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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR MORPHINE WITH ELECTROCHEMICAL DETECTION USING AN UNMODIFIED SILICA COLUMN WITH A NON-AQUEOUS IONIC ELUENT

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SUMMARY

A quick, sensitive and accurate high-performance liquid chromatographic method for morphine measurement in plasma has been developed using an unmodified silica column with a non-aqueous ionic eluent. Small plasma samples (0.4 ml) were extracted with a liquid–liquid extraction column and dihydromorphine was used as internal standard. The limit of quantitation was 2 ng/ml. The electrochemical detector was capable of detecting 150 pg of morphine. A calibration line with a correlation of 0.9994 ± 0.00026 was produced over the range 2—80 ng/ml.

INTRODUCTION

The pharmacokinetics of morphine are not fully elucidated and, due to its increasing use in treating chronic pain and as a drug of abuse, methods to measure it in biological fluids are of considerable interest for scientific, clinical and forensic purposes.

Radioimmunoassays can detect picogram quantities of morphine [1], but they often lack specificity due to cross-reaction of the antibodies with, for example, morphine-3-glucuronide, diamorphine and/or codeine [2]. Spectrophotofluorimetric and radiolabelled morphine assays have the same defect [3]. Until recently the only suitable methods for the selective measurement of morphine were (a) high-performance liquid chromatography (HPLC) with fluorescence detection [4] and (b) gas chromatography with either flame-ionization detec-

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tion [5], electron-capture detection [6] or mass spectrometry [7]. These methods involve relatively complex and long analytical procedures and some lack sensitivity.

White [8] was the first to report an HPLC assay involving morphine detection by means of electrochemical oxidation. Since then, a number of articles based on this technique (see Table II) have been published, reporting modifications and improvements in sensitivity and extraction methods. All make use of aqueous (methanolic) eluents and, excluding that of White [8] and a modified method by Bedford and White [9], rely on the use of C_{18} bonded-phase columns. One reason for this could be that a slightly better peak shape was obtained with these columns as compared with silica columns, which have a tendency to give tailing peaks under acidic and neutral conditions. The use of an unmodified silica column in combination with a non-aqueous ionic eluent has the advantage of providing a more flexible system for the analysis of various basic drugs [10], reducing baseline noise and allowing the use of higher electrode potentials if necessary. We undertook to develop such a method using an internal standard which should not be present in patient samples and a reproducible extraction procedure, which was similar to that of Todd et al. [11].

EXPERIMENTAL

Reagents and materials

Methanol and hexane were HPLC grade and glacial acetic acid and ammonia solution (35%) were Aristar grade. Sodium bicarbonate, sodium hydroxide, chloroform and isopropanol were Analar grade. The chemicals were purchased from BDH (Poole, U.K.). Clin-Elut CE1001 extraction columns were obtained from Analytichem International (Harbor City, CA, U.S.A.) and the 10-ml conical glass tubes were silanized with a 5% solution of dichlorodimethylsilane in toluene. Eppendorf 3810 test tubes were used.

Instrumentation and chromatographic conditions

The equipment used for this assay was a Hewlett-Packard 1081B high-performance liquid chromatograph, equipped with an oven, a one-piston pump and a variable-volume injector. A 25 cm × 4.5 mm I.D. Spherisorb S5W (5 μm) silica column was used (Jones Chromatography, Llanbradach, U.K.). A long (7.5 m) stainless-steel capillary tube was placed between the pump and the column to act as a pulsedamper. The HPLC system was combined with an LCA15 electrochemical detector (EDT Research, London, U.K.), equipped with a glassy carbon electrode. The potential was set at +0.75 V versus a reference electrode (Ag/AgCl) and the sensitivity was set at 10 nA full scale. The response signals were recorded on a Yokogawa 3047 chart recorder and processed by a Hewlett-Packard 3392A integrator. The pH of the different eluents investigated was measured with a Corning 145 pH meter equipped with a standard glass electrode and a standard calomel electrode, calibrated against aqueous buffers, and uncorrected for the fact that non-aqueous solutions were being measured.

