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Rapid and sensitive high-performance liquid chromatographic assay for nalbuphine in plasma

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Abstract

A simple and reliable reversed-phase high-performance liquid chromatographic method with adequate internal analog standardization and coulometric detection is described for the quantification of nalbuphine in plasma samples. The lower limit of detection was estimated to be 0.1 ng/ml. For routine analysis, the limit of quantification was set at 0.5 ng/ml and only a small plasma volume (500 μl) was required. The nalbuphine calibration curve was linear over the concentration range 0–100 ng/ml. The recoveries of nalbuphine and 6-monoacetylmorphine, used as internal standard, were close to 85%. Due to the small sample volume of blood required, this highly sensitive, accurate and specific method is suitable for pharmacokinetic studies of nalbuphine, and particularly for drug monitoring in neonates born from mothers treated with nalbuphine.

1. Introduction

Nalbuphine is a semisynthetic narcotic agonist–antagonist of the phenanthrene series. Structurally, it is closely related to naloxone, an antagonist of the opiate receptors and to oxymorphone, a narcotic agonist. Nalbuphine has been shown to be approximately equianalgesic to morphine [1], yet with a ceiling effect on ventilatory depression [2] and fewer adverse effects than pethidine or pentazocine [3]. Despite the increase in the use of epidural analgesia for pain relief in obstetrics, systemic analgesia with nalbuphine is still commonly used. However, several cases of severe perinatal cardiovascular and respiratory depression have been recently reported when nalbuphine was given to the mother either by the intramuscular (i.m.) or intravenous (i.v.) route [4–6]. These reports prompted us to develop a method for the assay of nalbuphine in plasma for therapeutic monitoring of this drug in obstetric analgesia.

A few methods have been described for the determination of the nalbuphine concentration in human plasma using high-performance liquid chromatography (HPLC) with electrochemical detection [7–11]. We describe here a sensitive, rapid and highly selective HPLC assay with coulometric detection for the determination of nalbuphine concentrations, using 6-monoacetylmorphine as internal standard. The results

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obtained for the extraction from human plasma under routine conditions are reported with respect to accuracy, precision, limit of detection, recovery and selectivity.

2. Experimental

2.1. Reagents

Nalbuphine free base was a gift from Dupont Pharmaceutical (Mississauga, Canada). 6-Monoacetylmorphine was purchased from Sanofi, Francopia (Paris, France). Hexane, dichloromethane, isopropanol, methanol, boric acid, potassium chloride, sodium hydroxide and orthophosphoric acid were Normapur quality products (Prolabo, Paris, France). Potassium dihydrogenphosphate was purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Chromatography

A Model 510 (Waters, Millipore, Guyancourt, France) pump was used for solvent delivery. Fifty μl of the 200-μl sample were injected through a Model 7125 Rheodyne valve onto a 150 × 4.6 mm I.D. Lichrospher C_{18} column (particle size 5 μm, Merck) at room temperature. The Model 5100 A Coulochem detector (Esa, Bedford, MA, USA) was equipped with a Model 5020 guard cell working at 0.50 V and a Model 5011 analytical cell operating in the oxidation screening mode, with the potential of the first electrode set at 0.06 V and the second electrode set at 0.40 V. The signal was amplified by setting the multiplier gain switch to 1000.

Nalbuphine and 6-monoacetylmorphine used as internal standard (I.S.), were eluted with a mobile phase of methanol–water (20:80, v/v) containing potassium dihydrogenphosphate (24 m*M*) and EDTA disodium salt (0.06 m*M*) adjusted to pH 3.4 with orthophosphoric acid, at a flow-rate of 0.8 ml/min. The mobile phase was degassed by vacuum before use then with a stream of helium and was recycled over 12 h at a flow-rate of 0.5 ml/min at room temperature. Chromatograms were recorded on a flat bed recorder at 2 mm/min using a 10-mV setting (Model BD 40, Kipp and Zonen, Netherlands).

