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Modified method of nalbuphine determination in plasma: validation and application to pharmacokinetics of the rectal route

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Abstract

A solid-phase extraction (SPE) procedure was developed for the quantification of nalbuphine in a small volume (500 μl) of human plasma with subsequent assay by high-performance liquid chromatography (HPLC) and electrochemical detection using 6-monoacetylmorphine as internal standard. Plasma was extracted using Bond Elute certified extraction columns (LCR: 10 ml, 130 mg) after conditioning with methanol and 0.2 *M* Tris buffer (pH 8). Elution was performed with a CH_2Cl_2–isopropanol–NH_4OH (79:20:1, v/v). The organic phase was evaporated to dryness and resuspended in HPLC mobile phase containing 2% isopropanol. Linearity was assessed over the 5–100 ng/ml concentration range and a straight line passing through the origin was obtained. Experiments with spiked plasma samples resulted in recoveries of 95±5.4% and 98±6.2% for nalbuphine and 6-monoacetylmorphine, respectively. The optimal pH conditions for the SPE were found at pH 8. The intra-day coefficients of variation (C.V.) for 5, 40, and 100 ng/ml were 5.3, 3.0 and 2.3% ($n=8$) and the inter-day C.V.s were 7.7, 3.2 and 3.5% ($n=10$), respectively. The detection limit for 500 μl plasma sample was 0.02 ng/ml and the limit of quantification 0.1 ng/ml (C.V.=12.4%). The ease of the proposed method of analysis, as well as its high accuracy and sensitivity allow its application to pharmacokinetic studies. A preliminary kinetic profile of nalbuphine after rectal administration in a pediatric patient is presented.

Keywords: Nalbuphine

1. Introduction

Nalbuphine (Nubain), which is a powerful narcotic agonist–antagonist analgesic opioid, structurally related to the phenanthrene series, has been shown to be approximately equianalgesic to morphine [1] but with a noteworthy ceiling effect on analgesia and respiratory depression [2]. Thus, it may represent a safe analgesic for paediatric surgery. Its efficacy has been shown in infants during the per- and post-operative period by intravenous (IV) or intrasmuscular (IM) routes [3,4]. More recently, in order to avoid a fright reaction in front of parenteral administration, the rectal route was successfully proposed in surgical premedication in children [5]. However, in spite of its effective utilization by this route, no pharmacokinetic studies on the rectal route have yet been carried out.

Several methods are now available for nalbuphine quantification in biological materials using gas chromatography with electron capture detection [6], high-

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performance liquid chromatography (HPLC) coupled with electrochemical detection [7–13] or photodiode-array detection [12]. All these HPLC techniques use liquid–liquid partitioning. None of them includes the liquid–solid approach which is yet considered to provide a more selective and more efficient separation mechanism than liquid–liquid extraction.

In view of the pharmacokinetic study of the rectal route in children, we developed a new extraction method using solid-phase extraction (SPE) which could be subsequently automated and, in addition, which significantly limits both health and environment risks. The HPLC separation as well as the electrochemical detection were the same as previously developed in our laboratory [13].

The validation of a modified method of nalbuphine quantification, involving a new extraction technique, is described and compared to the liquid–liquid extraction assay currently used in our laboratory. The method has also been successfully applied to human plasma.

2. Experimental

2.1. Reagents

Nalbuphine free base was provided by Dupont Pharmaceutical (Missauga, Canada). 6-Monoacetylmorphine free base was purchased from Sanofi, Francopia (France). Methanol, acetic acid, Tris (trishydroxymethylaminomethane), potassium dihydrogenphosphate were supplied by Merck (Darmstadt, Germany). Dichloromethane, ammonia, 2-propanol and orthophosphoric acid were Normapur quality products purchased from Prolabo (Paris, France). EDTA (ethylenediamine tetraacetic acid disodium salt) was from Sygma (St.-Quentin Fallavier, France).