The eluent contained 0.325 ml acetic acid (100%) and 0.55 ml ammonia

(35%) in 1 l of methanol—hexane (7:3). The pH was 8.30 (adjusted if necessary with 35% ammonia solution). The flow-rate was 2.0 ml/min and the oven temperature was 31°C.

In order to obtain a stable baseline in a short period of time (less than 0.5 h) the detector was left switched on overnight with a reduced solvent flow-rate (0.4 ml/min). When the detector was started from "cold" the potential was set at 1.0 V and left for conditioning at this potential for 1.5 h. The system was ready to be used after a further 0.5 h. When the detector was conditioned overnight this procedure was not required. The solvent was recycled when no injections were being made.

Extraction procedure

The material in the extraction column was slightly compressed with a plunger in order to achieve greater uniformity and was placed above a 10-ml conical silanized glass tube. Plasma (0.4 ml), internal standard (280 ng/ml dihydromorphine in water, 50 μl) and bicarbonate buffer (1.0 *M*; pH 9.3, adjusted with 10 *M* sodium hydroxide solution; 0.6 ml) were added to a 1.0-ml Eppendorf tube. The tube was closed, shaken gently and the contents were transferred onto the column and allowed to absorb for 3 min, after which 1.0 ml of the extraction solvent (chloroform—isopropanol, 95:5) was added. A further portion of the extraction solvent (2 ml) was added after 3 min and the column was tapped gently to remove any trapped air bubbles. Another portion of extraction solvent (3 ml) was added after a further 5 min and once elution was complete, the eluate was evaporated under stream of filtered air in a waterbath (55°C). The residue was redissolved in methanol (100 μl) and the solution was transferred to a vial for automatic injection (50 μl).

Instrument calibration

The standards (2, 5, 10, 20, 40, 80 ng/ml) were prepared in pooled human plasma (six drug-free volunteers) and were stored at −40°C in 0.5-ml aliquots. The morphine-to-dihydromorphine peak height ratios were calculated by the integrator.

RESULTS AND DISCUSSION

Electrochemical detection

The phenolic group is the oxidisable moiety in morphine at lower potentials. At potentials above 0.9 V codeine also reacts. In codeine the phenolic group is etherised and therefore the electroactive group is the *tert.*-amino group. The advantage of the electrochemical detector is that it is possible to introduce a degree of selectivity. There are therefore two opposing factors. Firstly, we want as high a response as possible so a "high" voltage should be used. Secondly, we want a selective method: phenolic groups are very easily oxidised and therefore a "low" potential would be advantageous so as to exclude responses from other groups such as amines. The noise-to-peak height ratio is a useful parameter to evaluate the optimum potential. A potential of 0.75 V was found to be the optimum voltage for the assay (Table I).

TABLE I

NOISE-TO-PEAK HEIGHT RATIOS OF INJECTIONS OF 1 ng MORPHINE IN METHANOL AT DIFFERENT POTENTIALS

Apart from the applied potential see text for the chromatographic conditions.

Applied potential (V)	Noise-to-peak height ratio (%)
0.80	14 0
0.75	5.0
0.70	5.7

Optimisation of the chromatographic system

A number of different eluents with varying amounts of supporting electrolyte were investigated. They were combinations of methanol and aqueous buffers, chloroform or hexane. It was observed that the presence of water in the eluent caused an increase in noise-to-peak height ratio and that the latter was also inversely proportional to the ionic strength of the eluent at low levels (below 20 m*M*). However, an increased ionic strength resulted in a decreased retention time.

A retention time of 4.5 min was thought desirable to achieve satisfactory resolution from the solvent front. The ionic strength and pH were found to be very critical. Flanagan and Jane [12] found that the pK_a values of basic analytes predicted the pH of maximum retention. This was found to be true for morphine which has a pK_a of 8.1 [13]. An increase in pH from neutral to basic values decreased the retention time considerably. Changes in pH not only alter the ionic state of morphine but also the ionisation state of the silica silanols. At pH values higher than the pK_a value the retention time rapidly became shorter. The explanation is that non-protonated basic analytes are not easily retained on silica, especially when the latter is itself ionized and is repulsing negatively charged phenolic moieties. When the pH was decreased from neutral to acidic values the retention time also shortened, but the changes were not as pronounced as those observed at basic pH values.

