ELSEVIER

Journal of Chromatography B, 694 (1997) 219–226

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic assay for methyl-β-cyclodextrin in plasma and cell lysate

Pierre Yves Grosse[a,b], Frédéric Pinguet[a], Jean Michel Joulia[a,b], Cécile Astre[a], Françoise Bressolle[a,b,*]

[a]*Laboratoire d'Onco-Pharmacologie, Centre Régional de Lutte contre le cancer, 34298 Montpellier Cédex, France*
[b]*Laboratoire de Pharmacocinétique, Faculté de Pharmacie, 34060 Montpellier Cédex 01, France*

Received 6 December 1996; revised 29 January 1997; accepted 3 February 1997

Abstract

This paper describes a high-performance liquid chromatographic method with fluorescence detection for the analysis of methyl-β-cyclodextrin (MEBCD) in plasma and cell lysate, after in situ complexation with 1-naphthol. The size-exclusion HPLC column packed with TSK 3000 SW gel, was equilibrated with an eluent mixture composed of methanol and purified water (2:98, v/v) containing 10^{-4} *M* 1-naphthol as a fluorophore. The detection is based on fluorescence enhancement caused by the formation of inclusion complexes and was performed at 290 and 360 nm for excitation and emission, respectively. The method involved a simple treatment of the samples with chloroform. Daunorubicin was used as internal standard. Limits of quantitation were 0.8 μ*M* in plasma and 0.5 μ*M* in cell lysate. Detection limits of 0.5 μ*M* (50 pmol) and 0.3 μ*M* (30 pmol) were obtained for MEBCD in the two media, respectively. Linear detection response was obtained for concentrations ranging from 1 to 100 μ*M* in plasma and cell lysate. Recovery from plasma proved to be more than 40%. Precision, expressed as C.V. was in the range of 4 to 11%. Accuracy ranged from 89 to 105%.

Keywords: Methyl-β-cyclodextrin

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides torus-shaped molecules consisting of 6, 7 or 8 glucopyranose units (α-, β-, γ-CD). Their particular spatial conformation enables them to form inclusion complexes with lipophilic compounds, changing their physical and chemical properties and enhancing their stability and solubility in water [1,2]. Since native CDs have low solubility in aqueous solvents, derivatives have been made by chemical modifications of the hydroxyl groups which greatly improve their solubility and their ability to dissolve hydrophobic compounds, as well as reducing their toxicity [1,3]. The use of β-CDs and their derivatives is now widespread in many fields like pharmaceutical and food industries, cosmetics, chemistry and biotechnology. In pharmaceutical research, they are used to improve drug stability, dissolution rate, bioavail-

*Corresponding author. Address for correspondence: Laboratoire de Pharmacocinétique, Faculté de Pharmacie, 34060 Montpellier Cedex 01, France.

0378-4347/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0378-4347(97)00103-5

ability and for reduction of side effects [1,2]. Recently, it has been reported that methyl-β-CD (MEBCD) improves nasal absorption of drugs [4,5] and enhances cellular membrane permeability to various molecules [6]. In a previous paper [7], we also reported that MEBCD could potentiate the cytotoxic activity of doxorubicin in a human cancer cell line. The major advanced mechanism is an interaction of MEBCD with cholesterol and lipids of the biological membranes [8]. After intravenous administration, β-CD and its derivatives are cleared by glomerular filtration [9].

In order to perform pharmacokinetic studies, sensitive analytical methods are required for the determination of MEBCD in plasma, urine, intratissular and intracellular media. A major obstacle to the development of such methods is the absence of a chromophore, electroactive or chemically reactive functional groups. While non-substituted CDs can be determined with a good sensitivity using pulsed-amperometric detection [10,11], substitution of hydroxyl groups by methylation results in a weak sensitivity using this mode of detection. High-performance liquid chromatographic (HPLC) methods with detection based on inclusion complex formation were described to quantify methylated CDs. One of them, using negative-colorimetric detection after post-column complexation with phenolphthalein, was described for the determination of CDs in biological fluids [12,13]. The complexity of this method, the high background and its lack of sensitivity limit its use for pharmacokinetic experiments. Another HPLC method [14] is based on fluorescence enhancement of a fluorophore (1-naphthol) added to the mobile phase, caused by the formation of inclusion complexes with MEBCD. This simple method was applied to the determination of MEBCD in urine and provided good results.

This paper describes a rapid, specific, reliable and sensitive analytical method for the quantitation of MEBCD in plasma and cell lysate based on complexation with the fluorophore 1-naphthol. For both media, the procedure involves the addition of an internal standard (daunorubicin). This method was validated according to Good Laboratory Practice guidelines [15–17]. Moreover, stability tests under various conditions have been performed.

