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High-performance liquid chromatographic method for the simultaneous determination of nalbuphine and its prodrug, sebacoyl dinalbuphine ester, in dog plasma and application to pharmacokinetic studies in dogs

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Abstract

For the determination of nalbuphine and its long acting prodrug, sebacoyl dinalbuphine ester (SDN), in biological samples, a reversed-phase high-performance liquid chromatographic method using dual detectors was established. Ultraviolet and fluorescence detectors were connected in series for determining SDN and nalbuphine, respectively. The two analytes and internal standard were extracted from plasma by alkaline liquid–liquid extraction using *n*-hexane–isoamyl alcohol (9:1, v/v). The calibration curve for nalbuphine was linear over the range from 10 to 2500 ng/ml, while the range was 25 to 2500 ng/ml for SDN. The within- and between-day precision and accuracy were all within 10% for both nalbuphine and SDN over these concentrations. The method was applied successfully to a pharmacokinetic study of SDN administered at 20 mg/kg to two beagle dogs. Pharmacokinetic analysis revealed that SDN followed a linear one-compartment model with an elimination half-life of 74.7 min. Formation of nalbuphine after intravenous administration of SDN was observed in the first time point sample (5 min). These results indicate that SDN is rapidly metabolized to its active moiety, nalbuphine, in dogs and no other metabolites are detected in plasma.

Keywords: Nalbuphine; Sebacoyl dinalbuphine ester

1. Introduction

Nalbuphine is a semi-synthetic narcotic kappa receptor agonist/mu receptor antagonist [1] and is an effective analgesic for relief of moderate to severe pain [2–6]. However, nalbuphine undergoes extensive first-pass metabolism after oral administration. The oral dosing interval of nalbuphine is 3–6 h. This causes major inconvenience in clinical therapy like other narcotic analgesics. SDN is a novel synthetic di-ester prodrug of nalbuphine which prolongs its duration of action (Fig. 1). Previous testing of the ester prodrug indicates that the safety profile and pharmacological actions are similar to the parent compound [7–10].

To study the in vivo metabolism of the prodrug, a

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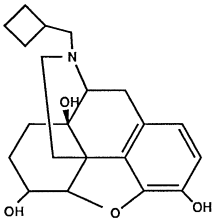
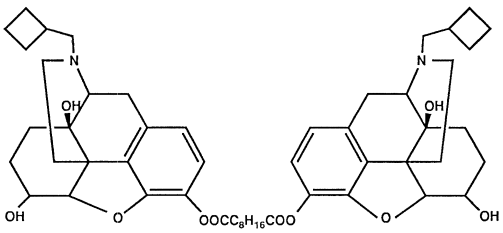


Nalbuphine



Sebacoyl dinalbuphine ester

Fig. 1. Structures of sebacoyl dinalbuphine ester (SDN) and nalbuphine.

sensitive, precise and accurate assay method is essential. The sample preparation process should be as simple as possible to avoid any decomposition of the prodrug. Furthermore, the pH of the extraction procedure is especially critical for an ester since the prodrug may hydrolyze when the sample pH is not controlled. Although many analytic methods have been reported for the analysis of nalbuphine in biological fluids [11–15], none of these methods is suitable for the analysis of nalbuphine and SDN simultaneously. We have developed a simple, rapid, sensitive, and selective high-performance liquid chromatography (HPLC) method with a one-step extraction procedure for simultaneous determination of nalbuphine and its di-ester prodrug and have used this method successfully in an intravenous pharmacokinetic study of SDN in dogs.

2. Experimental

2.1. Materials

Sebacoyl dinalbuphine ester (SDN) was prepared by Yung-Shin Pharmaceutical (Taiwan, ROC) by a method obtained from the Pharmaceutical Research Institute, National Defense Medical Center. To a 100 ml round-bottomed flask containing 40 ml methylene chloride, 5 g nalbuphine was added and dissolved. Then, 4.4 ml triethylamine was added, and 1.51 g sebacoyl chloride in 2 ml methylene chloride was added dropwise over 30 min. The suspension was stirred at ambient temperature for 1 h and then cooled to 0–4°C. After filtration, the product was washed three times with 5 ml water and twice with 5% citric acid solution and then dried over magnesium sulfate. The solid was dissolved in hot methanol and recrystallized in an ethyl acetate and isopropyl alcohol (4:1, v/v) mixture. The structure of SDN was identified by high-resolution mass spectrophotometry, ^{13}C-NMR, and IR spectrophotometry. Nalbuphine and ethylmorphine (HPLC internal standard; I.S.) were purchased from the Narcotic Bureau, Department of Health, Executive Yuan, ROC. All chemicals and solvents were analytical or HPLC grade. Sodium carbonate was purchased from Riedel-de Haen (Seelze, Germany). Acetonitrile, methanol, *n*-hexane, and isopropyl alcohol were obtained from Merck (Darmstadt, Germany). Water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Chromatography

