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Ion-pair extraction and liquid chromatographic analysis of morphine in rat brain and plasma

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ABSTRACT

A highly efficient and reproducible two-step liquid–liquid ion-pair extraction technique for the isolation of morphine from biological samples is described. A rapid normal phase high-performance liquid chromatographic procedure coupled with amperometric electrochemical detection has also been developed for subsequent quantification of morphine. Extraction involves the disruption of brain tissue or plasma in methanol, centrifugation, evaporation and reconstitution in ethyl acetate containing 10 m*M* di-(2-ethylhexyl) phosphoric acid, a liquid cation-exchanger, and back-extraction into 170 m*M* orthophosphoric acid. An acidic eluent consisting of acetonitrile–76 m*M* orthophosphoric acid–ammonia buffer (pH 3.0) (15:85, v/v) in combination with a strong cation-exchange column allows complete separation of morphine and the internal standard, nalbuphine. The limit of detection for morphine is 1.3 ng on-column.

INTRODUCTION

High-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED) has arguably become the most popular technique for the determination of morphine in biological samples. White [1] was the first to employ HPLC–ED for the detection of morphine, and subsequent reports have focused on obtaining higher sensitivity or lower interferences from matrix components during analysis.

Various separation methods for the determination of morphine have been employed; however, in most instances, reversed-phase or ion-pair reversed-phase separations on C_{18}-bonded silica columns have been reported [2–5].

Major attempts have been made to simplify and improve the extraction and isolation of morphine from samples prior to analysis, while maintaining sensitivity, selectivity and adequate column protection. Similarly, the use of expensive equipment must be avoided during routine extraction in order for the method to be feasible. Early investigators [1–3,6] reported complex purification steps that involved the buffering of the initial sample (usually plasma or blood) at pH *ca.* 9.0, extraction with various organic mixtures,

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back-extraction with acid, and subsequent alkalinization and re-extraction with an organic phase.

Solid-phase extraction procedures have also been widely reported [7–12], wherein disposable columns absorb the sample prior to exposure to an eluting organic mixture that is subsequently evaporated, with the resultant residue being redissolved for injection. These methods have been shown to be highly efficient and reproducible; however, the recurring costs may be considered to be excessive, especially in instances where two cartridges are used for one sample [7,11].

There have been several attempts to determine morphine concentrations from biological tissues with little or no sample purification. Kim *et al.* [13] reported the direct injection of an aliquot of a morphine-containing supernatant following homogenization and centrifugation of brain tissue. These authors stated the necessity to replace the guard column every 250 analyses, but did not report the effect of repeated injections on the column itself. Similarly, Tagliaro *et al.* [14] described a system that allowed the direct injection of filtered and centrifuged plasma or serum. The main problem with the described method was a gradual build-up of pressure after several injections, requiring the removal and cleaning of the frit between the injector and the column. Furthermore, the method is limited to the analysis of blood or blood products.

This paper describes a procedure involving a highly selective two-step liquid–liquid extraction of morphine from rat brain and plasma, using the liquid cation-exchanger di-(ethylhexyl)phosphoric acid (DEHPA) [15,16]. In addition, a normal-phase HPLC method has been developed for the subsequent separation of morphine isolated from rat brain and plasma. The method has been used to study the time-course of the distribution of morphine after intraperitoneal administration of pharmacologically relevant doses of morphine into the rat.

EXPERIMENTAL

Material

Morphine hydrochloride was supplied by Glaxo-Macfarlane Smith (Edinburgh, UK). Nalbuphine hydrochloride was obtained from Du-Pont Pharmaceuticals (Wilmington, DE, USA). Di-(2-ethylhexyl)phosphoric acid (DEHPA) was purchased from Sigma (St. Louis, MO, USA). All solvents used were of analytical or LC standard. Water was doubly glass-distilled prior to use.

Animals

Male Glaxo Wistar rats, supplied by the Victorian College of Pharmacy Animal House, were maintained in a temperature-controlled environment (22°C) and subjected to a 12 h:12 h light/dark cycle. Food and water were available to animals *ad libitum*.

Instrumentation

Chromatography was performed using a BAS PM-60 liquid chromatograph (Bioanalytical Systems, West Lafayette, IN, USA). All samples were introduced into the column by means of a Rheodyne 7125 injector fitted with a 100-μl loop. Detection was achieved using a BAS LC-4B dual-electrode electrochemical detector fitted with a TL-5A glassy carbon working electrode (Bioanalytical Systems) operated at 0.95 V against a Ag/AgCl reference. Outputs were recorded on an Omniscribe recorder (Houston Instruments, Austin, TX, USA).

