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Determination of nalbuphine in human plasma by high-performance liquid chromatography with electrochemical detection
Application to a pharmacokinetic study

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Abstract

A high-performance liquid chromatographic procedure has been developed for the measurement of nalbuphine in plasma. Separation is performed on a reversed-phase analytical column (Ultrasphere ODS, 250×4.6 mm I.D., particle size, 5 μm). Mobile phase consisted of methanol–phosphate buffer (20:80, v/v) (pH 3.4). Electrochemical detection was performed using an ESA Coulochem II Model 5200 electrochemical detector equipped with a Model 5020 guard cell working at 550 mV and a Model 5021 analytical cell operating in the oxidation screening mode, with the potential of the first electrode set at 60 mV and the second electrode set at 450 mV. The method involves sample clean-up by liquid–liquid extraction using a chloroform–isopropanol mixture. After centrifugation, the organic phase was back-extracted with 17 m*M* phosphoric acid and then the aqueous phase was injected onto the column. The limits of quantitation and detection were 0.3 and 0.1 ng/ml, respectively. The extraction recovery was 91.1±3.7%. The intra- and inter-assay coefficients of variation were below 10%. Stability tests under various conditions have been performed. This method has been used to determine the pharmacokinetic parameters of nalbuphine in children.

Keywords: Nalbuphine

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1. Introduction

Nalbuphine, (−)-17-(cyclobutylmethyl)-4,5α-epoxymorphinan-3,6α,14-triol (Fig. 1), is a narcotic partial-agonist with analgesic efficacy similar to that of morphine [1]. It is structurally related to the

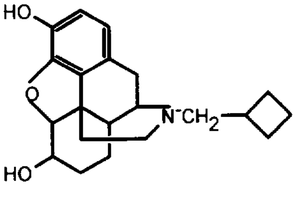

Fig. 1. Chemical structure of nalbuphine.

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narcotic analgesic oxymorphone and to the antagonist naloxone. Nalbuphine is an agonist at kappa receptors and an antagonist at other opioid receptors [2].

Nalbuphine undergoes an important hepatic metabolism in humans. The major metabolite is N-hydroxycetocyclobutylmethyl nornalbuphine, and other metabolites correspond to hydroxylated forms. This intensive biotransformation of the drug explains the importance of the first-pass effect. Nalbuphine can therefore be characterized as a perfusion-limited drug, and its hepatic clearance will be mainly dependent on hepatic blood flow [3,4]. Hepatic blood flow is high in children [4,5] while in 65-year-old patients it was equal to only 40 to 45% of the hepatic blood flow that was measured in young subjects (25 years of age) [4,6]. With respect to analgesic efficacy and respiratory depressant effects, nalbuphine has been shown to be approximately equipotent to morphine [7]. This drug is still commonly used in obstetrics after intravenous administration and in children after intravenous and rectal administrations.

A few methods have been described to detect nalbuphine in plasma; they include gas chromatography coupled to electron-capture detection [8] or mass spectrometry [9] and high-performance liquid chromatography (HPLC) with electrochemical detection [10–15]. The GC methods [8,9], although sensitive, involved expensive equipment and time-consuming preparation of samples and are not easily available for routine drug monitoring. The HPLC method of Lake et al. [10] does not provide adequate sensitivity, and while the methods published by Keegan and Kay [11], Lo et al. [12] and Wetzelsberger et al. [14] are sensitive (limit of detection of 0.05 to 0.3 ng/ml) they required 2–3 ml of plasma, hampering its use for drug monitoring in children. Moreover, most of the published techniques demonstrated several interferences when applied to clinical samples. However, the HPLC method proposed by Nicolle et al. [15] used a small sample volume of plasma and had a good sensitivity.

The purpose of this study was to develop a rapid, reproducible, reliable and highly selective HPLC method with electrochemical detection for the determination of nalbuphine in plasma. This method was validated with respect to accuracy, precision, selectivity and limits of quantitation and detection according to Good Laboratory Practice Guidelines [16,17].