The HPLC system consisted of a pump (Spectroflow 400, Applied Biosystem, Ramsey, NJ, USA), a 712 WISP autosampler (Waters, MA, USA), and two detectors, a SPD-10A variable-wavelength UV detector set at 210 nm at a sensitivity of 0.001 a.u.f.s. and a RF-551 fluorescence detector (excitation at 210 nm and emission at 345 nm), both from Shimadzu (Japan). The two detectors were connected in series such that the sample passed the UV detector first and then the fluorescence detector for the determination of SDN and nalbuphine, respectively.

Chromatographic separations were obtained using a μProsil column (300×3.9 mm, 10 μm particle size, Waters) and a 15×3.2 mm, 10 μm particle pre-column (Applied Biosystems, San Jose, CA, USA). The column was maintained at 40°C in a column oven. Nalbuphine, SDN, and ethylmorphine were eluted isocratically with a mobile phase consisting of 5 m*M* sodium acetate buffer (pH 3.75) and acetonitrile (15:85, v/v) at a flow-rate of 1.3 ml/

min. Peak heights were recorded by a Hem-Win integrator (Scientific Information Service, Taipei, Taiwan, ROC).

2.3. Sample preparation

Plasma samples were obtained from blood by centrifugation at 0°C to inhibit enzymatic hydrolysis of the prodrug. The extraction solution consisted of 20 μl I.S., 1 ml 0.5 *M* sodium bicarbonate and 3 ml of a mixture of *n*-hexane–isoamyl alcohol (9:1, v/v). Hydrolysis of the prodrug during extraction was further reduced by the addition of plasma into ice-cold extraction solution as soon as possible after collection. Analytes were extracted on a rotary shaker for 30 min at 100 rpm. After centrifugation for 10 min at 3000 rpm (1080 *g*), the aqueous layer was frozen by placing it in a freezer at −80°C for 20 min. The organic layer was then transferred to clean tubes and evaporated to dryness under vacuum. The sample was reconstituted in 300 μl of acetonitrile and aliquots of 200 μl were injected onto the column.

2.4. Calibration graphs

Stock solutions of 500 μg/ml of nalbuphine and SDN were prepared separately in acetonitrile. Ethylmorphine (IS) was prepared at 40 μg/ml in acetonitrile. Calibration standards were prepared by adding known amounts of nalbuphine (10–2500 ng/ml) and SDN (25–2500 ng/ml) and 20 μl of internal standard to 0.5 ml of plasma. Sample extraction and HPLC analysis were carried out as described above. Concentrations of nalbuphine and SDN were calculated from a linear least-squares-fitted line of peak-height ratios of nalbuphine and SDN to the internal standard versus standard concentrations using $1/x^2$ weighting.

2.5. Repeatability, precision and accuracy

The repeatability of the method was estimated by comparing the linear regression slopes and correlation of the standard graphs from plasma. Precision and accuracy were determined by back calculation of spiked plasma samples at eight concentrations with respect to a calibration graph prepared each day. The precision was expressed as the within-day and between-day coefficient of variation (%). Accuracy was calculated as the mean deviation of each concentration from the theoretical value.

2.6. Pharmacokinetic study

SDN solution for intravenous administration was prepared in sesame oil. Following the guidelines of the American Association for the Accreditation of Laboratory Animal Care, two male beagle dogs (12 and 13.5 kg) were administered 20 mg/kg intravenous SDN after an overnight fast. A 10-ml blood sample was obtained predose from a foreleg vein and 1 ml of blood was obtained at 5, 10, 15, 20, 30, and 45 min and at 1, 1.5, 2, 3, 4, 5, 6, and 7 h after intravenous administration of the drug. Blood samples were centrifuged immediately to separate plasma, which was stored at −80°C pending analysis by the analytical method described above. Plasma concentrations were fitted to a one-compartment open model using the computer program Kinetica 2.0 (MicroPharm International), with reciprocal concentration weighting. Pharmacokinetic parameters such as half-life, clearance, apparent volume distribution and area under the plasma concentration–time curve were calculated for each dog by standard methods [16].