In order to dampen the noise caused by the pumpstrokes a high flow-rate (2.0 ml/min) was used. A constant temperature was found to be essential in order to obtain a stable baseline. The oven set at 31°C served this purpose very well.

The eluent finally chosen, together with the chromatographic conditions indicated (see above), gave an elution time of 5.0 min for morphine and 10.8 min for dihydromorphine. The buffering capacity of the eluent prevented a possible drift in retention time due to absorption of atmospheric carbon dioxide by the basic solvent.

Chromatograms of a standard and sample extract are illustrated in Fig. 1. The volunteer (a healthy female) was injected with 5 mg morphine intravenously and a plasma sample was taken 2 h later. No interfering peaks were found in fifty plasma samples taken from seven volunteers, who received this dose of morphine (5 mg, intravenously). There was a peak at ca. 14.5 min, which was due to a contaminant eluted from the polyethylene of the Clin-Elut extraction column. Addition of hexane to the eluent (30%) was found necessary

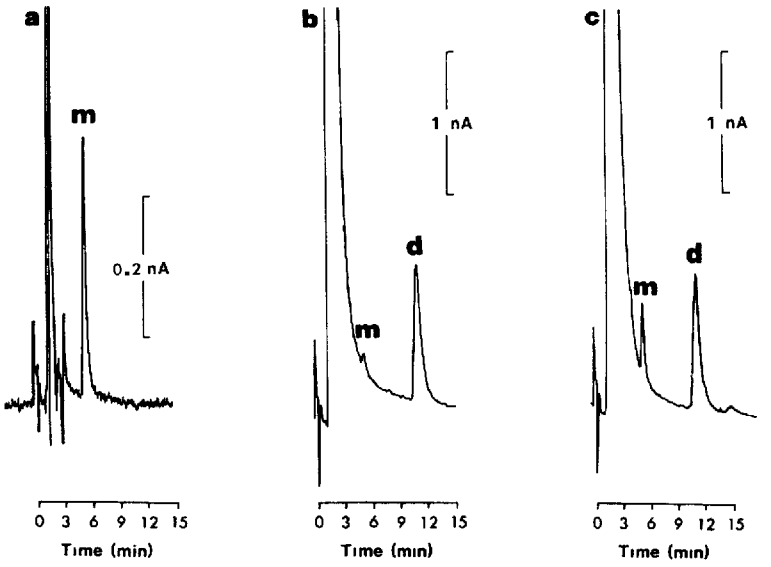

Fig. 1. (a) Chromatogram of 1 ng morphine (m) in methanol. (b) Chromatogram of an extracted plasma standard (0.4 ml) containing morphine (m, 2 ng/ml) and dihydromorphine (d, 35 ng/ml) as internal standard. (c) Extraction of a plasma sample (0.4 ml) from a volunteer containing morphine (m, 9.8 ng/ml) and dihydromorphine (d, 35 ng/ml) as internal standard. For chromatographic conditions see text.

in order to avoid another contaminant from the extraction columns interfering with the morphine peak. This interfering peak was moved forward into the solvent front by adding hexane. Addition of hexane also reduced the baseline noise by 15—20%, without affecting the peak heights.

A comparison is made between the current method and previously reported HPLC assays for morphine with electrochemical detection (Table II).

Internal standard

An internal standard was sought which would be unlikely to be found in patient samples. Compounds such as normorphine, a metabolite of morphine, dextrorphan, a cough suppressant, nalorphine and naloxone, both narcotic antagonists, were therefore unsuitable. Dihydromorphine, which is not in therapeutic use, was found to be ideal for our chromatographic system.

The retention characteristics of dihydromorphine were similar to those of morphine and there were no peaks present in plasma extracts which interfered with it. The response at the electrochemical detector at +0.75 V was close to that of morphine and the extraction characteristics were also very similar (Fig. 2).

A fractionated extraction was performed on a plasma sample containing 32 ng morphine and 80 ng dihydromorphine. The experiment showed that 6 ml of solvent were sufficient to achieve complete elution of both compounds from the extraction column (Fig. 2). Only 5 ml were actually collected in the tube for evaporation, as the last 1 ml was not eluted from the column.

