine, biological samples as small as 0.1 to 0.4 ml can be quantified with an average relative precision of 4.1 ± 3.9% over the concentration range 1—200 ng/ml. The potential clinical importance of the assay is demonstrated using a time course distribution study of morphine in the cerbrospinal fluid and plasma of a Rhesus monkey.

INTRODUCTION

In recent years, several sensitive methods for the determination of low levels of morphine in biological fluids have been reported. Radioimmunoassays (RIA) are capable of detecting picogram amounts of morphine, but they lack specificity and therefore cannot distinguish between morphine and compounds which are structurally related to morphine [1, 2]. Spectrofluorometric and radiolabeled morphine assays also have low specificity unless morphine is purified by chromatography prior to quantitation [3—5]. Assays using gas

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chromatography (GC) with electron-capture detection (ECD) require extraction and formation of a volatile morphine derivative prior to measurement [6—8]. The sensitivity of GC—ECD methods is comparable with that of radioimmunoassay [7]. Recently, Wallace et al. [9] reported the use of high-performance liquid chromatography (HPLC) with electrochemical detection (ElCD) to precisely determine morphine concentrations as low as 1 ng/ml in 1—2 ml of plasma with sensitivity exceeding that of GC—ECD methods [9].

In order to determine the pharmacokinetics of morphine in cerebrospinal fluid (CSF) and plasma after epidural administration of morphine sulfate, repeated sampling of the CSF and blood is required. GC—ECD and HPLC—ElCD methods require a minimum of 1 ml of CSF for each sample. The repeated sampling of such volumes from a small animal model cannot be performed without seriously impairing the physiological state of the animal.

We report on an HPLC—ElCD system which can accurately and precisely determine morphine levels as low as 1 ng/ml in 0.1—0.4 ml of CSF or plasma after extraction by a modification of Wallace et al.'s procedure [9]. An alternative extraction method is also presented which has the advantage of reduced sample preparation time. The application of the assay to the analysis of CSF and blood samples drawn following epidural administration of morphine sulfate is discussed.

EXPERIMENTAL

Reagents and materials

Methanol, acetonitrile, chloroform, isobutanol, and isopropanol were HPLC grade and used as received. Potassium monobasic phosphate (primary standard grade), boric acid and sodium borate (ACS grade) required no further purification. Clin-Elute CE-1001 extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). Morphine sulfate pentahydrate and nalorphine hydrochloride were purchased from Merck (Darmstadt, G.F.R.).

Chromatographic apparatus and conditions

The HPLC—ElCD system was composed of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery pump, Rheodyne injector equipped with a 200-μl sample loop, and a Waters μBondapak C_{18} column, 300 mm × 4 mm I.D., was used in conjunction with an amperometric detector system (LC-4A, Bioanalytical Systems, Lafayette, IN, U.S.A.). A guard column packed with Corasil-C_{18} was placed before the reversed-phase column. The electrochemical cell contained a glassy carbon working electrode modified as described by Moyer and Jiang [10], a stainless-steel auxiliary electrode, and a Ag/AgCl reference electrode. The working electrode was maintained at an applied potential of +0.79 V.

The chromatography was performed at ambient temperature using an isocratic mobile phase composed of 0.07 *M* KH_2PO_4 containing 0.5 m*M* EDTA and modified with 5% acetonitrile and 8% methanol. The flow-rate was 1.0 ml/min.

Determination of cell potential

The applied cell potential was determined by constructing a hydrodynamic voltammogram for the oxidation of morphine sulphate in the mobile phase.

Sample collection and preparation

All blood samples were drawn from an indwelling catheter with disposable syringes and transfered to Veneject® collection tubes containing Na_2EDTA. The tubes were centrifuged at 1600 *g* for 20 min and the plasma transfered to a polypropylene test tube, which was capped and stored at −80°C. CSF samples were collected in chilled polypropylene test tubes and stored at −80°C.