3. Results and discussion

3.1. Chromatography

Representative chromatograms of plasma extract from the same sample are shown in Fig. 2. Fig. 2A and B represent the chromatograms for the determination of SDN obtained by UV monitoring at 210 nm. Fig. 2C and D are the chromatograms of nalbuphine obtained using the fluorescence detector. Fig. 2A and C were obtained from blank plasma extracts and show no interfering peaks. Fig. 2B and D are from plasma samples spiked with SDN, nalbuphine, and internal standard. With the described system, the retention times for nalbuphine, internal standard, and SDN are 10.3, 18.2, and 31.1 min, respectively, and the sample was completely eluted in 45 min. The low limits of quantitation for SDN

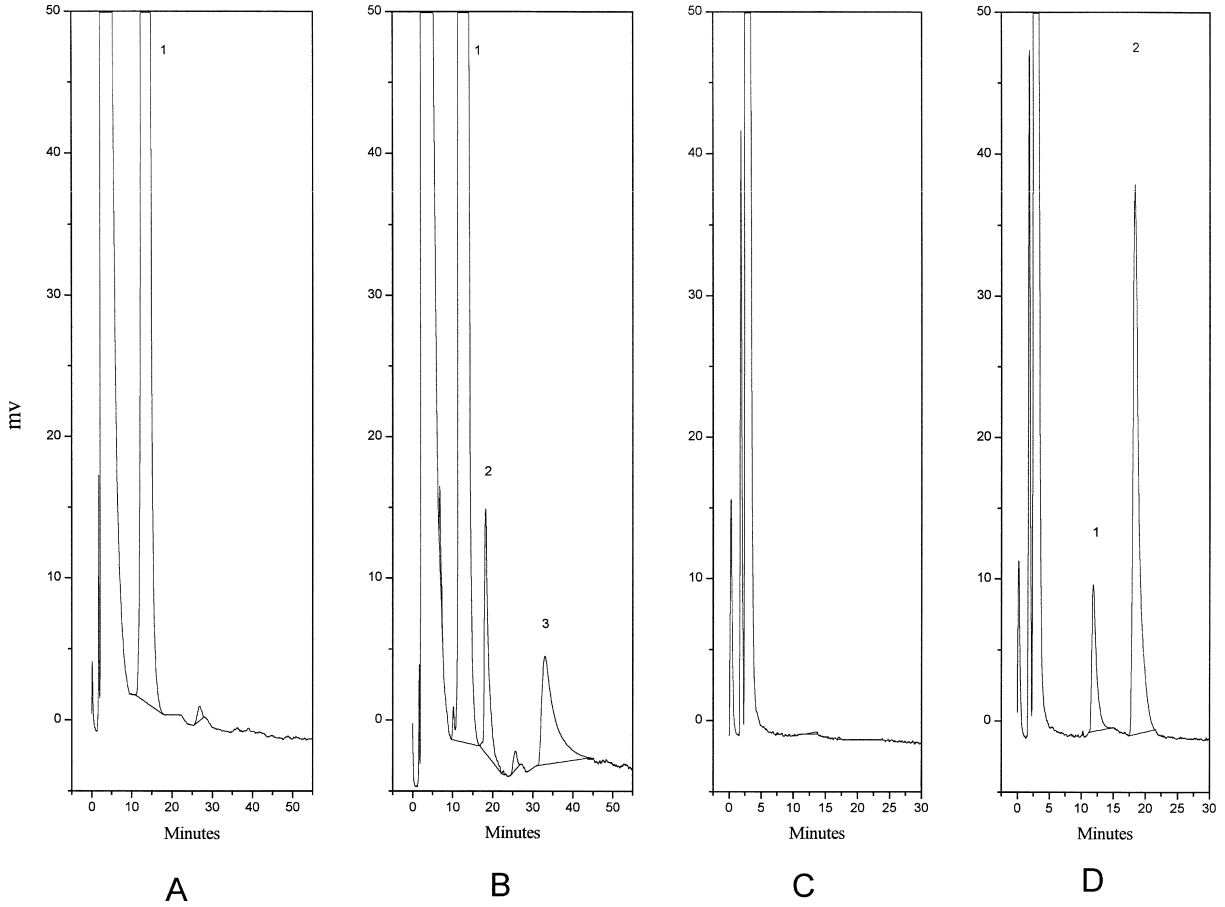


Fig. 2. Chromatograms of extracts from blank plasma (A and C) and plasma spiked with 50 ng/ml SDN and 50 ng/ml nalbuphine (B and D). (A) and (B) were detected by UV. (C) and (D) were detected by fluorescence. Peaks of (A) and (B): 1 = impurity, 2 = IS, 3 = SDN. Peaks of (C) and (D): 1 = nalbuphine, 2 = IS.

and nalbuphine in 0.5 ml plasma were 25 and 10 ng/ml, respectively.