Stock standard solutions were prepared dissolving nalbuphine (5 mg) or 6-monoacetylmorphine (5 mg), used as internal standard (I.S.), in 50 ml methanol and stored at −20°C. Working solutions were prepared daily by adequate dilutions in water.

2.2. Patients and sample collection

Samples were taken from children (aged 2–7 years) in the Department of Anaesthesiology of the A. Michallon Hospital. The procedure has been approved by the local Ethical Committee. Heparinized blood samples were collected at regular time intervals during and after anaesthesia: 15, 30, 40, 50, 120, 180, 240, 360 and 480 min after the rectal administration of 0.3 mg/kg nalbuphine (range: 4–7.2 mg) and then centrifuged for 10 min at 4°C (2000 *g*). Plasma samples of 500 μl were stored at −20°C and analyzed within 7 weeks. The stability of nalbuphine in plasma stored for 18 weeks at −20°C was previously assessed by Lo et al. [8].

2.3. Extraction procedure

Plasma samples were purified and extracted with solid-phase extraction using Bond Elut Certify extraction columns (10 ml, 130 mg; Varian, Sample Preparation Products, Harbor City, CA, USA). The extraction cartridges were positioned on a Vac Elut vacuum manifold (Varian) and were conditioned with 2 ml of methanol, then 2 ml of 0.2 *M* Tris buffer (pH 8), without column drying. The plasma (500 μl) was diluted with 2 ml of 0.2 *M* Tris buffer (pH 8) and working solution of 6-monoacetylmorphine was added at the final concentration of 20 ng/ml. The specimen was put up to the top of the packing and drawn slowly through the column (>2 min). The column was washed sequentially by 2 ml of deionised water, 2 ml of 10 m*M* acetic acid (pH 3.3) and completely dried. The sorbent bed was rinsed by 3 ml of methanol. Sample elution was performed twice with 2-ml volumes of CH_2Cl_2–isopropanol–NH_4OH (79:20:1, v/v) extemporaneously prepared. The organic phase was collected in a glass tube and evaporated to dryness under a nitrogen stream, at 40°C. The sample residue was resuspended in 200 μl of the HPLC mobile phase system containing 2% of isopropanol.

2.4. HPLC procedure

Nalbuphine and 6-monoacetylmorphine used as internal standard were chromatographed by HPLC with electrochemical detection, according to a modified procedure previously described by Wetzelberger et al. [10]. A manual injection of 50 μl volume was carried out through a Model 7125 Rheodyne valve, onto a 150×4.6 mm I.D. LiChrospher C_{18} column (particle size 5 μm, Merck). Elution was performed isocratically by a Model 590 solvent delivery system (Waters, Milford, MA, USA) at a flow-rate of 0.8 ml/min, with a mobile phase of methanol–water (20:80, v/v) containing 24 m*M* KH_2PO_4 and 0.06 m*M* EDTA (pH 3.4). The mixture was filtered and degassed with a gentle stream of helium during use. The Model 5100 A Coulochem detector (ESA, Bedford, MA, USA) equipped with a Model 5020 guard cell (+0.50 V) and a Model 5011 dual-electrode analytical cell operating in the oxidation screening mode with potentials set at +0.06 V and +0.40 V for the first and the second electrode, respectively. The detector gain was set to 4000. Under these conditions, typical retention times of 6-monoacetylmorphine and nalbuphine were approximately 8.4 and 13.6 min, respectively. Chromatograms were recorded on a flat bed recorder at 2 mm/min using a 10 mV setting (Model BD 40, Kipp and Zonen, Netherlands).

2.5. Calibration curve

The standard curve was established by plotting peak-height ratio (nalbuphine/6-monoacetylmorphine) versus nalbuphine concentration (ng/ml) and was performed daily. Nalbuphine standard concentrations were obtained by spiking drug-free human plasma with various amounts of diluted nalbuphine stock solutions (0.05 μg/ml and 0.25 μg/ml) and 40 μl of internal standard solution (25 μg/ml) to cover a 5–100 ng/ml concentration range. This concentration range was selected to give a close approach of the patient concentrations we obtained, preliminarily in the framework of our pharmacokinetic study.

