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DETERMINATION OF NALOXONE IN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive, selective and reproducible reversed-phase high-performance liquid chromatographic method is described for the quantification of naloxone in small blood samples of premature infants. Naloxone and the internal standard, naltrexone, are extracted from alkalinized blood into diethyl ether and subsequently back extracted into 0.05% (v/v) phosphoric acid before chromatographing on a reversed-phase system. The mobile phase comprises 85 parts of acetonitrile and 15 parts of 0.06% (v/v) triethylamine in an aqueous phosphoric acid solution at pH 5 and is pumped at 1.5 ml/min. The retention times of naloxone and naltrexone were observed to be 5.4 and 7.5 min respectively. Ultraviolet detection at 214 nm enabled a limit of detection of 1 ng to be achieved. The reproducibility of the method was good at both 100 ng (C.V. = 3.4%; n = 9) and 10 ng (C.V. = 5.1%; n = 6). The high sensitivity and speed with which this assay can be performed makes it especially useful for the estimation of naloxone in small volumes (0.3—0.6 ml) of blood. It is thus particularly valuable for the determination of naloxone blood concentration—time profiles in premature infants where the minimization of the volume of blood collected is of paramount importance.

INTRODUCTION

Naloxone is a pure narcotic antagonist with no agonist activity which is commonly used to reverse narcotic-induced respiratory and cardiovascular depression following surgery and narcotic overdose [1]. In obstetric practice, parenteral administration of naloxone to mothers shortly before delivery or to

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the neonate after birth [2, 3] has been reported to reverse neonatal depressant effects of pethidine, acquired transplacentally during parturition.

Several analytical methods for the determination of naloxone in biological fluids have been described previously, including the detection by scintillation counting of radioactively labelled naloxone after its administration [4], ultraviolet (UV) spectrophotometry [5], radioimmunoassay [6] and gas–liquid chromatography (GLC) [5, 7, 8]. Only the radioimmunoassay procedure [6] and the GLC method, employing an electron-capture detector (ECD–GLC) [8], had sufficient sensitivity to enable quantification of naloxone in biological fluids after conventional doses in man. However, the radioimmunoassay [6] lacked specificity in that the antiserum reacted, not only with naloxone, but also with its reduction product naloxol. The more specific ECD–GLC method [8] suffered from the disadvantage that the perfluoroalkyl ester derivatives of naloxone and the internal standard, naltrexone, were unstable and, therefore, peak height ratios varied with time.

In view of the growing use of naloxone in premature infants and adults, a rapid, sensitive, selective and reproducible high-performance liquid chromatographic (HPLC) method has been developed for the quantification of naloxone in blood. This method involves the use of the structurally related compound, naltrexone, as internal standard (Fig. 1) and a relatively simple extraction procedure prior to chromatography.

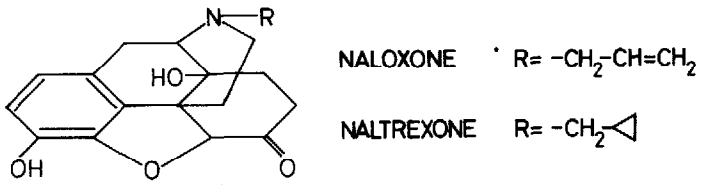

Fig. 1. Structures of naloxone and internal standard, naltrexone.

EXPERIMENTAL

Reagents and materials

Naloxone hydrochloride and naltrexone hydrochloride were gifts from Endo Labs. (Garden City, NY, U.S.A.). Aqueous standard solutions of these compounds were stored at 4°C. Triethylamine and phosphoric acid (Ajax Chemicals Unilab, Syndney, Australia) and acetonitrile (Waters Assoc., Milford, MA, U.S.A.) were used without further purification. Diethyl ether (Anaesthetic Grade, BP) was freshly distilled. Carbonate buffer, 1 *M* (pH 10.0), was prepared by dissolving 5.3 g of sodium carbonate and 4.2 g of sodium hydrogen carbonate in 100 ml distilled water. Glass tubes, cleaned in 2% (v/v) Extran 300 (Merck, Rahway, NJ, U.S.A.) were used in all steps of the analysis.

HPLC instrumentation

A Varian Aerograph (Palo Alto, CA, U.S.A.) Model 8500 HPLC pump equipped with a 100-μl loop injection system (Valco, Houston, TX, U.S.A.) and a 30 × 0.39 cm I.D. μBondapak phenyl column (10 μm) (Waters Assoc.)

was used. Naloxone and naltrexone were detected at 214 nm by means of an Altex Model 160 fixed-wavelength ultraviolet detector equipped with a zinc lamp (Altex Scientific, Berkeley, CA, U.S.A.). A single-pen recorder (Cole-Parmer, Chicago, IL, U.S.A.) with input set of 10 mV and chart speed of 20 cm/h was used.

