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DETERMINATION OF NALBUPHINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION: APPLICATION TO CLINICAL SAMPLES FROM POST-OPERATIVE PATIENTS

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SUMMARY

A rapid, selective and reproducible high-performance liquid chromatographic assay with electrochemical detection was developed for the determination of nalbuphine in human plasma. The method involves extraction with chloroform–isopropanol at pH 9.4, back-extraction into dilute phosphoric acid and reversed-phase chromatography on a μBondapak phenyl column. The recovery of nalbuphine and naltrexone (internal standard) was greater than 90%. Calibration curves were linear over a concentration range of 3–36 ng/ml with coefficients of variation, within-day or between-day, not exceeding 8% at any level. Although the limit of detection was 0.3 ng/ml based on a signal-to-noise ratio of 3, the reliable limit of quantitation was 1 ng/ml (coefficient of variation 12%) using 1 ml of plasma. The dual-electrode detector was operated in the screening mode of oxidation (electrode 1, 0.3 V and electrode 2, 0.6 V), providing a greater specificity and reducing background noise. This procedure was applied to a large number of clinical samples in an intravenous dose-range pharmacokinetic study in patients.

INTRODUCTION

Nalbuphine, (−)-17-(cyclobutylmethyl)-4,5α-epoxymorphinan-3,6α,14-triol, is a narcotic partial-agonist with analgesic efficacy similar to that of morphine

[1]. Its antagonist activity provides for a significantly reduced risk of respiratory depression [2]. Nalbuphine is structurally related to the agonist morphine and the antagonist naloxone (Fig. 1). Current reports of analysis of nalbuphine in biological fluids include a gas chromatographic (GC) method [3] and two high-performance liquid chromatographic (HPLC) methods with electrochemical detection (ED) [4,5]. The GC method of Weinstein et al. [3] although sensitive, is much too time-consuming to be used in a pharmacokinetic study involving serial blood samples in a large patient population. The HPLC method of Lake et al. [4] does not provide adequate sensitivity, and while the method published by Lo et al. [5] is rapid and sensitive down to 0.1 ng/ml, it requires 3 ml of plasma and demonstrates several interferences when applied to the clinical samples. We have therefore developed a rapid, sensitive and highly selective HPLC method with ED for the determination of nalbuphine in plasma. The method is suitable for intravenous dose-range pharmacokinetic studies in patients receiving the drug post-operatively.

EXPERIMENTAL

Materials

Nalbuphine hydrochloride and naltrexone hydrochloride (used as internal standard) (Fig. 1) were supplied by Dupont Pharmaceutical Canada (Mississauga, Canada). Methanol, acetonitrile (J.T. Baker, Phillipsburg, NJ, U.S.A.) and chloroform, isopropanol (Fisher Scientific, Fairlawn, NJ, U.S.A.) were of HPLC grade. Trimethylchlorosilane was purchased from Chromatographic Specialties (Brockville, Canada). All other solvents and chemicals were of analyti-

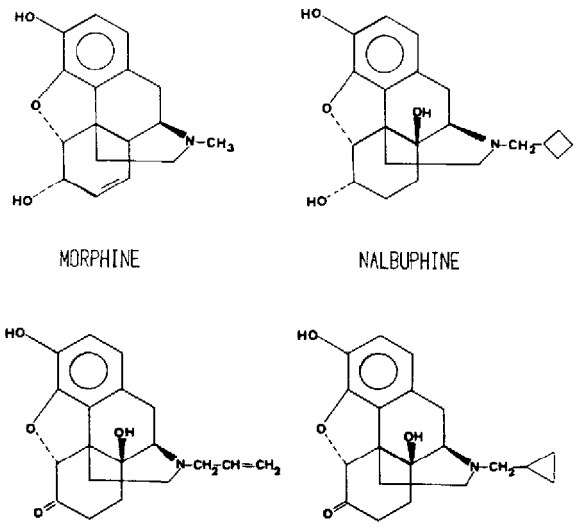

Fig. 1. Structures of nalbuphine and related morphinans.

cal-reagent grade. All aqueous solutions were prepared using double-distilled water.