High-performance liquid chromatography

Separation of morphine was achieved by cation-exchange chromatography on a strong cation-exchange column (Whatman Partisil 10 SCX, 25 cm × 4.6 mm I.D.; 10μm) protected by an SCX Newguard pre-column cartridge (Brownlee Labs, Santa Clara, LA, USA). The mobile phase was acetonitrile–76 m*M* orthophosphoric acid–ammonia buffer (pH 3.0), (15:85, v/v). The flow-rate was 3 ml/min, and the column temperature was maintained at 28°C. To ensure baseline stability, the mobile phase was continuously recycled and freshly prepared fortnightly.

Preparation of morphine hydrochloride for injection

Morphine hydrochloride was dissolved in isotonic saline to give a final concentration of 10 mg/ml (8.9 mg/ml of morphine free base).

Procedure for the extraction of morphine from brain tissue and plasma

Animals were sacrificed by decapitation at 0 (control), 5, 10, 15, 30, 60 and 120 min following the intraperitoneal administration of 10 mg/kg of morphine HCl (n = 4 or 5 for each time interval). Trunk blood was collected into 10 ml Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) containing 143 USP units of lithium heparin, and immediately mixed. Plasma was obtained by centrifugation at 3000 g for 10 min at 4°C in a Sorvall 6000B refrigerated centrifuge. The brain was rapidly removed and placed at 0°C. Regions of the brain to be examined were then rapidly dissected according to the method of Glowinski and Iversen [17].

An accurately weighed sample of brain tissue (50–100 mg) or plasma (0.1 ml) was placed in a 10-ml polypropylene Sorvall centrifuge tube, and 2.6 ml methanol containing 30 ng/ml of the internal standard nalbuphine hydrochloride was added. The brain tissue was initially homogenized in the centrifuge tube using a closely fitting 13 mm O.D. teflon pestle (Thomas Scientific, Svedesbaro, NJ, USA). The brain homogenate or plasma–methanol mixture was then sonicated using a Soniprep 150 Ultrasonic Disintegrator (MSB Scientific Instruments, Sussex, UK). The probe was operated at an amplitude of 23 micrometer for 60 s. After disruption of the tissue, the homogenates were centrifuged at 15 000 g in a Sorvall high-speed centrifuge, Model RC-2B (DuPont, Newtown, CN, USA) for 30 min at 4°C. The supernatant was then transferred to a 10-ml conical glass centrifuge tube and evaporated to dryness in a Jouan RC 1010 centrifugal evaporater linked to a Dynavac high-vacuum freeze-drying unit (Dynavac, Melbourne, Australia). The residue was reconstituted in 3 ml of ethyl acetate containing 10 mM DEHPA. Following washing with 1 ml of 50 mM sodium phosphate buffer (pH 7.0), the tube was centrifuged at 3000 g at 4°C for 10 min. A 2.8-ml aliquot of the organic phase was transferred to a 10-ml conical glass centrifuge tube containing 500 μl of 170 mM orthophosphoric acid. After vortex mixing for 30 s, the tube was centrifuged at 3000 g at 4°C for 10 min. The organic layer was carefully removed and discarded. The pH of the aqueous phase was adjusted to 2.5–3.0 by the addition of 24 μl 11.2 M ammonia solution. A 100-μl aliquot of the aqueous phase containing morphine was then injected into the HPLC column.

Preparation of standard extracts

Samples (50–100 mg) of brain tissue (including cortex and corpus striatum) and plasma (0.1 ml) obtained from saline-pretreated control animals were placed in polypropylene Sorvall centrifuge tubes. The tissue samples were spiked with amounts of morphine hydrochloride in the range 0–26 ng (0–23 ng of morphine free base). Following the addition of the internal standard nalbuphine hydrochloride, the tissues were homogenized and the morphine extracted as described above. Overall percentage recoveries were determined by a comparison of the peak heights with those of appropriate standards.