Selection of mobile phase

An aqueous solution of naloxone hydrochloride was initially chromatographed to select a suitable mobile phase. Binary mixtures comprising varying proportions of either acetonitrile and distilled water, acetonitrile and 0.05% (v/v) phosphoric acid in distilled water or acetonitrile and 0.06% (v/v) triethylamine in distilled water acidified to pH 5.0 with phosphoric acid (triethylamine phosphate solution) were initially investigated as potential mobile phases. All aqueous components of the mixtures were passed through a 0.45-μm HA filter (Millipore, Bedford, MA, U.S.A.) prior to mixing with acetonitrile. The binary mixture which gave a desirable retention time and peak shape for naloxone was then adjusted to provide satisfactory resolution between naloxone and the internal standard, naltrexone.

The mobile phase used for quantification of naloxone in blood was acetonitrile—0.06% (v/v) triethylamine phosphate solution, pH 5 (85:15). The flow-rate was set at 1.5 ml/min resulting in an inlet pressure of approximately 34.5 bar. All chromatographic separations were carried out at ambient temperature.

Collection of biological samples

Blood samples were obtained at various times (up to 4 h) after the administration of a single intravenous dose of naloxone hydrochloride to premature apnoeic infants. Blood samples (0.3—0.6 ml) were collected by heel-prick into 1-ml heparinized plastic capillary collecting tubes (Walter Sarstedt, Edwards Labs., Sydney, Australia). A maximal aliquot of each blood sample was accurately measured and transferred to an extraction tube. The blood was then diluted with an equal volume of distilled water before freezing (—22°C). Analyses for naloxone were carried out on whole blood because sample volumes in these subjects were necessarily minimal. A sample of blood was obtained from each subject, before drug administration, to serve as control.

Extraction procedure

Diluted blood (0.6—1.2 ml) together with 100 ng of naltrexone hydrochloride (100 ng per 100 μl) as internal standard, and 250 μl of 1 *M* carbonate buffer (pH 10.0) were vortexed with 5 ml diethyl ether for 1 min. After centrifugation for 5 min at 1000 *g*, the ethereal layer was transferred to a 7-ml glass tube with a tapered base containing 100 μl of 0.05% (v/v) phosphoric acid, pH 2.35. Both naloxone and naltrexone were then back-extracted to this acidic aqueous phase by vortexing for 1 min. After further centrifuging about 80—90 μl of the phosphoric acid extract were injected directly onto the HPLC column.

Calibration and reproducibility

Control blood samples were supplemented with known quantities of

naloxone hydrochloride ranging from 1 ng to 200 ng (0.9—180 ng naloxone base) and analysed as outlined above. A calibration curve was constructed by calculating the naloxone:naltrexone peak height ratio and plotting this ratio against the amount of naloxone added to the sample. Naloxone concentrations in the unknown blood samples were determined by referring the observed peak height ratios to a calibration curve established on the same day.

The reproducibility of the method was assessed by performing the assay on replicate blood samples which contained either 100 ng (n = 9) or 10 ng (n = 6) of naloxone hydrochloride (equivalent to 90 ng and 9 ng of naloxone base, respectively).

RESULTS AND DISCUSSION

With reversed-phase HPLC, the chromatographic behaviour is usually quite predictable from the pH of the eluent and the pK_a and partition coefficient of the eluate. When naloxone was chromatographed on a reversed-phase column using a non-acidified mobile phase, it was strongly retained. A capacity factor (k') of 2 was obtained with an eluent of acetonitrile—water (85:15). When the mobile phase was acidified, a similar capacity factor was obtained with an eluent of acetonitrile—0.05% (v/v) phosphoric acid (10:90). However, broad and tailing naloxone peaks were obtained with these two systems. Asymmetric peaks are reported to be a recurrent problem in the separation of many weak organic bases [9]. This is generally attributed, at least in part, to ion-exchange or adsorption phenomena with unreacted silanol groups on the solid support. The slow kinetics of desorption from these sites are responsible for the broad and tailing peaks.