Chromatography

The chromatographic system consisted of a Model SP8700XR solvent-delivery system, an SP8780XR autosampler and an SP4290 computing integrator from Spectra-Physics (San Jose, CA, U.S.A.), and completely automated by means of the LABNET software (Spectra-Physics). An ESA Coulochem® Model 5100A electrochemical detector (Chromatographic Sciences, Montreal, Canada) equipped with the Model 5010 dual-electrode analytical cell was operated in the oxidation screening mode, with the potential of the first electrode set at 0.3 V and the second electrode set at 0.6 V. The signal of the electrodes was amplified by setting the multiplier gain switch to 25. Chromatographic experiments were performed on a μBondapak phenyl column (300 mm × 3.9 mm I.D., particle size 10 μm) (Waters Assoc., Milford, MA, U.S.A.). The mobile phase, acetonitrile–dilute 0.3% phosphoric acid, pH 3.5 (20:80, v/v), was degassed by vacuum before use. The mobile phase was recycled over 24 h at a flow-rate of 1.0 ml/min at ambient temperature (ca. 22°C) yielding a back-pressure of 9.0 MPa.

Standard solutions

Nalbuphine. A stock solution of 1.0 mg/ml nalbuphine free base was prepared in methanol and a standard solution of 300 ng/ml in water was made by serial dilutions. Aliquots of this standard solution (100–1200 μl) were added to aliquots of blank plasma (8.8–9.9 ml) to give final concentrations of 3, 9, 18 and 36 ng/ml of plasma.

Internal standard. A 1.0 mg/ml methanolic solution of naltrexone free base was prepared and further diluted to give a working solution of 200 ng/ml in water. A 50-μl aliquot (10 ng) was added to each 1-ml aliquot of plasma standard or specimen.

Sample preparation

To 1 ml of plasma placed in a 15-ml glass capacity culture tube, fitted with a PTFE-lined screw cap, were added 50 μl of internal standard solution and 1 ml of carbonate buffer pH 9.4. The samples were extracted with 7 ml of a mixture of chloroform–isopropanol (98:2, v/v) by mixing for 15 min on a Roto-rack. After centrifugation at 25°C for 10 min at 1120 *g*, the aqueous phase was aspirated and discarded. The remaining organic phase was transferred to another 10-ml culture tube and back-extracted with 200 μl of 0.05% phosphoric acid by agitating for 30 s on a vortex mixer. Following centrifugation using the above conditions, a 75-μl aliquot of the aqueous phase was injected onto the column.

All screw-capped culture tubes were washed with 50% nitric acid and then silanized using 3% trimethylchlorosilane in toluene. Residual silanizing agent was removed by thoroughly rinsing with toluene followed by methanol.

Calibration

Calibration curves were obtained by assay of duplicate extracts of blank plasma samples spiked with nalbuphine to cover a concentration range of 3–36 ng/ml

and the internal standard. The concentration of nalbuphine in unknown samples was determined by using the linear regression line (unweighted) of peak-height ratios versus concentration of calibration standards.

Application of the method

This method of analysis has been employed in a pharmacokinetic/pharmacodynamic study of intravenous nalbuphine conducted in 60 patients. A continuous infusion of nalbuphine at a rate of 12.5, 25.0 or 50 μg/kg/h was administered post-operatively over a 24-h period. Serial blood samples were collected during the 24-h infusion period and after stopping the treatment to characterize the pharmacokinetic parameters of the drug.

RESULTS AND DISCUSSION

Electrochemistry

The electrochemical detector used for this method consisted of two coulometrically efficient porous graphite electrodes working in series. The screen mode of operation was selected in order to improve the detector selectivity without compromising sensitivity. The operating potentials were optimized by generating current–voltage curves using cyclic voltammetry for the oxidation of nalbuphine and the internal standard under the HPLC conditions described above. From the cyclic voltammograms, the first electrode potential was set at +0.3 V resulting in a reduced background noise from the impurities of the mobile phase and the sample extracts. An applied potential of +0.6 V was chosen for the second electrode (test electrode) which provided a very sensitive signal for nalbuphine without increasing the noise level. The background noise was also decreased by recirculating the mobile phase. The recirculation process results in many impurities being irreversibly reduced. However, there was a tendency for the background current to increase if the mobile phase was recycled for more than 24 h, most probably due to accumulation of impurities from the several biological sample extracts injected. A guard cell, Model 5020 (ESA), installed between the HPLC pump and the injector further reduced the background noise and allowed a longer recycling time for the mobile phase. In spite of several precautions taken to eliminate electroactive contaminants, a gradual increase in back-pressure and noise level was observed over a period of time. In order to maintain optimum detector performance, the analytical cell was cleaned according to a procedure described by the manufacturer, and in-line filters were replaced, either on a monthly basis or after analysis of approximately 500 samples.

Selectivity and specificity

Fig. 2 shows typical chromatograms of an extract of blank human plasma (A), a plasma standard spiked with 18 ng/ml nalbuphine and the internal standard (B) and a plasma sample obtained from a patient receiving intravenous nalbuphine (C).