2. Experimental

2.1. Materials and reagents

Nalbuphine was obtained from Sigma (St. Louis, MO, USA). Methanol, isopropanol and chloroform were Chromasol grade (SDS, Peypin, France) and used without further purification. Potassium dihydrogen phosphate and ethylenediaminetetraacetic acid (EDTA) were all analytical grade (Merck, Darmstadt, Germany). Buffer pH 9 (boric acid, potassium chloride, sodium hydroxide, bidistilled water) was obtained from Merck. Orthophosporic acid (17 m*M*) was prepared in purified water (Laboratoires Fandre, Ludres, France). The buffer (pH 3.4) consisted of 24 m*M* potassium dihydrogen phosphate and 0.03 m*M* EDTA adjusted to pH 3.4 with 17 m*M* orthophosphoric acid.

Stock solution of nalbuphine (0.1 mg/ml) was prepared in methanol and was stored at −30°C. Working solutions were freshly prepared before analysis by dilution of the stock solution with purified water in a ratio of 1:100 and 1:1000. They were used to spike the plasma samples prior to extraction. An unextracted working standard solution (8 ng/ml) in purified water was prepared daily to check the resolution of the chromatographic system.

For validation of the method, drug-free human plasma was obtained from pooled blood samples collected from healthy volunteers. Coagulation was prevented by adding EDTA-sodium salt. Then, the blood was centrifuged at 2000 *g* for 10 min. The obtained drug-free plasma was stored at −30°C before use.

2.2. Instrumentation

The chromatographic system consisted of a Model LC-9A solvent-delivery system (Shimadzu, Kyoto, Japan), an automatic sample injection system (Model 231; Gilson Medical electronic, Villiers le Bel, France) with a Rheodyne loading valve (Model 7010) fitted with a 50-μl sample loop, an oven (Du Pont de Nemours, Les Ulis, France), a guard column

(20×4.6 mm I.D.; SFCC, Neuilly Plaisance, France) packed with Hypersil ODS (5 μm) placed just before the inlet of the analytical column, and a stainless-steel column (250×4.6 mm I.D., Beckman, Fullerton, CA, USA) packed with Ultrasphere ODS (particle size, 5 μm). An ESA Coulochem II Model 5200 electrochemical detector (Touzart Matignon, Vitry sur Seine, France) was equipped with a Model 5020 guard cell working at 550 mV and a Model 5021 analytical cell operating in the oxidation screening mode, with the potential of the first electrode set at 60 mV and the second electrode set at 450 mV. The sensitivity of the detector was set at 2 nA. The signal was amplified by setting the multiplier gain switch to 100. All the chromatographic conditions were controlled using the GME 712 Gilson software.

2.3. Chromatographic conditions

Methanol and the phosphate buffer pH 3.4 were filtered through a membrane filter (0.45 μm; Millipore, Molsheim, France). The mobile phase containing methanol and phosphate buffer (20:80, v/v) was degassed by vacuum before use, then with a stream of helium during use. The flow-rate was 1.0 ml/min, which corresponds to a pressure of about 15 MPa. The oven temperature was 50°C. Before analysis the mobile phase was recycled over 12 h at a flow-rate of 1.0 ml/min then recycled during use over 40 h. Using such conditions, the baseline was stable for five days. The following two days, the chromatographic system was washed using a mixture of methanol–water (50:50, v/v); then, the guard cell was removed and flushed with methanol for 15 min. Before a new analysis the system was equilibrated with the mobile phase. Each month, the analytical cell was removed and flushed with deionized water for 1 h.

2.4. Sample processing

To 0.5 ml of plasma placed in a 15-ml glass centrifuge tube, fitted with a PTFE-lined screw cap was added 1 ml of borate buffer pH 9. The samples were extracted with 10 ml of chloroform–isopropanol (98:2, v/v) by mechanical shaking for 15 min. Subsequently the samples were centrifuged at 2000 *g* for 10 min. The lower layer was separated using phase separators silicone treated filter paper Whatman 1 PS (Prolabo, Paris, France) and the aqueous phase was discarded. The organic phase was transferred to another 15-ml glass tube and back-extracted with 200 μl of 17 m*M* phosphoric acid. After mechanical shaking for 2 min, the samples were centrifuged at 2000 *g* for 5 min. The aqueous phase was separated using Whatman filter and 50 μl of this solution were injected onto the column.

Calibration standards for control plasma were prepared using concentrations of 1, 2, 4, 6, 8, 10 and 14 ng/ml. The volume added was always smaller than or equal to 2% of the total volume of the samples, so that the integrity of the plasma was maintained.

2.5. Data analysis

Peak-height of nalbuphine was used as the assay parameter. Unweighted least squares linear regression of the peak heights as a function of the theoretical concentrations was applied to each standard curve.