2.6. Linearity

Linearity was checked on three different days from spiked blank human plasma. Five different fortification levels (5, 20, 40, 60 and 100 ng/ml), corresponding to the domain of patient concentrations, were analyzed. To cover a wide range of concentrations potentially useful in other fields of application than pharmacokinetic studies, linearity was extended up to 500 ng/ml, using five other concentration levels: 50, 125, 250, 400 and 500 ng/ml.

2.7. Statistical analysis

The proposed method for nalbuphine quantification has been validated according to SFSTP Committee regulations [14]. Parameters of validation have been statistically analysed by ANOVA [15] (m=mean, S.D.=standard deviation, C.V.=coefficient of variation).

3. Results

3.1. Extraction procedure recovery of nalbuphine and 6-monoacetylmorphine

The extraction efficiency was determined by comparing the peak heights between extracted plasma samples spiked with intermediate stock solutions and unextracted standard solutions (Table 1).

The extraction recoveries were 95±5.4% (mean±S.D., $n=12$, C.V.=5.7%) and 98±6.2% (mean±S.D., $n=7$, C.V.=6.3%) for nalbuphine and 6-monoacetylmorphine, respectively.

The pH buffer was shown to play a major role in the extraction recovery. Fig. 1 illustrates the variations of the recovery according to pH buffer values checked: 6, 7, 8, 9 and 9.5. Best results for nalbuphine and 6-monoacetylmorphine extraction were reached with a pH 8 phosphate buffer, used both for the conditioning step and dilution of plasma samples.

The proportion of isopropanol introduced in the HPLC mobile phase for the dissolution of dry sample residue also had a significance for the recovery. The

Table 1
Extraction procedure recoveries for nalbuphine and 6-monoacetylmorphine

Concentration (ng/ml)	Recovery (mean±S.D.) (%)	C.V. (%)
Nalbuphine (n=12)		
Blank	ND[a]	–
5	94±4.3	4.6
40	95±3.9	4.1
100	97±8.1	8.4
6-Monoacetylmorphine (n=7)		
Blank	ND[a]	–
10	98±6.2	6.3

[a] Not detectable.