2.3. Standard solutions

Nalbuphine (10 mg, free base) and 6-monoacetylmorphine (10 mg, free base) were dissolved in 100 ml of methanol to yield stock standard solutions at a final concentration of 100 μg/ml. The solutions were stored in glass-stoppered bottles at −25°C. Working solutions were freshly prepared before analysis by dilution with distilled water in a ratio of 1:200 and 1:500 for nalbuphine and 6-monoacetylmorphine, respectively. Drug free plasma was obtained from healthy volunteers and stored at −25°C.

2.4. Sample preparation

Blood samples were collected in heparinized tubes and centrifuged for 10 min at 4°C (2000 *g*). The plasma was stored at −25°C until analysis. Working solution of 6-monoacetylmorphine (100 μl) was added to 500 μl of plasma (final concentration 40 ng/ml) in a 20-ml screw-topped tube, then 2 ml of boric acid (0.1 *M*)–KCl (0.1 *M*) adjusted to pH 9 with 0.1 *M* NaOH were added to the sample. Subsequently, samples were extracted with 10 ml of hexane–dichloromethane–isopropanol (69:30:1, v/v) by mechanical shaking for 15 min at 60 rpm (ARTM, Firlabo, Lyon, France). After shaking, samples were centrifuged for 5 min at 4°C (2000 *g*). The upper organic layer was transferred to a second screw-capped tube and reextracted by shaking for 2 min with 200 μl of 17 m*M* phosphoric acid (SMI Vortexer, American Dade, Miami, FL, USA). The samples were centrifuged for 5 min at 4°C (2000 *g*) and the lower layer transferred into a conical vial. An aliquot of 50 μl was injected onto the HPLC system.

2.5. Calibration

Calibration standards for nalbuphine were prepared in 500 μl of drug-free plasma by spiking 20, 40, 60, 80, and 100 μl of nalbuphine working solution (0.5 μg/ml) and 100 μl of I.S. solution (0.2 μg/ml) to cover the plasma con-

Table 1
Calibration curve of nalbuphine in plasma

Plasma sample	Nalbuphine/6-monoacetylmorphine peak-height ratio				
	20 ng/ml	40 ng/ml	60 ng/ml	80 ng/ml	100 ng/ml
1	0.22	0.48	0.73	0.98	1.21
2	0.22	0.50	0.72	0.98	1.21
3	0.23	0.49	0.72	0.98	1.19
4	0.23	0.48	0.71	0.97	1.21
Mean ± S.D.[a]	0.23 ± 0.01	0.49 ± 0.01	0.72 ± 0.01	0.98 ± 0.01	1.21 ± 0.01
C.V. (%)	2.6	2.0	1.1	0.5	0.8

Equation of the regression line: $y = -0.012 + 0.012x$ ($n = 20$, $r = 0.99$).
[a] $n = 4$.

centration range 0–100 ng/ml. The calibration curve was obtained by linear regression of the peak-height ratio: nalbuphine/6-monoacetylmorphine (y) vs. nalbuphine concentrations (x) (Table 1).

3. Results

3.1. Electrochemical conditions

The hydrodynamic voltammogram of nalbuphine presented in Fig. 1 was performed with the ESA Coulochem electrochemical detector under the same chromatographic conditions as sample analysis. The test electrode (second electrode) was set at a potential of 0.40 V, approximately corresponding to the half-wave potential.

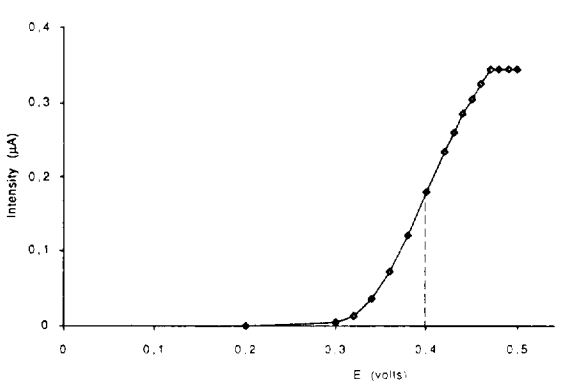

Fig. 1. Hydrodynamic voltammogram of nalbuphine.