The linearity of the method was confirmed by comparing the slopes and the intercepts of linear calibration curves with zero, and the correlation coefficients with 1. Moreover, the Kolmogorov–Smirnov test was used to compare the distribution of the residuals (difference between nominal and back-calculated concentrations) to the expected one (N(0,1)).

2.6. Specificity

To evaluate the specificity of the method, several pre-dose human plasma samples from different healthy subjects and patients were tested for the absence of interfering compounds. The retention times of endogenous compounds in plasma were compared with that of nalbuphine.

The interference from the metabolites of nalbuphine was also checked.

Plasma samples from patients receiving drugs that could be used in combination with nalbuphine were analysed for interference. The following drugs were checked: codeine, caffeine, naloxone, theobromine and theophylline.

2.7. Precision and accuracy

Inter-day and intra-day repeatabilities of the assay were assessed by performing replicate analyses of spiked samples at high, middle and low concentrations in plasma (2, 8 and 12 ng/ml) against a calibration curve. The procedure was repeated on different days on the same spiked standards to determine inter-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate the same day.

The accuracy was evaluated as error percent [(mean of measured−mean of added)/mean of added]×100, while the precision was given by the inter-day and intra-day coefficients of variation.

2.8. Determination of the limits of quantitation (LOQ) and detection (LOD)

The LOQ was determined from the peak and the standard deviation of the noise level (S_N). The LOQ was defined as the sample concentration of nalbuphine resulting in a peak height of 10 times S_N. The estimate S_N was determined by extrapolation to zero. To determine the analytical error in the LOQ, spiked samples were used.

The LOD was defined as the sample concentration resulting in a peak height of 4 times S_N.

2.9. Recovery

The extraction efficiency (recovery) was determined by comparing peak heights from drug-free plasma spiked with known amounts of drug (2, 6, 8, 10 and 12 ng/ml), assayed accordingly, versus peak heights of the same concentrations prepared in purified water injected directly onto the analytical column. Each sample was determined in triplicate.

In order to study the effect of co-extracted biological material, recoveries were also computed by comparison of extracts from spiked samples with blank extracts spiked after the extraction.

2.10. Stability study

The stability of nalbuphine in methanol (stock solution) and in water (working solution) was assessed over 4 weeks at −30°C and 12 h at 4°C, respectively.

For stability studies in a biological matrix, control human plasma samples were spiked with 2, 8 and 12 ng/ml of nalbuphine. The short-term stability was assessed after 1, 2, 4 and 6 h of storage at both ordinary laboratory conditions (20°C at daylight exposure) and at 4°C. The stability of nalbuphine in frozen human plasma (−30°C) was determined by periodic analysis over a span of 4 months; prior to their analyses, samples were brought to room temperature and vortex-mixed well. Spiked samples were analysed immediately after preparation (reference values) and after storage. Each determination was performed in triplicate.

The freeze–thaw stability was also determined. Spiked plasma was analysed immediately after preparation and on a daily basis after repeated freezing–thawing cycles at −30°C on three consecutive days.

The stability of nalbuphine in acidic extract was inspected during 20 h at 20°C and over 4 days at −30°C.

3. Results

3.1. Retention time and specificity

The retention time of nalbuphine is 13.4 min. In drug-free plasma samples, no peak interfered at the retention times of nalbuphine.

No interference was found with the metabolites of nalbuphine and with all drugs tested that could be co-administered.

Representative chromatograms are shown in Fig. 2.

3.2. Linearity

Peak height of nalbuphine varied linearly with concentration over the range used. The correlation coefficients (r) for calibration curves were equal or better than 0.992. Intra-day reproducibility was determined for calibration curves prepared the same day in replicate ($n=6$) using the same stock solutions. Inter-day reproducibility was determined for calibration curves prepared on different days ($n=12$). Results are given Table 1. For each point of cali-