Extraction method

This procedure is a modification of the procedure of Wallace et al. [9]. CSF (0.1—0.4 ml) or plasma (0.4 ml) was added to a 14-ml polypropylene centrifuge tube containing borate buffer (0.1 *M*, pH 8.9, 0.5 ml) sodium chloride (0.25 g) and nalorphine (25 ng) as an internal standard. The morphine was extracted by adding chloroform—isobutanol (95:5, 9 ml) and the mixture was shaken for 30 min on a mechanical shaker. Organic and aqueous layers were separated by centrifugation (5 min, 900 *g*) and the aqueous layer removed by aspiration. Phosphate buffer (2 *M*, pH 10, 0.5 ml) was then added to the organic extract. The mixture was shaken for 10 min, centrifuged and the aqueous layer was removed by aspiration. This washing procedure was repeated a second time. Morphine was extracted from the organic phase by adding 0.5 *N* hydrochloric acid (3 ml) and shaking the mixture for 15 min. Following centrifugation, the aqueous layer was transfered to a polypropylene centrifuge tube (12 ml) and the pH adjusted to 8.9 ± 0.2 with solid potassium carbonate. Morphine was extracted from the aqueous phase with chloroform—isopropanol (95:5, 9 ml) by shaking the mixture for 30 min. The samples were centrifuged and the aqueous layer removed by aspiration. The organic extract was transfered to a 12 × 75 mm glass test tube and the solvent evaporated to dryness at 55°C under a stream of filtered air. The residue was reconstituted in the HPLC mobile phase (0.20 ml) and 10—100 µl injected into the HPLC system or the sample was stored at −80°C.

Extraction method II

Borate buffer (pH 8.9, 0.4 ml), nalorphine hydrochloride (25 ng) and plasma or CSF (0.1—0.4 ml) were applied to a Clin-Elute CE-1001 extraction column. After waiting 3 min for the sample to adsorb onto the column packing, chloroform—isopropanol (95:5, 5 ml) was added to the column and the eluate collected in a glass test tube. The extract was evaporated to dryness in a water bath maintained at 55°C under a gentle stream of filtered air. The residue was reconstituted in 0.2 ml of the HPLC mobile phase and 10—100 µl injected or the sample was stored at −80°C.

Determination of morphine in unknown samples

Morphine sulfate standards (1—200 ng/ml) were prepared with pooled human plasma. The plasma standards were then extracted and the peak height

ratios of morphine/nalorphine (M/N) obtained from chromatogams were plotted against the original morphine concentrations. Standard curves were determined by linear regression. Unknown samples were spiked with the same amount of internal standard and assayed. The morphine concentration of the unknown sample was determined from the calibration curve using the calculated peak height ratio (M/N).

Animal study

Rhesus monkeys were anesthetized with ketamine and equipped with indwelling catheters in the femoral vein and in the upper lumbar region of the spinal column. Morphine sulphate pentahydrate was dissolved in 0.9% saline and injected epidurally into the caudal space of the spinal column. Blood (3—5 ml per sample) was drawn for 6 h and CSF was continuously collected for 24 h. The samples were processed as described above.

RESULTS AND DISCUSSION

Determination of the electrochemical cell potential

The hydrodynamic voltammogram was established with two concentrations of morphine sulfate (Fig. 1). A voltage range was determined from a cyclic voltammogram of morphine sulfate dissolved in the mobile phase. The cyclic voltammogram contained an oxidation wave between +0.6 and +0.8 V using a pair of platinum electrodes. Allowing for different oxidation responses between platinum and glassy carbon electrodes, the potential range was expanded from +0.5 to +0.9 V for construction of the hydrodynamic voltammogram. The optimum applied cell potential was found to be +0.79 V.