Of the different reversed-phase columns tested, the μBondapak phenyl column exhibited a greater selectivity. The retention times of nalbuphine and the internal

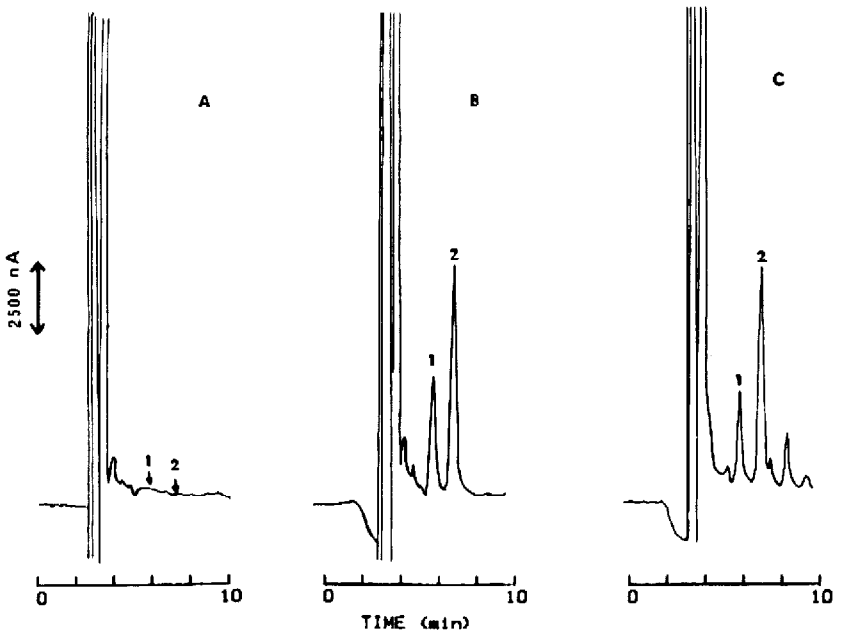

Fig. 2. Chromatograms of extracts from (A) blank human plasma, (B) spiked plasma (nalbuphine 18 ng/ml) and (C) plasma sample from a patient (nalbuphine 23.2 ng/ml). Peaks: 1 = internal standard, naltrexone; 2 = nalbuphine.

standard were approximately 6.9 and 5.9 min, respectively. No interfering peaks were detected in the control human samples or the clinical samples from patients who had received several drugs such as morphine, fentanyl, atropine, pancuronium or naloxone as part of their general anaesthesia protocol or for post-operative analgesia. Some unidentified peaks eluted after that of nalbuphine (Fig. 2C) but did not interfere. Based on a signal-to-noise ratio of 3, the detection limit of the assay from 1 ml of plasma was 0.3 ng/ml. However, the lower practical limit of quantitation was set at 1 ng/ml based on a coefficient of variation of less than 12%. This method provided adequate sensitivity and specificity for the pharmacokinetic study.

Linearity and reproducibility

Calibration curves were linear over the concentration range 3–36 ng/ml with correlation coefficients $\geqslant 0.995$ and minimal intercept (Table I). Reproducibility and accuracy of the method were determined by processing spiked plasma samples at four concentrations with respect to a calibration curve run each day. Nine or ten samples were analyzed for each concentration. Within-day coefficients of variation ranged from 2.8 to 7.9%. The day-to-day variation of samples analyzed on 10 different days over a period of 33 days was 3.5–6.7% (Table II).

In inter-day assay variation was also estimated by comparing the linear regression slopes of twelve standard curves. Over a period of 47 days, the slope averaged 0.1122 with a coefficient of variation of 10.6% (Table I). The accuracy of the method expressed as the mean deviation of all concentrations from the theoret-

TABLE I

STANDARD CURVE REPRODUCIBILITY FOR NALBUPHINE

Standard curve No.	Slope	Intercept	r
1	0.1044	−0.0043	0.998
2	0.1034	0.0231	0.996
3	0.1093	−0.0080	0.995
4	0.1232	−0.0073	0.999
5	0.1128	−0.0295	0.997
6	0.1112	0.0229	0.999
7	0.1111	−0.0234	0.998
8	0.1140	0.0024	0.999
9	0.1050	0.0314	0.999
10	0.1180	−0.0370	0.998
11	0.1236	−0.0272	0.999
12	0.1102	0.0263	0.999
Mean	0.1122		
Coefficient of variation (%)	10.6		

TABLE II

PRECISION AND ACCURACY

Known concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Accuracy (% mean deviation)
Within-day (n = 9)			
3.0	2.9 ± 0.2	7.9	−3.3
9.0	9.2 ± 0.5	5.1	2.6
18.0	18.1 ± 0.5	2.8	0.7
36.0	35.7 ± 2.3	6.3	−0.7
Day-to-day (n = 10)			
5.0	5.0 ± 0.3	6.7	0.0
10.0	10.2 ± 0.5	4.8	2.0
20.0	20.5 ± 0.7	3.5	2.5
30.0	29.6 ± 2.0	6.6	−1.3

ical value ranged from −3.3% to 2.6% (Table II). These results demonstrate that this method is very reproducible and highly accurate.