3.2. Precision and accuracy

Data concerning precision and accuracy of the results are presented in Table 2. Intra-day reproducibility was assessed by using eight samples at three different concentrations, i.e. 5, 40 and 100 ng/ml, and analysed on the same day. The coefficients of variation (C.V.) were 2.6, 1.8 and 2.7%, respectively. Day-to-day reproducibility was determined ten times with three different quality control samples, within two weeks. The C.V.s at 5, 40 and 100 ng/ml were 5.9, 2.7 and 3.1%, respectively.

3.3. Linearity

The linearity of the method was tested on extracted plasma samples, in the concentration range 0–100 ng/ml of nalbuphine. As shown in Table 1, a linear relationship was observed with an equation of the regression line: $y = -0.012 + 0.012x$ ($n = 20$, $r = 0.99$).

3.4. Limits of quantification (LOQ) and detection (LOD)

The LOQ is the lowest concentration on the standard curve which can be measured with acceptable accuracy (C.V. $< 20\%$). The lower practical limit of quantification was set at 0.5 ng/ml based on a coefficient of variation of 3.8% ($n = 5$). Under procedural conditions the LOD using 500 μl of plasma and a 50-μl volume

Table 2
Precision and accuracy of nalbuphine assay in plasma

Nominal concentration (ng/ml)	Actual concentration (ng/ml)	Precision (C.V., %)	Accuracy	
			Actual/nominal (%)	Actual − nominal (ng/ml)
Intra-assay (n = 8)				
5	4.95 ± 0.13	2.6	99.00	−0.05
40	40.06 ± 0.73	1.8	100.15	0.06
100	100.06 ± 2.71	2.7	100.06	0.06
Inter-assay (n = 10)				
5	4.97 ± 0.30	5.9	99.40	−0.03
40	40.75 ± 1.09	2.7	101.88	0.75
100	101.02 ± 3.17	3.1	101.02	1.02

injected was estimated to 0.1 ng/ml based on a signal-to-noise ratio greater than 3:1.

3.5. Recoveries

The overall recoveries (Table 3) were calculated by comparing the peak heights of a series of nalbuphine and 6-monoacetylmorphine spiked samples after extraction from plasma, to the peak heights of a series of unextracted reference standards. Using this method, the actual recovery found was 85.7 ± 2.6% (mean ± S.D., $n = 12$, C.V. = 2.9%) for nalbuphine and 84.9 ± 2.9% (mean ± S.D., $n = 12$, C.V. = 5.6%) for internal standard.

3.6. Plasma interference and selectivity

Typical chromatograms are shown for human drug-free plasma (Fig. 2), for plasma from a patient receiving nalbuphine and spiked with I.S. (Fig. 3), and for plasma spiked with nalbuphine,

Table 3
Recoveries of nalbuphine and 6-monoacetylmorphine

Concentration (ng/ml)	Peak height		Recovery (%)
	Authentic standards	Standards extracted from plasma	
Nalbuphine			
Blank	N.D.[a]	N.D.	
5	0.95	0.80	84.2
40	7.75	6.60	85.2
100	19.45	17.00	87.6
Mean ± S.D.[b]			85.7 ± 2.6
6-Monoacetylmorphine			
Blank	N.D.	N.D.	
40	15.00	12.75	84.9
Mean ± S.D.[b]			84.9 ± 2.9

[a] N.D. = not detectable.
[b] $n = 12$.