3.2. Repeatability, precision and accuracy

The calibration curves ($n = 6$) for within- and between-day analyses were obtained by plotting the peak height ratio versus concentration. The value of peak height of the I.S. was read from the fluorescent trace since the interference peak may be observed in the UV trace. Over the concentration range examined, the calibration curves for both within- and between-day analysis were linear and the mean correlation coefficients were all >0.996 for both drugs (Table 1). Precision and accuracy were determined by analyzing spiked plasma samples at eight concentrations for nalbuphine (10–2500 ng/ml) and SDN (25–25 000 ng/ml) with respect to a calibration graph and the results are shown in Table 1. For within-day analysis, the coefficients of variation were all within 10% for both analytes and the deviation from the expected concentration, as a measurement of accuracy, ranged from -7.9 to $+3.3\%$ for SDN and -5.5 to $+8.7\%$ for nalbuphine. For between-day analysis, the coefficients of variation were also all within 10% for both drugs and the deviation from the expected concentration ranged from -6.3 to $+2.6\%$ for SDN and from -5.8 to $+7.0\%$ for nalbuphine. These results indicate that the method is precise and accurate.

Table 1
Precision and accuracy of SDN and nalbuphine determination in plasma by the HPLC method[a]

Conc. (ng/ml)	SDN			Nalbuphine		
	Conc. found (ng/ml)	C.V. (%)	Accuracy (% mean deviation)	Conc. found (ng/ml)	C.V. (%)	Accuracy (% mean deviation)
Within-day ($n = 6$)						
10	–	–	–	9.5±0.2	2.4	−4.5
25	25±1	4.4	0.3	27±1	3.4	8.7
50	52±4	6.9	3.2	54±2	3.9	7.9
100	92±4	4.4	−7.9	97±5	4.9	−2.7
250	248±21	8.6	−0.6	241±14	5.9	−3.6
500	517±20	4.0	3.3	473±26	5.6	−5.5
1000	1005±51	5.1	0.5	1023±51	5.0	2.3
2500	2533±137	5.4	1.3	2438±226	9.3	−2.5
Between-day ($n = 6$)						
10	–	–	–	9.6±0.3	2.6	−3.8
25	25±1	4.8	1.1	27±1	3.2	7.0
50	51±4	7.7	0.9	53±4	6.9	6.2
100	94±5	5.3	−6.3	101±5	5.2	1.1
250	247±23	9.4	−1.2	237±22	9.4	−5.3
500	510±20	4.0	2.0	471±33	7.0	−5.8
1000	1009±65	6.4	0.9	1035±29	2.8	3.5
2500	2565±110	4.3	2.6	2429±217	8.9	−2.8

[a] Within-day. Linear regression line, $1/x^2$ weighting, all data: SDN, $y = 0.000367x - 0.00293$, $r = 0.9966 \pm 0.0014$; nalbuphine, $y = 0.000269x - 0.000087$, $r = 0.9962 \pm 0.0006$. Between-day. Linear regression line, $1/x^2$ weighting, all data: SDN, $y = 0.000369x - 0.00258$, $r = 0.9967 \pm 0.0024$, slope 0.000369 ± 0.000043, C.V. 11.6%; nalbuphine, $y = 0.00027x - 0.00027$, $r = 0.9960 \pm 0.00007$, slope 0.000270 ± 0.000008, C.V. 2.96%.

3.3. Recovery

The recovery was determined by comparing peak areas from unextracted standards with those of extracted standards, across the range of each standard curve. The mean recovery of the analytes was 76–96% for SDN and 78–98% for nalbuphine over the constructed calibration concentration ranges.