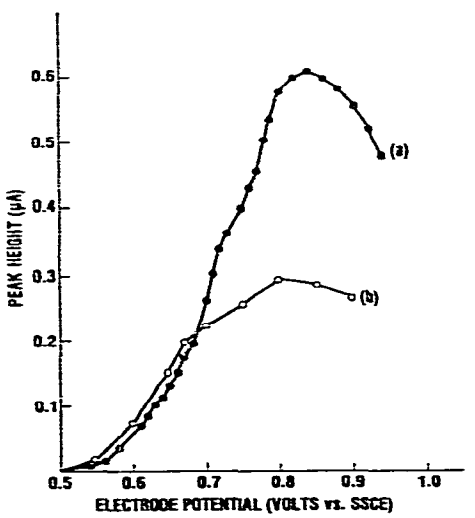

Fig. 1. Hydrodynamic voltammograms for morphine sulfate dissolved in the mobile phase at concentrations of (a) 1.32 μM and (b) 0.65 μM. Chromatographic conditions: column, μBondapak C_{18}; mobile phase, NaH_2PO_4 (0.07 M) with Na_2EDTA (0.5 mM)—methanol—acetonitrile (87:8:5, v/v), pH 4.5; flow-rate 1.0 ml/min; electrode potential, +0.79 V vs. Ag/AgCl reference electrode (SSCE); temperature, ambient.

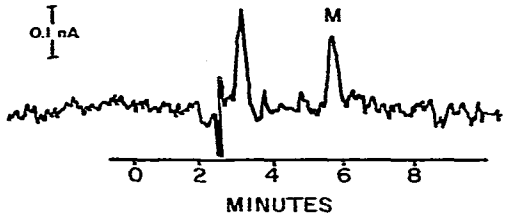

Fig. 2. Chromatogram of morphine sulfate (20 pg) injected into the HPLC system. Signal-to-noise ratio equals 4. Chromatographic conditions as in Fig. 1.

Sensitivity

The minimum amount of morphine sulfate detected by the electrochemical cell was 20 pg (signal-to-noise ratio 4, Fig. 2). When extracted from plasma (0.4 ml) the minimum quantifiable concentration of morphine was 1 ng/ml.

Electrochemical response

The glassy carbon electrode produced a linear response using a range of 20 pg to 10 ng of morphine sulfate when operated at the applied potential of +0.79 V. The electrode has remained stable for over five months without a significant decrease in sensitivity (<5%). However, an anomalous response to morphine sulfate was obtained with the glassy carbon electrode. A maximum cell response was reached at an applied potential of +0.84 V which decreased as the applied potential increased (Fig. 1). A similar electrochemical response is reported for the pesticide Aminocarb [11]. This hysteresis is reported to be the result of pacification of the electrode by polymerization of oxidation products onto its surface at the more positive potentials. At a lower concentration of morphine the hysteresis is still present but is not as pronounced. This observed phenomena may be due to less materials at the electrode surface.

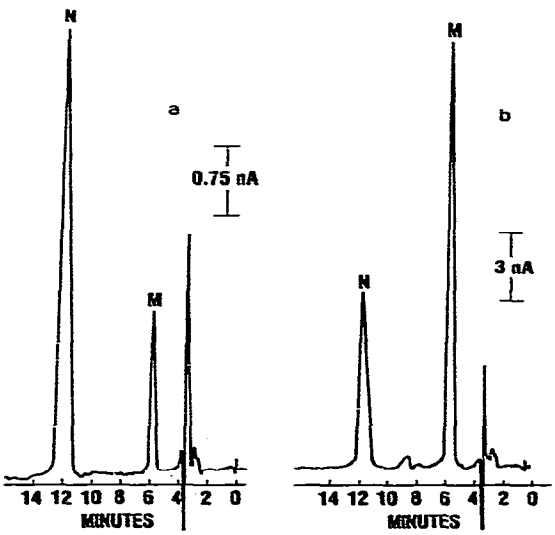

Fig. 3. Chromatograms of plasma (0.4 ml) extracted by method I: (a) plasma spiked with morphine (M, 10 ng/ml) and nalorphine (N, 25 ng) and (b) CSF drawn from a monkey 16 h after epidural administration of morphine sulfate (morphine concentration calculated to be 57.2 ng/ml). Chromatographic conditions as in Fig. 1.