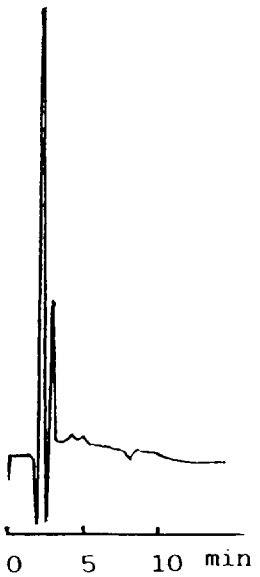

Fig. 2. Chromatogram of a drug free plasma sample.

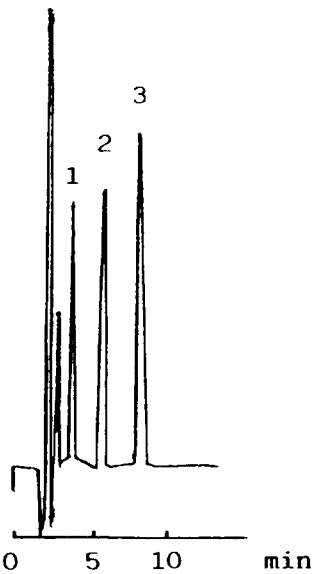

Fig. 4. Chromatogram of plasma spiked with 300 ng/ml of naloxone (peak 1), 40 ng/ml of 6-monoacetylmorphine (peak 2) and 100 ng/ml of nalbuphine (peak 3) standard solutions.

I.S. and naloxone (Fig. 4). The retention times of nalbuphine, 6-monoacetylmorphine and naloxone were approximately 8.5, 5.7 and 3.6 min, respectively. There was no interference from endogenous plasma constituents. Interference was studied with various compounds whose chemical structures or concurrent use could interfere with the assay. The following compounds could not be detected under the procedural conditions used: ethylmorphine, codeine, pholcodine, norcodeine, fentanyl, methadone, dextromoramide and dextropropoxyphene. Morphine and nalorphine, which are electroactive at the potential of 0.40 V, eluted early in the system (<3 min) due to their higher polarity compared to nalbuphine and 6-monoacetylmorphine. Naloxone and naltrexone have identical retention times (3.6 min). On the other hand, buprenorphine which is also electroactive at the working potential but less polar than the other opioids tested, did not show a signal over the time of analysis [12]. This compound probably gives a very late response under the HPLC conditions used.

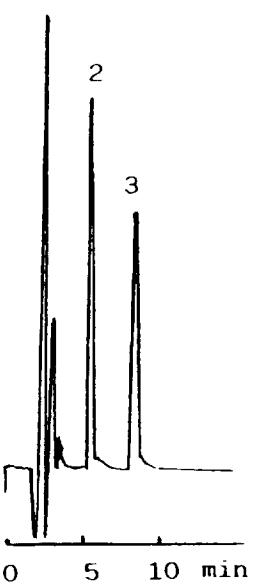

Fig. 3. Chromatogram of a treated patient plasma containing 82 ng/ml of nalbuphine (peak 3) and spiked with 50 ng/ml of 6-monoacetylmorphine (peak 2).

4. Discussion

An HPLC assay with amperometric detection was described by Lake et al. [7], but this was not

suited for pharmacokinetic studies because it lacked sensitivity and rapidity. The methods developed by Lo et al. [8] and Keegan and Kay [9] were faster and more sensitive but required 2–3 ml of plasma hampering its use for drug monitoring in neonates.

HPLC measurement coupled with coulometric detection as described by Wetzelsberger et al. [10] allowed a significant improvement in the sensitivity for nalbuphine with a limit of detection close to 0.05 ng/ml. Most of these techniques resulted in emulsifications during the extraction step and led to several awkward interference peaks when applied to human samples. The method reported by Dubé et al. [11] gave an acceptable limit of detection (1 ng/ml) but required large quantities of chloroform as extraction solvent leading to a significant amount of toxic waste and unsatisfactory nalbuphine peak shape.