Recovery

The extraction recovery of nalbuphine and the internal standard was determined at all levels of the calibration curve by comparing the peak heights obtained by direct injection of standard aqueous solutions to those obtained after the whole extraction procedure (Table III). The absolute recoveries of nalbuphine and internal standard were greater than 90% and independent of nalbu-

TABLE III

ABSOLUTE RECOVERIES OF NALBUPHINE AND NALTREXONE (INTERNAL STANDARD) FROM SPIKED PLASMA SAMPLES ($n=8$)

Drug	Concentration (ng/ml)	Recovery (%)	Coefficient of variation (%)
Nalbuphine	3.0	102.0	8.7
	9.0	99.3	9.1
	18.0	99.2	5.2
	36.0	92.5	9.3
Naltrexone	10.0	92.1	4.6

TABLE IV

STABILITY OF NALBUPHINE IN FROZEN PLASMA

Initial concentration (ng/ml)	Concentration found (ng/ml)			
	7 days	28 days	40 days	49 days
5	5.4	5.3	5.1	5.1
10	10.9	10.0	9.3	10.1
20	21.2	20.3	19.9	19.4
30	28.0	30.6	29.2	30.0

phine concentration. Compared to other extraction mixtures such as ethyl acetate–toluene–isopropanol, diethyl ether or heptane, the mixture of chloroform–isopropanol gave greater recovery and chromatograms with less background from plasma samples.

The extraction procedure, involving only two steps, is rapid and allows analysis of at least 40 samples daily using the automated HPLC system.

Stability

The stability of nalbuphine in spiked plasma was determined weekly for seven weeks of freezing at −20°C. The results appearing in Table IV indicate that no significant degradation had occurred over the seven-week period. Lo et al. [5] have reported that nalbuphine was stable for at least eighteen weeks in frozen plasma.

Application of the method

This HPLC method has been used intensively in our laboratories and more than 2500 samples collected from patients receiving intravenous nalbuphine have now been analyzed. Fig. 3 shows the plasma concentration–time profile of a patient receiving 25 μg/kg/h of nalbuphine for 24 h. Nalbuphine concentrations reached a steady-state level of 17.7 ng/ml at approximately 4 h. After stopping

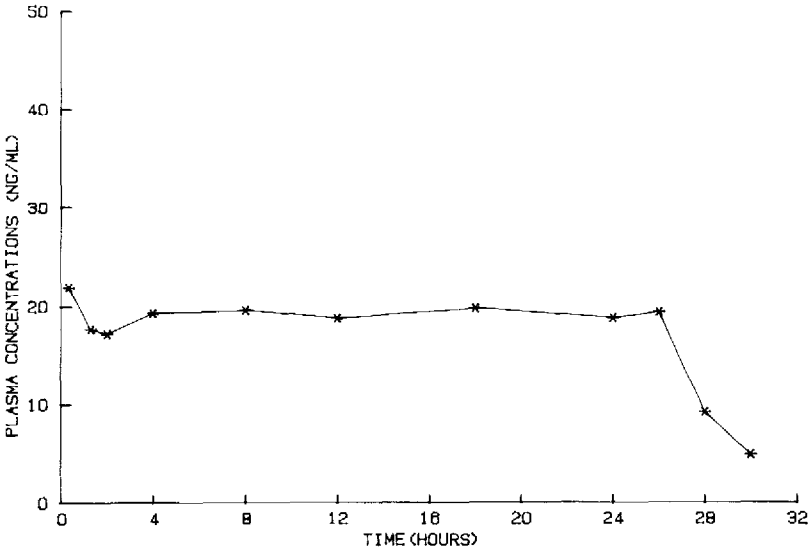

Fig. 3. Plasma concentration–time profile in a patient receiving 25 μg/kg/h of intravenous nalbuphine.

the infusion, plasma levels declined monoexponentially with a terminal half-life of 2.0 h. The results of the pharmacokinetic study conducted in the 60 patients will be reported separately.

CONCLUSION

The method described is sufficiently simple, sensitive, specific and rapid for the determination of nalbuphine in large numbers of plasma specimens. It was used in our laboratories to investigate the pharmacokinetics of the drug in patients.

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