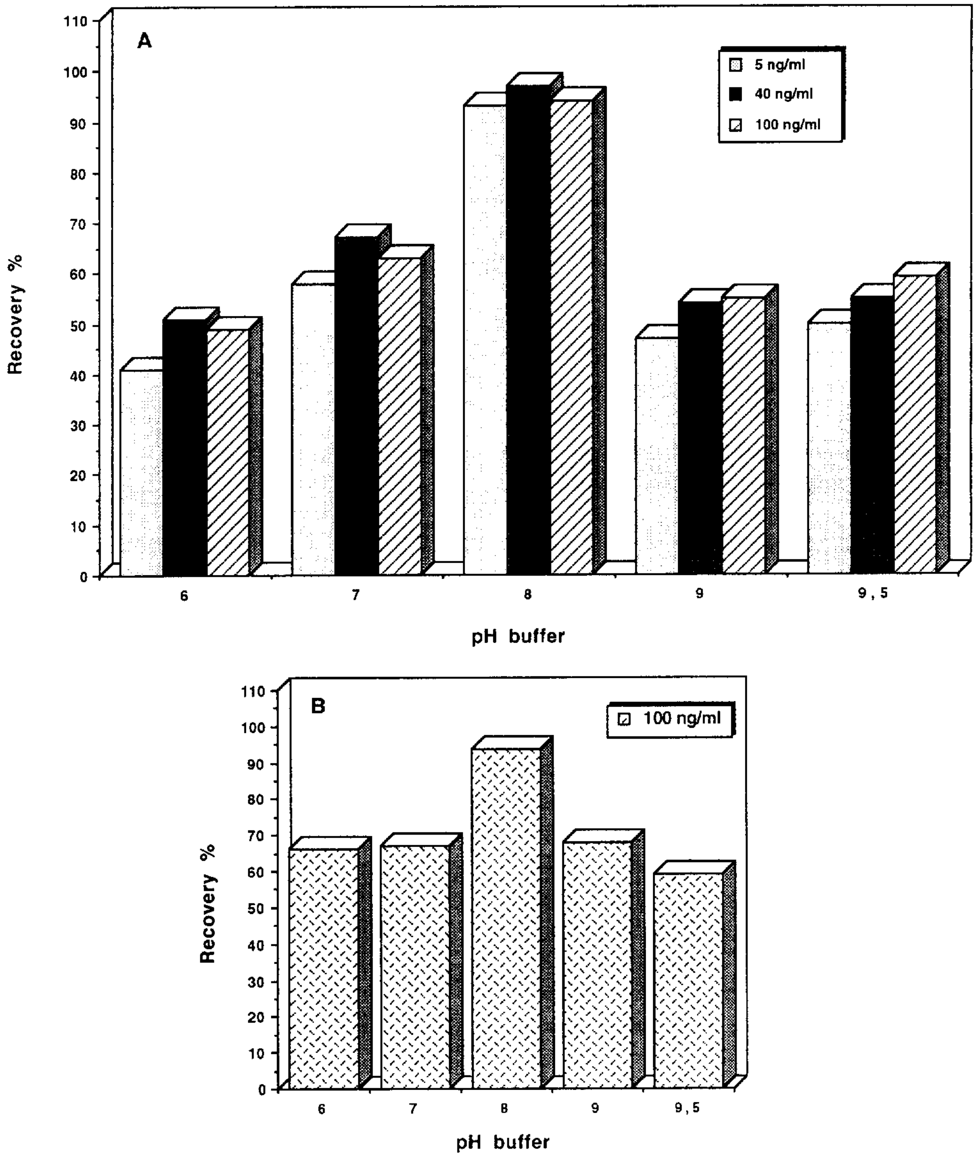

Fig. 1. Effect of buffer pH on recovery of nalbuphine (A) and 6-monoacetylmorphine (B).

results at the concentration 40 ng/ml for 0 and 2% of isopropanol were 65 and 95%, respectively.

3.2. Precision and accuracy

The intra- and inter-assay results were found by processing spiked plasma samples for three fortification levels with respect to a daily calibration curve covering concentration range 5–100 ng/ml of nalbuphine (Table 2). The intra-day coefficients of variation ($n=8$) of samples corresponding to concentrations 5, 40 and 100 ng/ml analyzed on the same day and under the same conditions were 5.3, 3.0 and 2.3%, respectively. The inter-day coefficients of variation ($n=10$) at three concentration levels: 5, 40 and 100 ng/ml analyzed on different days, over a 1-month period, were 7.7, 3.2 and 3.4%, respectively.

Table 2
Precision of the method

Theoretical concentration (ng/ml)	Observed concentration (ng/ml)[a]		C.V. (%)	
	Intra-assay[b]	Inter-assay[c]	Intra-assay[b]	Inter-assay[c]
5	5.18±0.27	5.59±0.43	5.3	7.7
40	38.93±1.18	39.80±1.27	3.0	3.2
100	97.05±2.20	99.32±3.39	2.3	3.4

[a] Mean±S.D.
[b] $n=8$.
[c] $n=10$.