RESULTS AND DISCUSSION

High-performance liquid chromatography

Chromatography performed on a strong cation-exchange column permitted the complete separation of morphine and the internal standard nalbuphine with an overall retention time of less than 7 min (Fig. 1). To determine the optimum conditions for the detection of morphine under the chromatographic conditions described, a hydrodynamic voltammogram was recorded following separation of 23 ng of morphine base (Fig. 2). The signal-to-noise (S/N) ratio was then determined at selected potentials. To achieve optimum sensitivity (maximum S/N ratio) ED was routinely carried out at 0.95 V. Attempts to use higher voltages, while resulting in a minor improvement in the S/N ratio, resulted in unacceptable baseline drift.

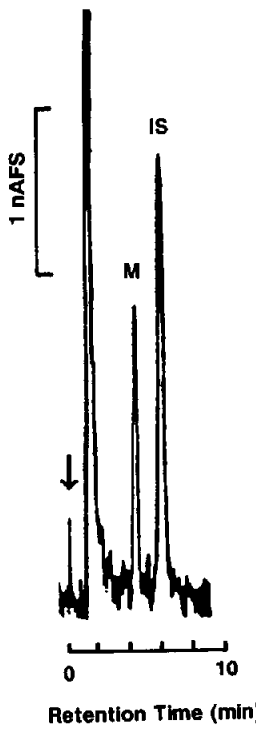

Fig. 1. Chromatogram illustrating the separation of morphine and the internal standard nalbuphine, on a Whatman strong cation-exchange column. A 60-μl aliquot of a mixture containing 100 ng/ml of morphine hydrochloride and 300 ng/ml of nalbuphine hydrochloride in 170 mM orthophosphoric acid was introduced into the column. HPLC was performed as described in Experimental. The time elapsed for the detection of both morphine and the I.S. is less than 7 min.

Sequential analysis of five 100-μl aliquots of a standard solution of morphine 35 ng/ml in 500 mM orthophosphoric acid (3.5 ng on-column) yielded coefficients of variation (C.V.) for the determination of morphine of less than 3.9%.

Extraction of morphine from brain and plasma

Protein precipitation and the initial extraction of morphine from 50–100 mg samples of brain tissue and from plasma (0.1 ml or 0.5 ml) were achieved by the disruption of samples of tissue in methanol (a water-miscible solvent in which morphine is known to have a high solubility) using a combination of homogenization and sonication. Following removal of the methanol by evaporation, the residue was resuspended in 10 mM DEHPA in ethyl acetate.

Morphine was isolated from the residue by ion-pair extraction using the liquid cation-exchanger DEHPA. In subsequent stages of the purification, polar impurities were removed by washing the organic phase with 50 mM sodium phosphate buffer (pH 7.0). The back-extraction of morphine from the organic phase, and its concomitant purification from non-polar impurities, was finally achieved using aqueous 170 mM orthophosphoric acid (500 μl). Following neutralization of the orthophosphoric acid with ammonia solution, aliquots (100 μl) of the aqueous phase containing morphine were injected directly into the HPLC system.

Optimization of the conditions for the ion-pair extraction of morphine

The optimum concentrations of DEHPA and orthophosphoric acid for the ion-pair extraction of morphine both from brain tissue extracts and from control samples processed in the absence of added tissues were systematically determined. The studies indicated that, in the absence of tissue, essentially quantitative overall recoveries of 107 ± 1.7% (mean ± S.E.M.; n = 3) of added morphine could be achieved using *ca.* 5 mM DEHPA (Fig. 3). It was found that, during the back-extraction of morphine from the organic phase, a concentration of 85 mM orthophosphoric acid (500 μl) was sufficient to ensure quantitative overall recoveries of morphine (Fig. 4).

In the presence of 100 mg of brain tissue, optimum recoveries were also attained at similar concentrations of DEHPA and orthophosphoric acid. However, in the presence of tissue, a significant ($p < 0.05$) reduction in the overall recovery of morphine was evident. It was noted that the addition of increasing concentrations of either the ion-pairing agent or the acid used in back-extraction did not further improve the overall recovery of morphine (Figs. 3 and 4).

The concentrations of DEHPA and orthophosphoric acid routinely selected for extraction of morphine from both brain tissue and plasma were 10 mM and 170 mM (500 μl), respectively.