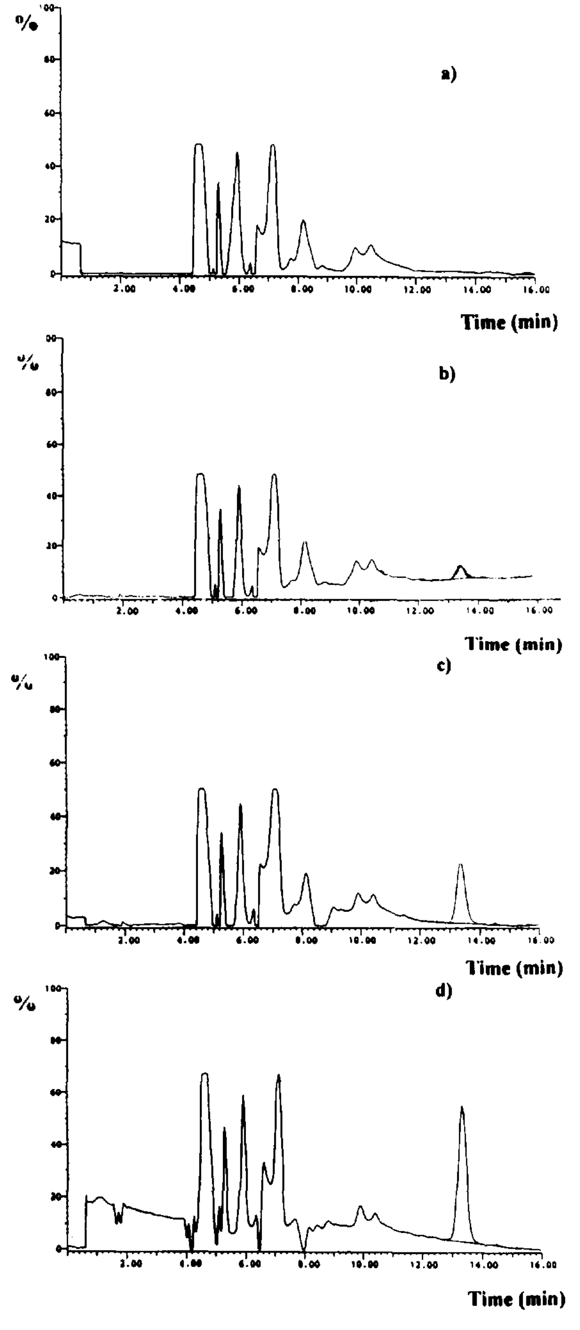

Fig. 2. Chromatograms of blank plasma (a) and of plasma spiked with 1 ng/ml (b), 6 ng/ml (c) and 14 ng/ml (d) of nalbuphine. For chromatographic conditions see Section 2.3.

bration standards, the concentrations were back-calculated from the equation of the linear regression curves (experimental concentrations) and the coefficients of variation (C.V.) were computed. Inter-day and intra-day variabilities at concentration of calibration standards are presented in Table 2. A linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to 0 (Student *t*-test).

For each calibration curve, the slope was significantly different from 0, and the intercept was not statistically different from zero. Moreover, the residuals (difference between nominal and back-calculated concentrations) were normally distributed and centred around zero (Kolmogorov–Smirnov test).

3.3. Precision and accuracy

For concentrations of calibration standards, the precision around the mean value did not exceed 10% (Table 2).

The results for accuracy, intra-day and inter-day precision are presented in Table 3.

3.4. Recovery

The mean recoveries averaged 91.1±3.7% (n= 15). The extraction efficiency is not statistically different over the range of concentrations studied. No effect of the co-extracted biological material was detected.

3.5. Limit of quantitation and limit of detection

The limit of quantification was 0.3 ng/ml. At this level the analytical error averaged 16.7±5.2%. The limit of detection was 0.1 ng/ml.

3.6. Stability

Stock and working solutions of nalbuphine were stable for at least 4 weeks and 12 h, respectively.

After storage at 20 and at 4°C, nalbuphine was stable in plasma for 6 h. Indeed, at 20°C, the percent recoveries averaged 95.5±2.5, 97.4±4.8 and 92.3±0.42% for 2, 8 and 12 ng/ml, respectively; they were 94.2±4.9, 100±5.1 and 99.1±4.3% at 4°C, respectively. Nalbuphine frozen at −30°C was

Table 1
Assay linearity

	Correlation coefficient of the linear regression analysis[a] ($r \pm$S.D.)	Slope (b) (mean±S.D.)	Intercept (a) (mean±S.D.)
Intra-day reproducibility ($n=6$)	$0.9942 \pm 3.01 \times 10^{-3}$ (C.V.=0.30%)	721.6±16.7 (C.V.=2.3%)	343.5±102.3 (C.V.=29.8%)
Inter-day reproducibility ($n=12$)	$0.9943 \pm 4.46 \times 10^{-3}$ (C.V.=0.45%)	678.2±28.0 (C.V.=4.13%)	349.9±171.9 (C.V.=49.1%)

r =correlation coefficient.