TABLE II

HPLC ASSAYS FOR MORPHINE WITH ELECTROCHEMICAL DETECTION

Reference	Column	Eluent	Flow-rate (ml/min)	Internal standard	Applied voltage (mV)	Detection limit	Sample size
Owen and Sitar [14]	μBondapak C_{18}	Methanol—water—ammonium hydroxide (50:50:0.1)	1.3	Normorphine	650	1 ng/ml plasma	0.5 ml plasma
White [8]	Silica	Methanol—ammonium nitrate buffer pH 10.2 (90:10)	1.0	Dextrorphan	600	0.5 ng injected	0.5 ml plasma
Vandenberghe et al. [15]	μBondapak C_{18}	Methanol—70 m*M* sodium phosphate buffer pH 5.8 (36:64) containing 0.5 m*M* heptane sulphonic acid	1.0	5-Hydroxy-quinoline	600	1 ng/ml plasma	0.1 ml serum
Bedford and White [9]	Spherisorb silica	(0.05 *M* perchloric acid pH 9.0 with sodium hydroxide)—(methanol—acetonitrile, 9:1) (3:7)	1.5	Dextrorphan	1100	250 pg	0.5 ml blood
Kim and Kats [16]	Ultrasphere ODS	(0.05 *M* citric acid—0.1 *M* Na_2HPO_4, 3:2)—methanol (80:20)	1.5	Nalorphine	800	1—2 ng/ml	0.1 ml serum
Peterson et al. [17]	μBondapak C_{18}	Methanol—water (40:60) containing 50 m*M* tetramethyl ammonium hydroxide pH 6.1 with phosphoric acid	2.0	Nalorphine	800	"100 pg"	1.0 ml serum
Wallace et al. [18]	LiChrosorb ODS	Methanol—0.01 *M* potassium phosphate buffer (85:15)	1.0	Nalorphine	1000	1 ng/ml plasma	2.0 ml plasma
Raffa et al. [19]	RSilC$_{18}$HL	Methanol—water (25:75) containing 50 m*M* tetramethyl ammonium hydroxide pH 6.1 with phosphoric acid	1.5	Naloxone	600	Not given	Rat brain
Ishikawa et al. [20]	Ultrasphere ODS	0.05 *M* citrate buffer pH 4.25 containing 1% tetrahydrofuran	0.85	3,4-Dihydroxy-benzylamine	725	1 ng injected	Rat brain
Todd et al. [11]	μBondapak C_{18}	0.07 *M* KH_2PO_4, 0.5 *M* EDTA, 5% acetonitrile, 8% methanol	1.0	Nalorphine	790	20 pg injected, 1 ng/ml plasma	0.4 ml plasma
Current method	Spherisorb silica	1 l methanol—hexane (7:3), 0.325 ml acetic acid (100%), 0.55 ml ammonia (35%) pH 8.30	2.0	Dihydromorphine	750	150 pg injected, 1—2 ng/ml plasma	0.4 ml plasma