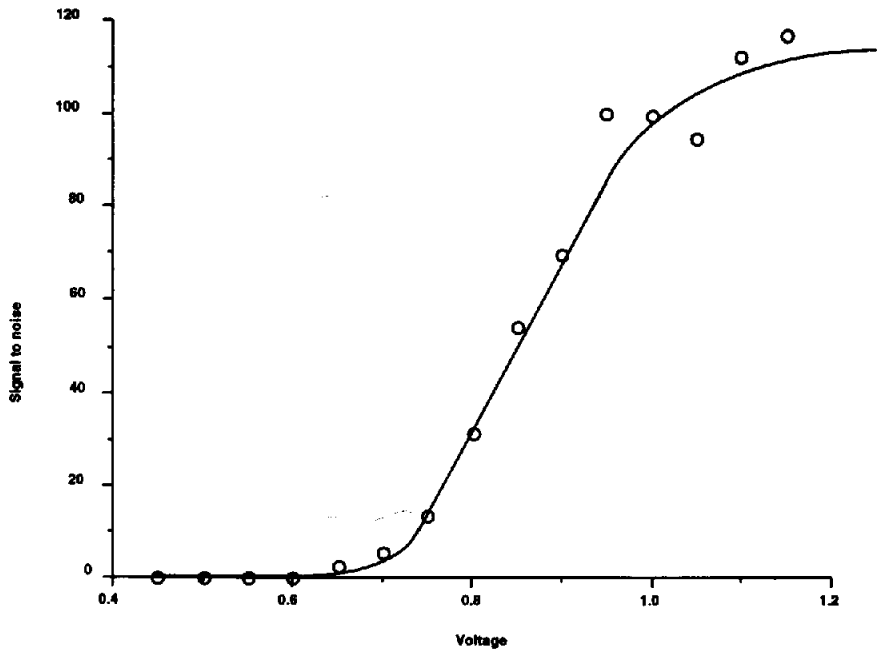

Fig. 2. Hydrodynamic voltammogram of morphine following its separation on a Whatman strong cation-exchange column. Aliquots (80 μl) of a standard solution of morphine hydrochloride were introduced into the column. Chromatograms were recorded at various potentials in the range 0.45–1.15 V, and the relationship between the signal-to-noise ratio for the detection of morphine and the applied potential was determined.

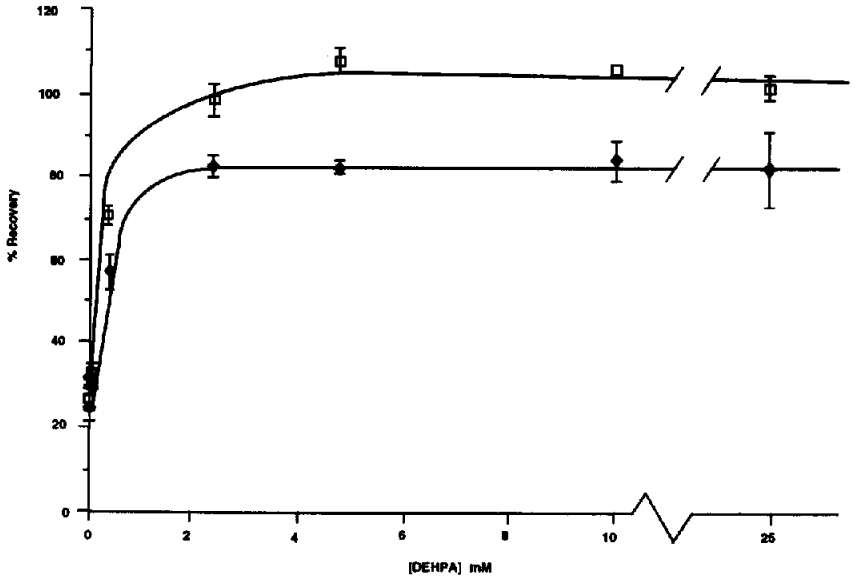

Fig. 3. Effect of the concentration of DEHPA on the overall efficiency of extraction of morphine in the absence (□) and in the presence (◆) of brain tissue. Samples (100 mg) of cortex spiked with 92 ng of morphine free base were extracted with 3.0 ml of ethyl acetate containing a range of concentrations of DEHPA. The back-extraction of morphine from the organic phase was achieved using 500 μl of 170 m*M* orthophosphoric acid. The overall efficiency of extraction of morphine was determined by comparison of the concentration of morphine recovered in the final extract with those of appropriate standards.