3.4. Pharmacokinetic study

This simple, precise and accurate HPLC method yields satisfactory results for the simultaneous determination of SDN and nalbuphine in plasma samples and has been used successfully in a pilot pharmacokinetic study of SDN in dogs following intravenous administration. The plasma concentration–time profiles of both SDN and nalbuphine in each dog are shown in Fig. 3. The pharmacokinetic parameters of both SDN and nalbuphine in dogs following intravenous administration are listed in Table 2. Pharmacokinetic analysis revealed that SDN concentrations fit a linear one-compartment model with an elimination half-life of 74.7 min after intravenous administration. The formation of nalbuphine following intravenous administration of SDN also followed a one-compartment model with a formation half-life of 21 min. More than 90% of the prodrug was converted to the active moiety, nalbuphine, within about 1 h of reaching the systemic circulation. Nalbuphine appeared in the plasma at the first time point of 5 min. Based on in vitro metabolism studies [17], the metabolic half-life of SDN in fresh human and dog plasma was reported to be 5.4 and 30.7 min, respectively. It is reasonable to predict that the conversion of SDN to nalbuphine will be much more rapid in human blood. This prodrug appears to fulfill several criteria proposed by Boder [18]. Basically, no unexpected compound (i.e., a metabolite derived from the non-active part of the prodrug) should be formed in vivo.

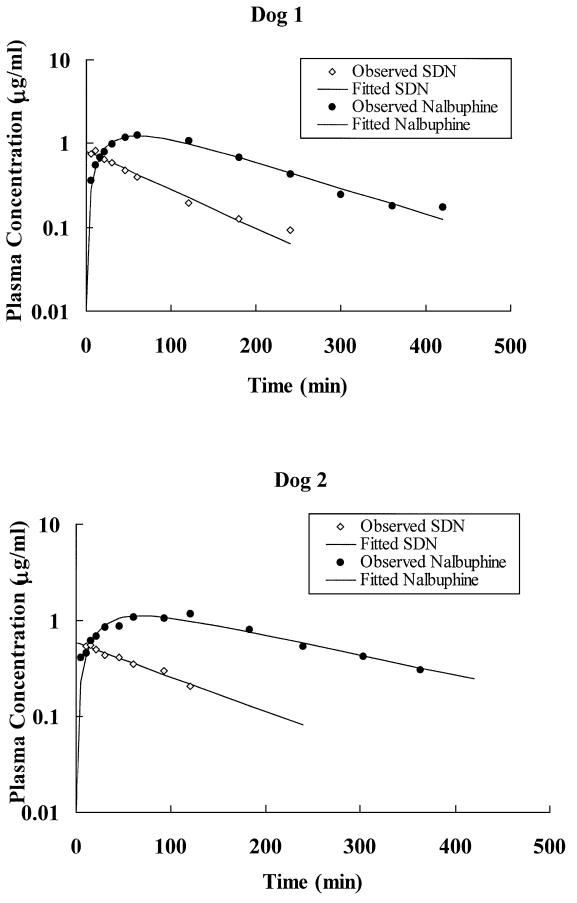


Fig. 3. Plasma concentration–time curve of SDN and nalbuphine after intravenous administration of 20 mg/kg SDN to dogs.

The elimination half-life of nalbuphine after i.v. and oral dosing in dogs was 1.2 and 2.2 h, respectively [19]. The elimination half-life of nalbuphine following i.v. administration of SDN averaged 2.1 h in this preliminary study. This result implies that the pharmacokinetic properties of nalbuphine converted from SDN in dogs appear to be unchanged.

A simple, sensitive, and accurate HPLC method has been described for the simultaneous determination of nalbuphine and its ester prodrug, SDN, in dog plasma using a one-step extraction. The method has been applied successfully to a pharmacokinetic study of SDN in dogs following intravenous administration, which demonstrated that SDN fulfilled the original prodrug design.

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Table 2
Pharmacokinetic parameters of SDN and nalbuphine following intravenous administration of SDN to dogs

Parameter	Dog 1		Dog 2	
	SDN	Nalbuphine	SDN	Nalbuphine
K_{el} (min^{-1})	0.0105	0.0070	0.0083	0.0047
K_f (min^{-1})[a]		0.0327		0.0247
$T_{1/2\,K_{el}}$ (min)	66.06	99.03	83.44	147.36
$T_{1,2\,K_f}$ (min)	21.22		20.90	
AUC_∞ (μg min/ml)	75.58	267.81	70.99	326.35
MRT (min)	95.27	142.87	120.38	212.60
V_d (l/kg)	25.21	10.67	33.91	13.02
Cl (ml/min/kg)	0.26		0.28	

[a] K_f is the the apparent first-order rate constant for nalbuphine formation.

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