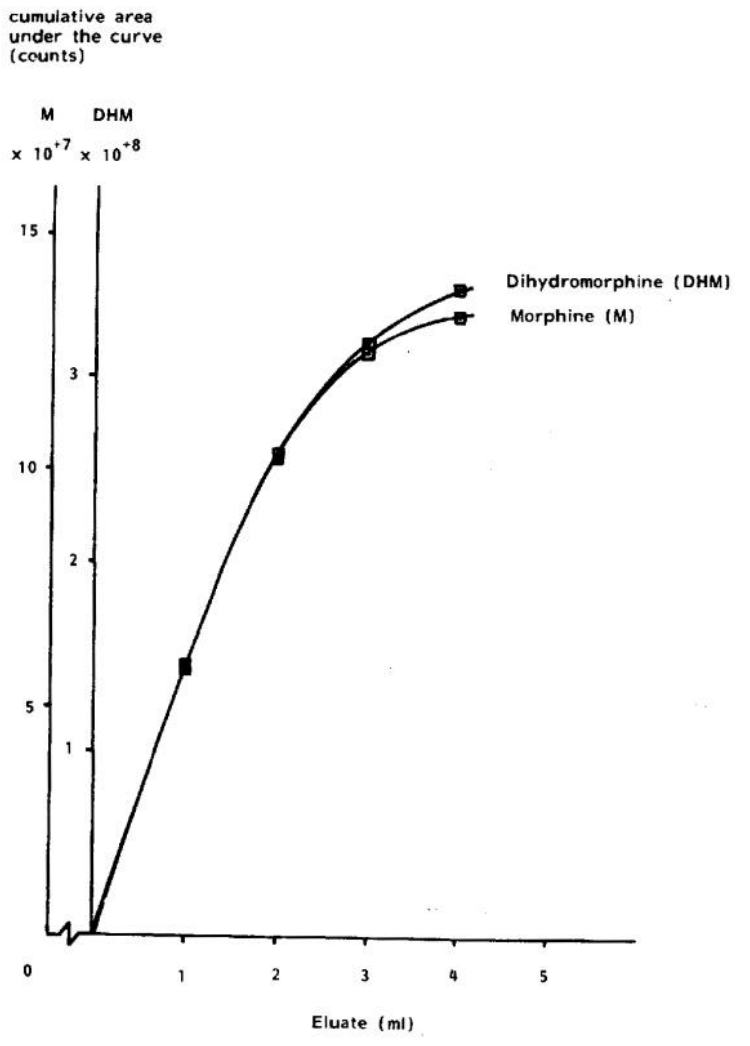

Fig. 2. Extraction profile of morphine (M, 32 ng) and dihydromorphine (DHM, 80 ng) using a Clin-Elut CE1001 extraction column with chloroform—isopropanol (95:5) as eluent. The area under the peaks was calculated by the integrator.

Extraction efficiency

The extraction efficiencies were determined by extracting a plasma sample containing 20 ng/ml morphine and 35 ng/ml dihydromorphine. The recoveries of morphine and dihydromorphine were 91.0 ± 4.9% (n=3) and 84.8 ± 6.7% (n=3), respectively. $x \pm dx$ were calculated from: $\frac{dx}{x} = \frac{dx_1}{x_1} + \frac{dx_2}{x_2}$ where dx_1 is the standard deviation of value from extraction method and dx_2 the standard deviation of value from methanol solution.

Sensitivity

The detection limit for morphine based upon the amount of material injected was 150 pg (signal-to-noise ratio = 3). This meant that the present assay

could certainly compete in this respect with the established methods (Table II). When extracted from plasma (0.4 ml), using a Clin-Elut CE1001 column, the minimum quantifiable concentration of morphine was 2 ng/ml.

Precision

The (inter-assay) relative standard deviations obtained were 4.4% for 2 ng/ml morphine in plasma (n=5) and 2.7% for 80 ng/ml morphine in plasma (n=4). The precision of the current method compared favourably with those reported previously (Table III).

TABLE III

COMPARISON OF RELATIVE STANDARD DEVIATIONS (R.S.D.) OF A NUMBER OF DIFFERENT MORPHINE HPLC ASSAYS

Reference	Morphine concentration (ng/ml)	R.S.D. (%)	Sample size (n)
Todd et al. [11]	5.6	14.0	4
Owen and Sitar [14]	2.0	31.5	?
Vandenberghe et al. [15]	25.0	7.8	12
Kim and Kats [16]	5.0	9.1	?
Wallace et al. [18]	5.0	6.5	3
Svensson et al. [21]	22.0	7.6	5
Gourlay et al. [22]	10.0	4.7	?
Current method	2.0	4.4	5

Linearity

The calibration line (consisting of eleven to fourteen points at six different concentrations over the range of 2—80 ng/ml) was linear with a regression coefficient of 0.9994 ± 0.00026 (n=3). The line started to be concave at over 80 ng/ml possibly due to electrostatic attraction between the negatively charged morphine and the positively charged working electrode, the concentration of the supporting electrolyte not having been sufficient to prevent this process.

CONCLUSIONS

The method described is accurate, sensitive and reproducible. The rapid and simple extraction procedure allows a large number of samples to be analysed in a relatively short period of time. The use of dihydromorphine as an internal standard reduces the possibility of interference from concomitantly administered opiate drugs in samples referred for clinical or forensic analysis.

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