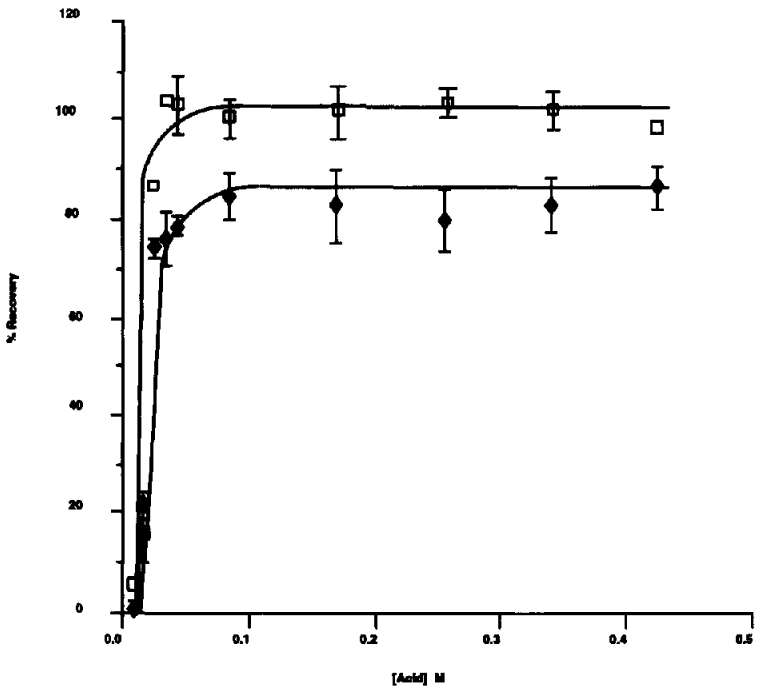

Fig. 4. Effect of the concentration of orthophosphoric acid on the overall efficiency of extraction of morphine in the absence (□) and in the presence (◆) of brain tissue. Samples of cortex (100 mg) spiked with 92 ng of morphine free base were extracted with 3.0 ml of ethyl acetate containing 10 m*M* DEHPA. Subsequent back-extraction was performed using 500 μl of various concentrations of orthophosphoric acid. The overall efficiency of extraction of morphine was determined by comparison of the concentration of morphine recovered in the final extract with those of appropriate standards.

This selection took into account the possibility that the amount of morphine present in tissues from treated animals may occasionally exceed the amount used during the study (92 ng). The overall recoveries of morphine from brain tissue and from plasma using the method described was 85% with C.V. values of 6.3% (n = 12). The average recovery of the internal standard, nalbuphine, from tissue samples was 96.0 ± 0.9% (mean ± S.E.M.; n = 12).

Establishment of standard curves for the quantitation of morphine in brain and plasma extracts

Brain tissue samples from untreated animals (100 mg) spiked with the I.S. (260 ng) and morphine hydrochloride in the range 0–130 ng (0–116 ng of morphine free base) were extracted and analysed as described above. The relationship between the peak-height ratio (morphine/I.S.) for processed standards, extracted in either the presence or the absence of tissue, and the amount of morphine added was found to be linear within the range of concentrations examined. The equation and coefficient of correlation for the calibration curve were $y = 0.009x - 0.003$ (r = 0.998). In view of the significant differences observed between the recoveries obtained in the presence and the absence of tissue, all quantitation of morphine concentrations in brain and plasma extracts was conducted with reference to standards processed in the presence of brain tissue or plasma where appropriate. The C.V. of the slope of the standard curve obtained from standards extracted from brain tissue on five consecutive days was 5.8%. The slope of the standard curve from extracted plasma samples determined on three consecutive days had a C.V. of 8.6%.

Chromatograms illustrating the analysis of an extract from the plasma and cortex of control animals not treated with morphine are presented

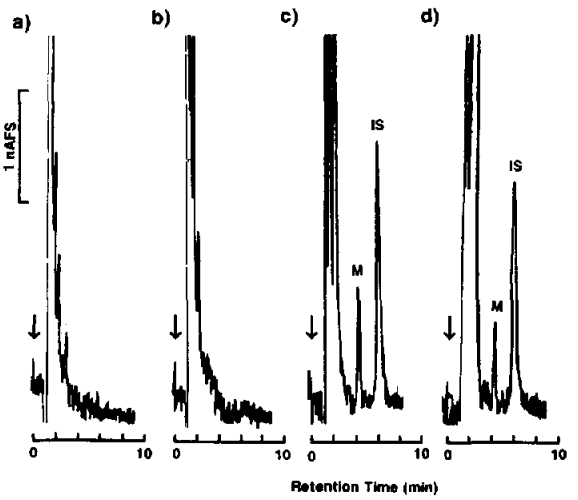

Fig. 5. Chromatograms obtained following the separation of rat tissue extracts prepared from (a) a sample of plasma from a saline-injected animal, (b) a sample of cortex from a saline-injected animal, (c) a sample of cortex from a control animal spiked with authentic morphine (13.8 ng), and (d) a 100-mg sample of cortex obtained from an animal 2 h after the i.p. administration of 10 mg/kg of morphine hydrochloride. The amount of I.S. used was 78 ng; the peak obtained in the treated rat corresponds to 123 ng/g of morphine.