3.3. Linearity

3.3.1. Linearity between 5 and 100 ng/ml

The linearity was examined and the results were linear over the range 5–100 ng/ml of nalbuphine. A straight line passing through the origin was obtained. The regression line calculated using the least square method was $y=-0.0202+0.0239x$ ($n=15$) with the correlation coefficient $r=0.9993$ (Table 3). As the use of the correlation coefficient is not always sufficient to assess linearity, an analysis of variance (ANOVA) was carried out. Verification of the linear model was assessed with a F Snedecor test. Its experimental value ($F_{\text{calculated}}=3.39$) was less than tabulated F Snedecor distribution $F_{(0.05;3;10)}=3.71$, consequently the model was linear. The Null hypothesis of the slope ($b=0$) was also tested with an F Snedecor test. $F_{\text{calculated}}=8866$ was greater than tabulated $F_{(0.05;1;13)}=4.67$, so the Null hypothesis was rejected. The proportionality test of the method (hypothesis $a=0$) was assessed with a student t test, $t_{\text{calculated}}=-1.42$ was less than the critical value $t_{(0.05;13)}=2.16$. Therefore, the Null hypothesis is retained and the independent term is not significantly different from zero.

Table 3
Linearity of the method between 5 and 100 ng/ml nalbuphine: peak-height ratio (nalbuphine/6-monoacetylmorphine)

Day	Peak-height ratio					
	Conc. (ng/ml)	5	20	40	60	100
1		0.14	0.47	0.94	1.42	2.38
2		0.12	0.44	0.90	1.38	2.36
3		0.12	0.46	0.88	1.40	2.44

Equation of the regression line: $y=-0.0202+0.0239x$ ($n^{a}=15$, $r=0.9993$, $P<0.005$).
[a] n=total number of points to establish the curve.

3.3.2. Linearity between 50 and 500 ng/ml

In the aim to extend the applications of this SPE method to other fields, we checked the linearity in the concentration range 50–500 ng/ml. The equation of the regression line was $y=0.0153+0.0579x$ ($n=15$, $r=0.9993$, $p=0.05$). The F Snedecor calculated test for assessing the linear model was 0.74 (less than $F_{(0.05;3;10)}=3.71$). In the test of the Null hypothesis of the slope ($b=0$), the F Snedecor test was found at 31799 (greater than tabulated $F_{(0.05;1;13)}=4.67$) allowing rejection of this hypothesis. The student t test for testing the proportionality of the method ($a=0$) was $t_{\text{calculated}}=2.14$ (less than the critical value $t_{(0.05;13)}=2.16$) allows to assess the independent term is not significantly different from zero.

So, the method was considered to be linear between 5–100 and 50–500 ng/ml of nalbuphine.

3.4. Limits of detection and quantification

The limit of detection (LOD) is defined as the lower signal of the analyte, expressed in concentration, that can be discerned from a blank plasma extracted in the same conditions. All the reagents were added to 500 μl blank human plasma, except the analyte, and then extracted by SPE procedure. With the gain of the detector set at 9900, the height (h_{max}) of the baseline, observed upon a distance of twenty-fold the nalbuphine peak width, at half-height, was determined. Then, from a nalbuphine spiked plasma, several successive dilutions were carried out to obtain the lower concentration giving an instrumental signal (H) three times as great as background noise ($H=3\times h_{\text{max}}$). In these procedural conditions, using 500 μl of plasma and 50-μl volumes injected, the LOD was estimated at 0.02 ng/ml ($n=8$).

The limit of quantification (LOQ) was calculated from the equation: $LOQ=(LOD/3)\times 10$ and found at 0.1 ng/ml (the 3 factor corresponding to a risk of 0.1% of concluding that the analyte is present, even though it is absent). The accuracy and precision at this concentration were estimated from plasma samples spiked with nalbuphine and extracted with SPE technique (actual concentration: mean±S.D.= 0.110±0.014 ng/ml, C.V.=12.4%, $n=8$). By increasing the amount of plasma placed on the SPE column or the quantity of extract injected, sensitivity can be increased further, if necessary.

3.5. Plasma interferences

By the following extraction and HPLC procedure, patient plasma samples receiving nalbuphine and spiked with internal standard gave only two chromatographic peaks (Fig. 2A). Blank plasma extractions showed neither impurities nor interfering peaks (Fig. 2B). Interference was studied with various phenolic compounds also electractive at the potential of +0.40 V: morphine, nalorphine, naloxone, naltrexone and norbuprenorphine. Morphine and nalorphine eluted with the solvent front. Naloxone and naltrexone had a close retention time of 5 and 6.5 min, respectively. A typical chromatogram of a pure solution mixture of naloxone, naltrexone, 6-monoacetylmorphine and nalbuphine is shown in Fig. 2C. Due to its very long retention time observed close to 60 min, norbuprenorphine is not perceptible in Fig. 2C. A graph of a calibration point at 60 ng/ml of nalbuphine is given in Fig. 2D.