Chromatography

Chromatogram obtained from samples of plasma and CSF after extraction (method I or II) show that both methods provide samples which are relatively free from interfering peaks (Figs. 3 and 4). Under the chromatographic conditions specified morphine and nalorphine eluted at 5.6 and 11.7 min, respectively; therefore a sample can be injected every 15 min.

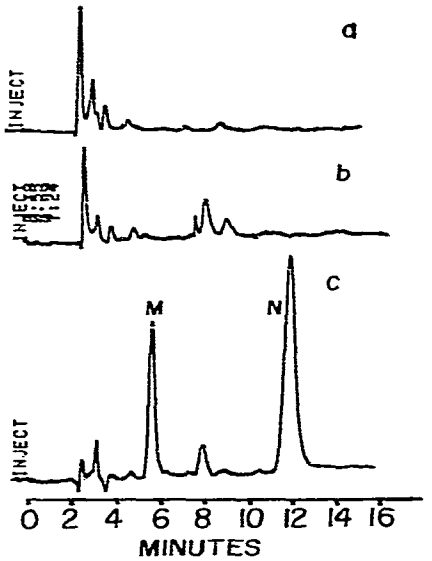

Fig. 4. Chromatograms of samples (0.4 ml) extracted by method II: (a) drug-free CSF, (b) drug-free plasma and (c) plasma containing morphine (M, 47 ng/ml) and nalorphine (N, 25 ng). Chromatographic conditions as in Fig. 1.

Extraction efficiency

Recoveries of morphine and nalorphine for each method were determined by extracting a plasma sample containing 100 ng/ml of morphine sulfate and 50 ng/ml of nalorphine hydrochloride. Six aliquots were assayed by each method and the peak heights compared to those obtained from a standard. The calculated mean recoveries for morphine and nalorphine were respectively 78.0 ± 4.7% and 79.0 ± 3.4% for method I and 84.8 ± 4.0% and 78.4 ± 2.2% for method II. The time required to prepare samples by each method for injection was compared. Using extraction method I 6 h were required to prepare 40 samples but when using extraction method II only 1.5 h were required.

Linearity

Calibration curves derived for morphine-spiked plasma prepared by extraction methods I and II were linear over the range 1—200 ng/ml (Table I and II). Correlation coefficients were typically 0.999. Accuracy decreased for samples containing less than 10 ng/ml of morphine when the above curves were used to quantify morphine. Therefore, to obtain optimal accuracy, the range of the calibration curve was decreased to 1—20 ng/ml.

TABLE I

PEAK HEIGHT RATIOS OF MORPHINE SULFATE (M) TO INTERNAL STANDARD, NALORPHINE HYDROCHLORIDE (N) OBTAINED WITH PLASMA SAMPLES EXTRACTED BY METHOD I

Values are expressed as the mean ± standard deviation.

Morphine concentration (ng/ml)	Peak height ratio (M/N, n = 4)	
1.25	0.04 ± 0.002	
2.50	0.10 ± 0.003	
10.00	0.38 ± 0.035	
20.00	0.71 ± 0.019	
50.00	1.75 ± 0.056	
100.00	3.41 ± 0.218	
200.00	6.84 ± 0.293	
	Range of curve (ng/ml)	
Linear regression analysis	1.25—20	1.25—200
Coefficient of correlation	0.999	0.999
Slope	0.035	0.034
y-Intercept	0.008	0.021

TABLE II

PEAK HEIGHT RATIOS OF MORPHINE SULFATE (M) TO INTERNAL STANDARD, NALORPHINE HYDROCHLORIDE (N) OBTAINED WITH PLASMA SAMPLES EXTRACTED BY METHOD II

Values are expressed as the mean ± the standard deviation.