Recently, Kintz et al. [13] described a procedure involving gas chromatography coupled to mass spectrometry (GC–MS) and an HPLC method coupled to a diode-array detector. The GC–MS technique showed improved specificity particularly for forensic applications, but required mass spectrometric equipment. The HPLC–photodiode-array analysis involved a tedious extraction procedure and resulted in a high detection limit (25 ng/ml) inadequate for pharmacokinetic studies in plasma.

In our assay, the operating potential was chosen from the hydrodynamic voltammogram of nalbuphine. The first electrode potential was set at the low value of 0.06 V to reduce background noise while avoiding oxidation of the analyte. The second potential of 0.40 V resulted in a high signal for nalbuphine, sufficiently sensitive for pharmacokinetic studies. Under these electrochemical conditions, performance of the detector was maintained over a one-week period of routine utilisation without increase in background noise and clogging of the cells.

Nalbuphine assays generally used naloxone [7,9] or naltrexone [10] as internal standards. These compounds are not suitable as internal standards for drug monitoring in obstetric analgesia since naloxone (Narcan) is used to reverse cardiorespiratory depression in neonates caused by nalbuphine administration to the mothers [14] and since naltrexone has the same retention time as naloxone under the conditions used. Lo et al. [8] employed a N-cyclopentylmethyl analogue of nalbuphine that is not commercially available. In the method presented here we used an internal standard, 6-monoacetylmorphine, which contains one oxidizable hydroxyl group (Fig. 5). The presence of an acetyl group in the 6-position increases the retention time of this compound leading to an excellent separation of all the analytes contained in the biological samples, i.e. nalbuphine, naloxone and 6-monoacetylmorphine (Fig. 4), without any interference with other opiate derivatives.

The stability of 6-monoacetylmorphine had to be studied since this compound is known to be unstable under alkaline conditions. Under the conditions used, the extraction step includes a contact of 15 min between this compound and an alkaline buffer (pH 9) which could lead to hydrolysis of the ester function of 6-monoacetylmorphine. Nevertheless, a previous study on the 6-monoacetylmorphine stability has demonstrated a negligible hydrolysis of the acetyl group after a contact of 30 min with a pH 9 buffered medium [15].

On the other hand, the use of hexane–dichloromethane–isopropanol (69:30:1, v/v) as the

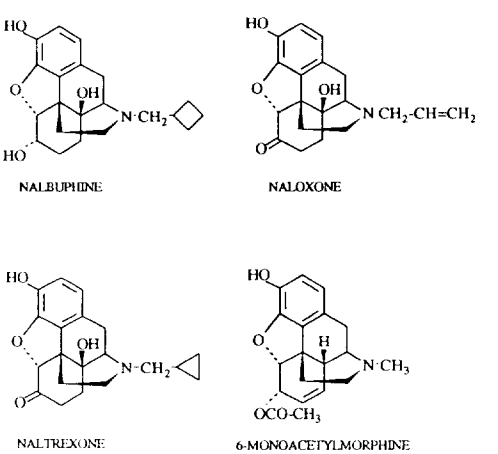

Fig. 5. Molecular structure of nalbuphine and related compounds.

extraction solvent avoids the emulsion formation which hampers the adequate removal of the organic layer. This choice gives a chromatogram completely free of interference from endogenous plasma constituents (Fig. 2). This solvent mixture, which does not have the chloroform related toxicity, was confirmed to be the best choice for the opiate derivatives involved in this assay, leading to a substantial improvement in the quality of the chromatograms and a suitable nalbuphine recovery of 85.7 ± 2.6%. In addition, the use of 1% isopropanol in the extraction solvent was found to significatively increase the recovery of nalbuphine (ca. 20%) and to give optimum peak resolution.

In conclusion, the present method allows a low limit of detection (0.1 ng/ml) with a high selectivity and reliability. The relative simplicity permits its use for pharmacokinetic studies or therapeutic drug monitoring. Moreover, the small volume of plasma (500 μl) required facilitates its application to neonates or infants.

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