[a] Linear unweighted regression, formula: $y=a+bx$.

Table 2
Intra- and inter-assay reproducibilities of the HPLC analysis

Theoretical concentration (ng/ml)	Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=12$)	
	Experimental concentration (mean±S.D.) (ng/ml)	C.V. (%)	Experimental concentration (mean±S.D.) (ng/ml)	C.V. (%)
1	1.12±0.087	7.77	1.04±0.099	9.52
2	2.18±0.068	3.12	2.04±0.16	7.84
4	4.18±0.97	2.32	4.33±0.37	8.54
6	5.91±0.26	4.40	6.21±0.25	4.02
8	8.19±0.32	3.90	8.03±0.30	3.74
10	10.3±0.30	2.91	9.92±0.50	5.04
14	13.6±0.26	1.91	13.8±0.23	1.67

stable over 4 months: the mean percent recovery was 99.9±2.95%.

Run-time stability at room temperature of processed samples after extraction (acidic extracts) was determined for each point of calibration standard. After 20 h no significant losses occurred. Likewise, after storage of these extracts at −30°C, no degradation occurred over a span of 4 days.

Table 3
Intra-day and inter-day precision and accuracy of the HPLC method

Theoretical concentration (ng/ml)	n	Experimental concentration (mean±S.D.) (ng/ml)	C.V. (%)	Mean recovery[a] (%)	Accuracy[b] (%)
Intra-day precision					
2	7	2.15±0.19	8.84	107.5	7.5
8	8	7.88±0.35	4.44	98.5	1.5
12	8	12.5±0.55	4.40	104.2	4.2
Inter-day precision					
2	7	1.98±0.18	9.09	99.0	1.0
8	6	7.97±0.59	7.40	99.6	0.4
12	6	12.8±0.91	7.11	106.7	6.7

[a] Mean recovery was computed using the following equation: (mean of measured/mean of added)×100.

[b] Accuracy was evaluated by calculating the mean percent differences between theoretical values and measured values.

At least three freeze–thaw cycles can be tolerated without losses higher than 10%.

4. Discussion and conclusion

The present HPLC method enables a rapid assay of nalbuphine in plasma with a run time lower than 14 min.

Nalbuphine assays generally used naloxone [10,11] or naltrexone [13,14] as internal standard. Naloxone is not suitable as internal standard since this drug is used to reverse cardiorespiratory depression in neonates caused by nalbuphine administration to the mothers [18]. On the other hand, previous assays have shown that, according to the drug free plasma used, several awkward peaks due to the matrix occurred at the retention time of naltrexone, consequently this drug has not been retained in our study. Moreover, using the same chromatographic conditions published by Wetzelsberger et al. [14], the retention time of naltrexone was not in accordance with the one reported by the authors, indeed in our finding it was lower than nalbuphine. Nicolle et al. [15] employed the 6-monoacetylmorphine as an internal standard; unfortunately it is difficult to obtain this drug, moreover, it is known to be unstable. The following other drugs have been tested: morphine, codeine, dextromoramide, diethylacetyl morphine, nalorphine, buprenorphine, sufentanil, pentazocine and pethidine. None of them give a good separation with peaks provided from endogenous compounds. Consequently, in the present study, the quantitation of nalbuphine was performed without internal standard.

Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and from the results of quality control samples. This method has been validated for concentrations ranging from 1 to 14 ng/ml which is currently thought to be the clinically relevant range for nalbuphine concentrations in plasma. This assay is selective, reliable and has a good efficiency in terms of run time or sample throughput, respectively. Recovery of 91% was in the range found by other authors [12–15]; however, during the optimisation step of the extraction procedure, using the same sample pre-treatment as that reported by Nicolle et al. [15], we have found a recovery lower than 50%. The day-to-day variations were always lower than 10%.

In conclusion, the present method with a low limit of quantitation (0.3 ng/ml) is sufficiently rapid, sensitive and specific for the determination of nalbuphine in a large number of plasma samples during therapeutic drug monitoring. It has been shown that the elimination rate of nalbuphine decreased as the age of the subjects increased [4]. Since the analgesic effect and the risk of side effects are dependent of the dose, it seems necessary to adjust the dose depending on the patients. Moreover, the relative simplicity of this method permits its use for pharmacokinetic studies.