Morphine concentration (ng/ml)	Peak height ratio (M/N, n = 4)	
1.07	0.02 ± 0.003	
5.60	0.10 ± 0.014	
9.90	0.18 ± 0.037	
22.80	0.36 ± 0.026	
47.60	0.71 ± 0.054	
104.00	1.51 ± 0.042	
200.00	3.00 ± 0.045	
	Range of curve (ng/ml)	
Linear regression analysis	1.07—22.80	1.07—200.00
Coefficient of correlation	0.999	0.999
Slope	0.016	0.015
y-Intercept	0.014	0.014

Precision and accuracy

The precision obtained with method I was determined in a blind study using a set of standards prepared by an independent laboratory. The precision, ex-

TABLE III

DETERMINATION OF THE WITH-IN RUN PRECISION OF EXTRACTION METHOD I WITH PLASMA SPIKED WITH MORPHINE SULFATE

Values are expressed as the mean and coefficient of variation.

Morphine concentration (ng/ml)		Coefficient of variation (%)
Theoretical	Calculated	
2.48	2.43	9.9
18.60	18.15	1.2
92.90	91.20	2.5
174.00	174.60	2.7

pressed as the average of the relative standard deviations obtained over the concentration range 2.5—200 ng/ml was 4.1% (Table III). Using standards prepared in our laboratory, the average precision obtained with method II over the concentration range 1.07—200.00 ng/ml was 9.2% with relative standard deviations of 7.1% at 22.8 ng/ml and 17.1% at 1.1 ng/ml. Analysis of a pooled plasma sample containing a morphine concentration of 7.29 ng/ml gave a value of 7.23 ± 0.34 ng/ml by method I (n = 5) and 7.34 ± 0.30 ng/ml by method II (n = 5).

Specificity

Plasma containing morphine and internal standard was spiked with codeine, acetominophen, or acetylsalicylic acid and assayed by methods I and II to determine whether these commonly used drugs interfere with the assay. At concentrations of 1 μg/ml no interference was found in the chromatography or quantitation of morphine. Retention times for the drugs tested were 8.7 and 8.8 min, respectively for acetominophen and codeine and no peak was detected within 20 min for acetylsalicylic acid.

Animal study

To illustrate the utility of the assay, unchanged morphine was quantitated in the plasma and CSF of Rhesus monkeys following an epidural administration of morphine sulfate. Blood and CSF samples were prepared for injection by extraction method I. The results obtained for one monkey are listed in Table IV.

Morphine entered the blood and CSF within 5—30 min. The maximum concentration of morphine in plasma occurred 10—15 min after epidural administration and declined rapidly ($t_{½}$, 0.5—1 h). Morphine fell below detectable limits after 4 h. In contrast, morphine reached maximal concentration in CSF within 30—35 min and disappeared more slowly ($t_{½} > 2$ h). Morphine was still detectable in CSF 23 h after administration of the drug. This seems to correlate with the clinical observation that morphine administered epidurally in man provides pain relief for 16—24 h while the same dosage given intramuscularly provides pain relief for only 4—6 h [1, 12—14].

TABLE IV

PLASMA AND CSF MORPHINE LEVELS IN ONE RHESUS MONKEY FOLLOWING AN EPIDURAL ADMINISTRATION OF MORPHINE SULFATE (2 mg/10 kg OF BODY WEIGHT)

Plasma (0.4 ml) and CSF (0.15 ml) samples were prepared with extraction method I.

Time (h)	Morphine concentration (ng/ml)	
	Plasma	CSF
0.00	n.d.*	n.d.
0.25	35.6	n.d.
0.50	27.4	3714.0
1.00	18.6	3173.0
2.00	14.4	1261.0
4.00	9.4	553.2
8.00	n.d.	125.1
16.00	n.d.	22.0
23.00	n.s.**	10.0
24.00	n.d.	n.d.

*n.d. = None detected (<1 ng/ml).
**n.s. = No sample.

CONCLUSION

In the development of pharmacokinetic profiles for drugs many samples must be drawn from the test animal; therefore it is necessary to utilize as small a sample as possible in order to not disrupt the physiological state of the animal. The precision and accuracy obtained with extraction method I makes it a most suitable method for analyzing small volumes of CSF and plasma. Method II has similar accuracy and precision but has the advantage of a faster analysis time. Extraction method II would be very useful to forensic laboratories requiring trace morphine analyses.

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