3.6. Comparison of the assay

Forty spiked plasma samples were quantified using both solid-phase extraction detailed herein and previ-

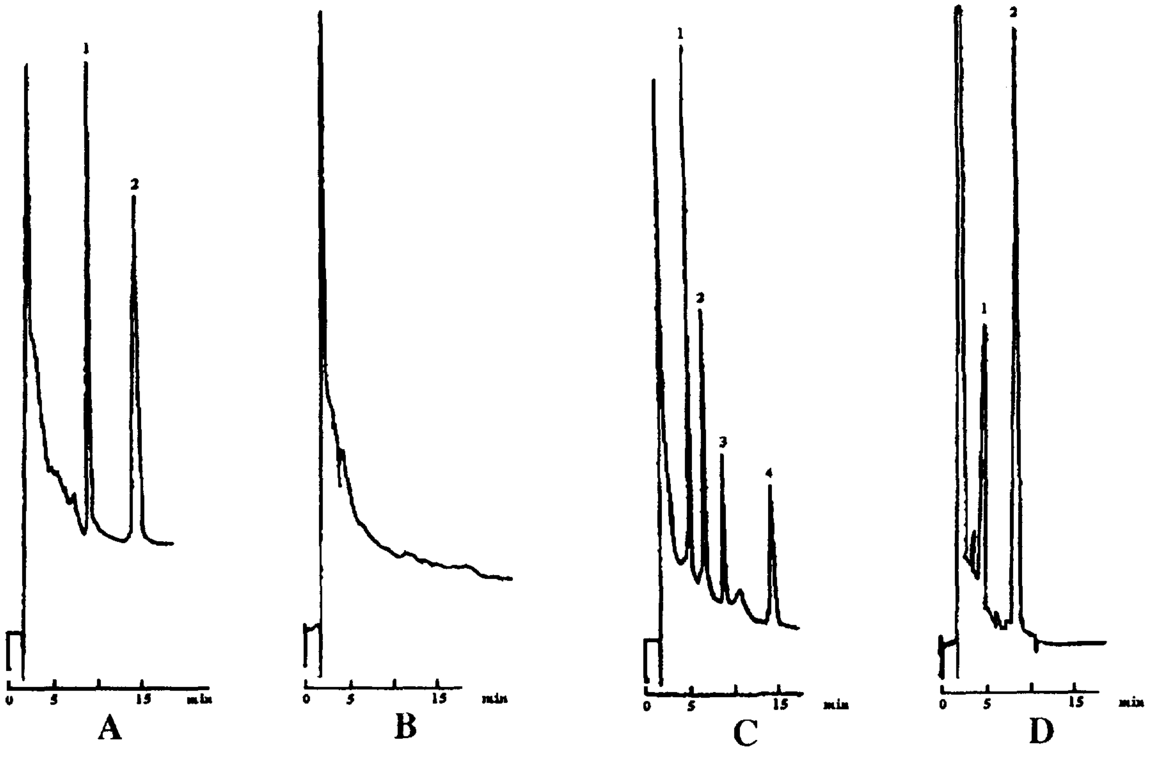

Fig. 2. HPLC profiles of (A) spiked 6-monoacetylmorphine (peak 1: 20 ng/ml) sample from a patient treated by rectal administration of nalbuphine (peak 2: 20 ng/ml), (B) blank plasma sample, (C) pure solution mixture containing naloxone (peak 1: 100 ng/ml), naltrexone (peak 2: 40 ng/ml), 6-monoacetylmorphine (peak 3: 6 ng/ml) and nalbuphine (peak 4: 10 ng/ml), (D) calibration point spiked with 6-monoacetylmorphine (peak 1: 20 ng/ml) and nalbuphine (peak 2: 60 ng/ml).