Triethylamine has frequently been incorporated into the eluent to mask the surface silanols and to competitively inhibit the adsorption of weak organic bases [9]. In this study, the incorporation of triethylamine in the eluent similarly improved the peak shapes and, consequently, the resolution of naloxone and the internal standard, naltrexone (Fig. 3b). However, improvement in naloxone peak shape was observed only when the eluent concentration of triethylamine was equal to or exceeded 0.06% (v/v). At triethylamine concentrations of less than 0.06%, a broad and tailing naloxone peak was observed. Concentrations in the range 0.06—0.2% (v/v) gave no further improvement in peak shape. This observation is presumably due to the limited adsorption capacity of the unreacted silanols which are then essentially completely occupied [10]. Thus, 0.06% (v/v) triethylamine was included in all subsequent analyses.

Lowering the pH of the triethylamine phosphate solution to 2.5 resulted in asymmetric peaks of naloxone. The peaks improved with increasing mobile phase pH up to 7.5. However, column life is shortened at elevated pH, thus pH 5 was selected as a satisfactory compromise.

The chromatographic parameters, k', α (selectivity factor) and R (resolution) for naloxone and naltrexone are summarized in Table I for mobile phases containing various proportions of acetonitrile in triethylamine phosphate solution. A mobile phase comprising acetonitrile—triethylamine phosphate solution (85:15) provided good resolution of naloxone and naltrexone. It is

TABLE I

EFFECT OF COMPOSITION OF THE MOBILE PHASE ON CHROMATOGRAPHIC PARAMETERS OF NALOXONE AND NALTREXONE

Mobile phase (acetonitrile—triethylamine phosphate solution)*	Capacity factor**		Selectivity factor (α)	Resolution*** (R)
	k'_1	k'_2		
40:60	3.6	4.2	1.17	0.20
50:50	3.7	4.6	1.24	0.22
60:40	3.8	4.8	1.26	0.38
70:30	3.9	5.1	1.31	0.45
85:15	4.0	5.8	1.44	0.60
90:10	6.0	—§	—	—

*0.06% triethylamine in distilled water, adjusted to pH 5.0 with phosphoric acid.
**Subscripts 1 and 2 refer to naloxone and naltrexone, respectively.
***Resolution calculated as quotient of retention time difference to average peak width.
§ Determination neglected because the naloxone peak was unacceptably broad.

interesting to note the unexpected observation that increasing the content of acetonitrile increased the retenton times of naloxone and naltrexone and improved the resolution between the compounds.

Since the mobile phase was rich in organic solvent, thus it was necessary to wash the column with water in order to avoid column pressure build-up. When another column from a different batch was obtained from the same manufacturer, the acetonitrile—triethylamine phosphate solution composition ratio had to be adjusted to 55:45 in order to obtain comparable retention and resolution.

Although the ultraviolet absorption peak of naloxone occurs at 284 nm, detection at this wavelength did not give satisfactory sensitivity. The recent availability of low-wavelength ultraviolet detectors fixed at 214 nm and of higher-purity acetonitrile (with UV cut-off at 190 nm) have enabled a new analytical approach for naloxone with excellent sensitivity. Detection at 214 nm increased the sensitivity of the assay approximately ten-fold relative to that at 284 nm. However, an excellent signal-to-noise ratio using the Altex Model 160 fixed-wavelength detector, allowed the use of 0.001 a.u.f.s. sensitivity setting for blood samples. At this setting, the signal-to-noise ratio obtained when quantifying 1 ng of naloxone hydrochloride (equivalent to 0.9 ng of naloxone base) was 10:1.

A typical chromatogram of extracted blank blood together with a chromatogram of blank blood to which 50 ng of naloxone hydrochloride and 100 ng of naltrexone hydrochloride had been added is shown in Fig. 2a and b. The retention times of naloxone and naltrexone were 5.4 and 7.5 min, respectively. The calibration curves of peak height ratio versus amount of naloxone added to the samples were linear over the concentration range from 0.9 ng to 180 ng of naloxone base ($Y = 0.0355X + 0.0134$; $r = 0.9994$). Separate calibration curves were established on each day that patient samples were analysed.

The analytical recovery was determined by comparing the chromatographic peak heights obtained from the analysis of nine replicate blank blood samples

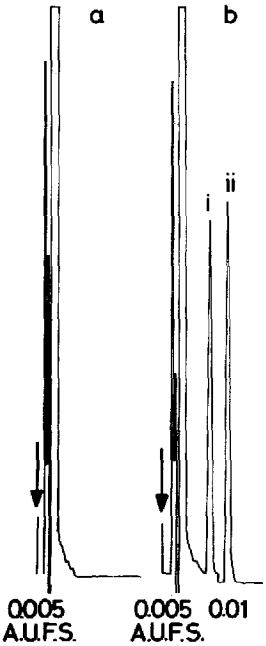

Fig. 2. Chromatograms of extracts of (a) blank blood and (b) blank blood spiked with 50 ng of naloxone hydrochloride (i) and 100 ng of internal standard, naltrexone hydrochloride (ii). The retention times of i and ii are 5.4 and 7.5 min, respectively. The arrows indicate the time of injections.