in Fig. 5a and b. Also presented is the chromatogram obtained from the extract of the cortex from a control untreated animal spiked with 13.8 ng of morphine base (Fig. 5c). A chromatogram obtained following the analysis of a similar extract, prepared from rat cortex 120 min after the intraperitoneal (i.p.) administration of 10 mg/kg of morphine hydrochloride, is presented in Fig. 5d. These chromatograms illustrate that there are no co-eluting compounds that might interfere with the determination of either morphine or the I.S.

The minimum detectable limit for morphine under the conditions described (determined two days after polishing the glassy carbon electrode) was 1.3 ng on column.

Analysis of morphine in brain tissue and plasma extracts

An application of the current method can be demonstrated by the pharmacokinetic study of morphine, following administration of a single i.p. dose of 10 mg/kg of morphine hydrochloride (Fig. 6). The peak level of morphine in plasma, 1211 ± 102.7 ng/ml (mean ± S.E.M.; $n = 4$), occurred 5 min following injection. In comparison, the peak level of morphine in the cortex was 181.2 ± 11.0 ng/g (mean ± S.E.M.; $n = 5$), 60 min following morphine administration. A measurable level of morphine was still present in plasma and cortex at 120 min.

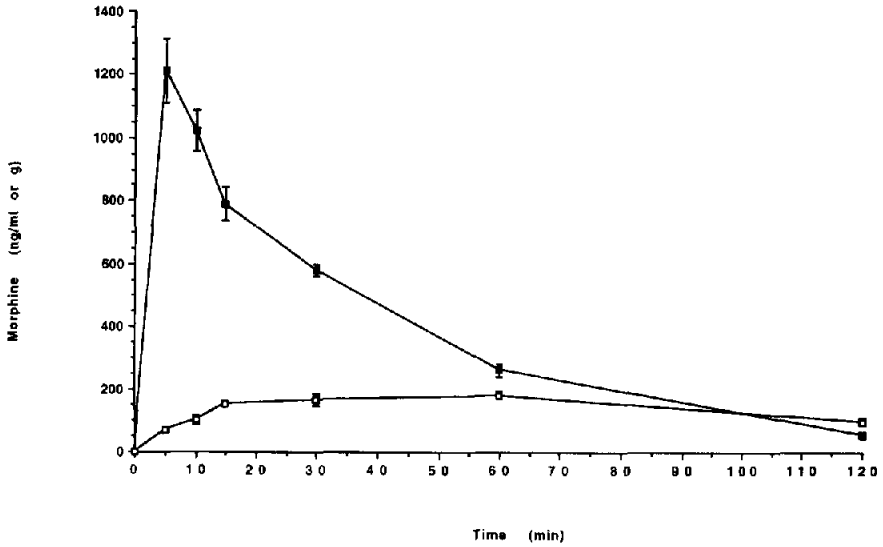

Fig. 6. Time course of morphine concentration in plasma (■) and cortex (□) of rats, expressed as ng/ml or ng/g. Morphine hydrochloride was injected intraperitoneally in a dose of 10 mg/kg. Results are the mean ± S.E.M. for four or five rats at each time interval.

CONCLUSION

The method described here for the liquid chromatographic analysis of morphine has proved to be simple, selective and reproducible. The presence of the ion-pairing agent, DEHPA, in the organic phase of the extraction procedure enables the sequence of events involving the preliminary acid back-extraction, alkalinization and organic re-extraction steps commonly used by previous authors to be omitted. Clearly, the ion-pairing of DEHPA to morphine is not exclusive; however, the presence of polar impurities will be drastically reduced compared with methods that do not use such an agent.

The selectivity involved with the extraction method is reflected by the simplicity and efficiency of the HPLC procedure. The use of a cation-exchange column and a simple acidic mobile phase results in clear separation of the injected components, allowing for a high flow-rate and short retention times for both morphine and nalbuphine. As a consequence, it is possible to analyse a large number of samples on a routine daily basis.

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