The reported method has been used for the determination of nalbuphine concentrations in plasma samples during pharmacokinetic investigation in children. After bolus intravenous administration, plasma concentrations of nalbuphine declined in a biexponential fashion. A representative plasma concentration–time curve obtained after administration of 3.3 mg at a child (age: 13 months, weight: 11 kg)

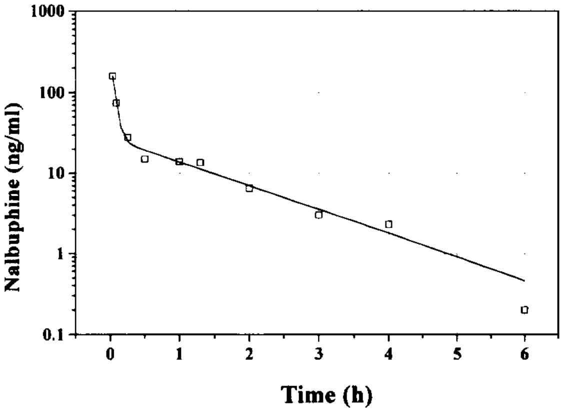

Fig. 3. Individual plasma concentration–time profile in a child receiving 3.3 mg nalbuphine intravenously. For concentrations higher than 14 ng/ml, plasma samples were diluted with drug-free plasma. To validate the use of diluted samples, QC samples were prepared under the same conditions. Six hours after drug administration, plasma concentration was 2 ng/ml (below the defined LOQ), in this case the extraction procedure has been performed using 1 ml of plasma instead of 0.5 ml. As the volume of the sample was increased, the selectivity of the method has been verified. Moreover, QC samples have been prepared under the same conditions to validate this procedure.

is shown in Fig. 3. The elimination half-life was 0.95 h; the total clearance was 5.3 l/h/kg and the volume of distribution was 7.2 l/kg.

References

[1] W.T. Beaver and G.A. Feise, Clin. Pharmacol. Ther., 24 (1978) 487.

[2] J.E. Hammond, Lancet, ii (1984) 1208.

[3] A.S. Nies, D.G. Shand and G.R. Wilkinson, Clin. Pharmacokin., 1 (1976) 135.

[4] P. Jaillon, M.E. Gardin, B. Lecocq, M.O. Richard, S. Meignan, Y. Blondel, J.C. Grippat, J. Bergnieres and O. Vergnoux, Clin. Pharmacol. Ther., 46 (1989) 226.

[5] P.L. Morselli, R. Franco-Morselli and L. Bossi, Clin. Pharmacokin., 5 (1980) 485.

[6] A.D. Bender, J. Am. Geriatr. Soc., 13 (1965) 192.

[7] J.K. Errick and R.C. Heel, Drugs, 26 (1983) 191.

[8] S.H. Weinstein, M. Alteras and J. Gaylord, J. Pharm. Sci., 67 (1978) 547.

[9] P. Kintz, A. Tracqui and P. Mangin, J. Chromatogr., 579 (1992) 172.

[10] C.L. Lake, C.A. DiFazio, E.N. Duckworth, J.C. Moscicki, J.S. Engle and C.G. Durbain, J. Chromatogr., 233 (1982) 410.

[11] M. Keegan and B. Kay, J. Chromatogr., 311 (1984) 223.

[12] M.W. Lo, G.P. Juergens and C.C. Whitney, Res. Commun. Chem. Pathol. Pharmacol., 43 (1984) 159.

[13] L.M. Dubré, N. Beaudoin, M. Lalande and I.J. McGilveray, J. Chromatogr., 427 (1988) 113.

[14] N. Wetzelsberger, P.W. Lücker and W. Erking, Arzneim. Forsch. Drug Res., 38 (1988) 1768.

[15] E. Nicolle, S. Michaut, F. Serre-Debeauvais and G. Bessard, J. Chromatogr. B, 663 (1995) 111.

[16] United States Pharmacopoeia XXXIII, The United States Pharmacopeial Convention, Rockville, MD, 1994, p. 1929.

[17] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 81 (1992) 309.

[18] R.R. Miller, Am. J. Hosp. Pharm., 37 (1980) 942.