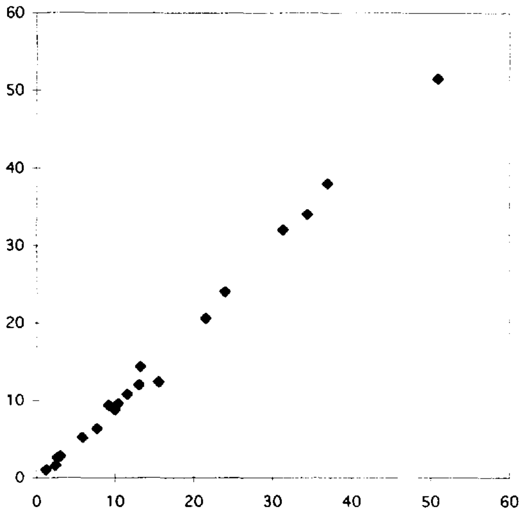

Fig. 3. Correlation between solid-phase extraction (x) and liquid–liquid extraction (y). Nalbuphine concentrations are expressed in ng/ml.

ously published liquid–liquid extraction followed by an identical HPLC analysis and coulometric detection [13]. Linear regression analysis (Fig. 3) gave a coefficient of correlation of 0.998 and an equation of the regression line $y=-0.815+1.025x$ ($P=0.0001$; $n=38$).

3.7. Application

The plasma concentration–time profile in one pediatric patient, following intra-rectal administration of nalbuphine (0.3 mg/kg), before anaesthesia, is presented in Fig. 4.

4. Discussion

The determination of nalbuphine plasma concentrations by means of HPLC with electrochemical detection is well documented [7–13]. Recently, we proposed a method of analysis involving a liquid–liquid extraction [13] which provided satisfactory selectivity, reproducibility and efficiency but included exacting handling of organic solvents and biological materials giving rise both to toxic and infectious risks.

Besides, in view of a large routine utilization in a clinical laboratory, it was interesting to develop a new extraction procedure easier than liquid–liquid extraction, easily automated, and affording more safety. Solid-phase extraction (in as much as the physical manipulations, solvent requirements and biological sample handling are much more limited than for liquid–liquid systems) seems to be a method of choice for nalbuphine determination in plasma. SPE procedure was never reported for nalbuphine determination in biological fluids. However, in the meantime of our study, an SPE technique followed by a gas chromatographic method coupled with mass-spectrometry was developed for the measurement of nalbuphine concentrations in drug abuser's urine [16]. This assay has not been extensively validated and gave a high cut off level of 0.1 μg/ml, which is not sufficient for pharmacokinetic studies.

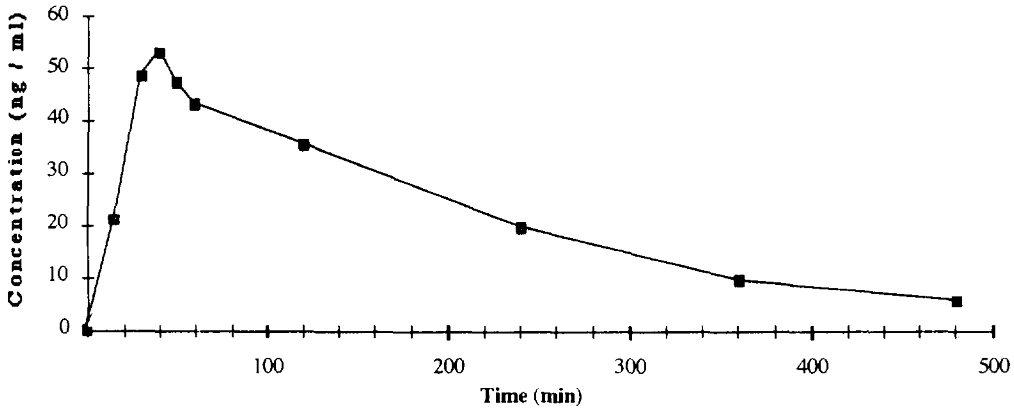

Fig. 4. Plasma concentration–time profile in one pediatric patient after a rectal administration of 7.2 mg nalbuphine.