to which had been added 100 ng of naloxone hydrochloride, with the peak heights resulting from the direct injection of 100 ng of naloxone hydrochloride contained in aqueous standards. The overall recovery of naloxone from blood was 78 ± 3.2% (n = 9). There was no effect on extraction recovery or on peak height ratio when different volumes of blood were extracted. The reproducibility of the method is good as indicated by the coefficients of variation at 100 ng (3.4%; n = 9) and at 10 ng (5.1%; n = 6).

Fig. 3 shows a typical chromatogram obtained by the analysis of a blood sample collected from an apnoeic, preterm infant who had received 0.4 mg of naloxone hydrochloride intravenously. Also shown is the chromatogram from the analysis of a pre-dose blood sample. An endogenous peak was also observed in these blood samples (Fig. 3). However, no interference occurred with peaks of naloxone or naltrexone in these samples or those from other subjects participating in the study. A typical blood naloxone level—time profile is shown in Fig. 4.

The selectivity of the assay was further investigated by examining the retention characteristics of several drugs commonly administered to women in premature labour or to premature neonates. The drugs examined included betamethasone, salbutamol, diazepam, ritodrine, pethidine, folic acid, theophylline, vitamins D and K, gentamycin, ampicillin and frusemide. None of these drugs interfered with the analysis of naloxone.

In summary, this HPLC method is sensitive, selective and reproducible. It

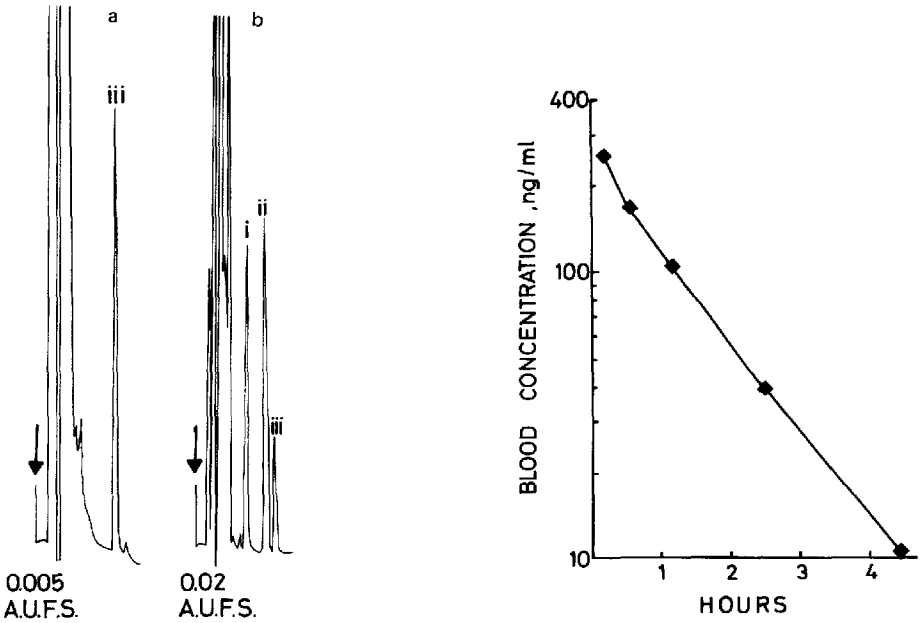

Fig. 3. Chromatograms of extracts of blood from preterm infant (a) just prior to dosing and (b) 35 min after dosing with 0.4 mg of naloxone HCl intravenously. Peaks: i, naloxone; ii, naltrexone; and iii, endogenous peak. The arrows indicate the time of injections.

Fig. 4. Blood naloxone concentration—time profile in a preterm infant (weight = 0.83 kg) after the intravenous administration of 0.4 mg of naloxone HCl.

can be rapidly performed — ten blood samples can be assayed in 90 min — since it does not involve a time-consuming evaporation step prior to chromatographic analysis and no derivatization is necessary. The assay therefore represents an improvement over the less specific radioimmunoassay method [3] and the more complex ECD—GLC method [8] which requires multiple extractions, followed by evaporation and derivatization. The HPLC method reported here has proven particularly useful for pharmacokinetic studies of naloxone in preterm infants and the results of those studies will be reported elsewhere.

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