In the present paper, we describe and validate an SPE procedure followed by a HPLC-electrochemical detection method. The coulometric detection was chosen for its high sensitivity and selectivity. As we noted in a previous work, the potential of +0.40V, that corresponds approximately to the half-wave oxidation potential of nalbuphine, provides a good compromise between sensitivity and difficulties inherent in the use of a high applied potential: baseline instability, noise and more frequent cleaning of the electrode [13].

Due to its retention time of 8.4 min, 6-monoacetylmorphine used as internal standard was well resolved from the solvent front. It allowed an easier reading of the graphs than the usually used naltrexone (3.9 min) and seemed to be the best choice for the internal standardization of the assay. Thus, the method described herein led to an effective, simple and rapid nalbuphine determination with chromatograms clear of interfering peaks at the retention time of nalbuphine and internal standard.

The recoveries of nalbuphine and 6-monoacetylmorphine were better than those previously reported by our laboratory: 95% vs. 86% and 98% vs. 85% for nalbuphine and 6-monoacetylmorphine, respectively. The recovery of nalbuphine was optimised with respect to the use of a phosphate buffer adjusted to pH 8, for columns conditioning and dilution of the plasma sample. As a matter of fact, two different ionizable groups exist in nalbuphine with two pK_a values associated, at 8.71 for the amine group, and 9.96 for the phenolic group.

Bond Elute Certify sorbent is a mixture of C_{18} phase and cationic resin intended for the improvement of morphinic compound recoveries. At pH 8, the majority of phenolic groups are in their unionized form and retained by the apolar C_{18} packing, while the protonated amine groups are not retained. Besides, the cationic phase provides fixation of the phenolic groups remaining ionized and, so, improves the extraction efficacy. Thus, compounds of interest adsorbed on the phase can be washed by water and methanol in acidic medium (acetic acid 10 m*M*, pH 3.3). Subsequently, nalbuphine is eluted by organic solvents in a strongly basic medium (NH_4OH 1%) which converts the phenolic group to an unretained ionized form. Moreover, the addition of 2% isopropanol in the mobile phase at the time of dry residue dissolution, substantially increased the nalbuphine recovery (ca. 20%). Isopropanol, indeed, is well known to improve the recovery of morphinic compounds, due to their adsorption on the glass material.

It is noteworthy that with this SPE technique, a constant extraction recovery is obtained in a concentration range between 5–100 ng/ml (Fig. 1A). Moreover, the linearity was proved between 5–100 ng/ml and also between 50–500 ng/ml, that represents a domain of linearity much larger than the one previously reported by our laboratory in liquid–liquid partitioning. In this previous method, the extraction seemed to be limited for medium concentrations, over 100 ng/ml [13].

Thus, this SPE method is more effective, even for high concentrations of nalbuphine, allowing applications in other fields than pharmacokinetic study in children. Besides, in the concentration range of 0–50 ng/ml, corresponding to the concentration range of studied patients, the nalbuphine assay described here compared well with the liquid–liquid extraction method (Fig. 3).

5. Conclusion

The high efficacy of the solid–liquid systems leads to accurate reading of nalbuphine levels, even at very low concentrations (LOQ: 0.1 ng/ml), from a very small volume of plasma (500 μl) and small volume injected (50 μl). In addition to its high sensitivity and reliability, this rapid assay offers several interesting advantages for routine clinical practice, especially the use of non-toxic solvents, and the perspective of an easy subsequent automatization. So this technique represents an improvement over the liquid–liquid extractions previously described, demonstrating its usefulness to obtain meaningful pharmacokinetic results.

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