2. Experimental

2.1. Materials and reagents

Methyl-β-cyclodextrin (MEBCD, degree of substitution: 10.5–14.7, Fig. 1), 1-naphthol, daunorubicin and PBS (phosphate buffer saline, pH 7.3) were obtained from Sigma (St. Louis, MO, USA). Chloroform and methanol were of LC grade (both from Prolabo, Paris, France). Potassium hydroxide pellets were purchased from Merck (Darmstadt, Germany). Pooled drug-free plasma samples from healthy volunteers were used for the validation of the method. HL-60 human leukemia cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). RPMI 1640 culture medium was purchased from Polylabo (Paris, France).

Stock solutions of MEBCD and internal standard were prepared in purified water (Laboratoires Aguettant, Lyon, France) at concentrations of 10^{-1} *M* for MEBCD and 0.5 mg/ml for daunorubicin and stored at −20°C. Standard solutions of MEBCD were obtained from stock solution by dilution with purified water 100-, 1000- and 10 000-fold, extemporaneously. They were used to spike the plasma and the cell lysate samples prior to extraction. An

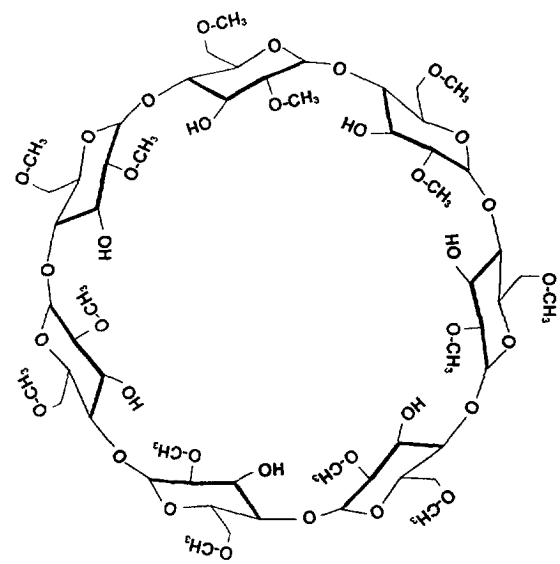

Fig. 1. Structural formulae of MEBCD used for the analytical method (degree of substitution: 14).

unextracted working standard solution of MEBCD (50 μ*M*) in purified water was prepared daily to check the efficiency of the column.

2.2. Instrumentation

The chromatographic system consisted of a Shimadzu Model LC9A pump (Kyoto, Japan), a Shimadzu Model RF-535 fluorescence detector, a Rheodyne loading valve (Model 7010) fitted with a 50-μl sample loop (Touzart–Matignon, Paris, France), a stainless-steel analytical column (300×7.5 mm I.D., Beckman, Paris, France) packed with exclusion gel TSK 3000 SW and a Shimadzu integrator Model C-R5A (chart speed, 0.5 mm/min).

2.3. Chromatographic conditions

The eluent mixture consisted of purified water–methanol (98:2, v/v) containing 10^{-4} *M* of 1-naphthol and was degassed ultrasonically and filtered prior to use. The mobile phase flow-rate was 1 ml/min, which corresponds to a pressure of about 3 MPa. The volume injected was 50 μl. Chromatography was performed at ambient temperature (20–22°C). Excitation and emission wavelengths were 290 and 360 nm, respectively.

2.4. Plasma extraction procedure

In 5-ml glass tubes, drug-free plasma samples (0.5 ml) were spiked with appropriate volumes of MEBCD standard solutions in order to obtain concentrations of 1, 5, 10, 50 and 100 μ*M*. All tubes were treated with 10 μl of KOH (5 *M*), and after a brief shaking, 1 ml of chloroform was added. Extraction was performed by full speed Vortex-mixing for 1 min. Then the vials were centrifuged at 3000 *g* for 10 min at 4°C. The lower organic phase was carefully removed and poured into another glass tube, spiked with internal standard solution (10 μg) and then evaporated under a nitrogen stream. The residue was dissolved in 250 μl of mobile phase and filtered through a 0.45-μm filter (Millipore, Bedford, MA, USA). A 50-μl sample was injected onto the column.

2.5. Treatment of cell samples

In 5-ml glass tubes, samples (1 ml) containing 10^6 cells in suspension in RPMI 1640 culture medium were added. Cells were centrifuged at 1500 *g* for 5 min and washed twice with cold PBS (4°C). After a final centrifugation at 1500 *g*, the supernatant was removed and the cell pellet was spiked with various concentrations of MEBCD (1 to 100 μ*M*). Daunorubicin (10 μg) was added as an internal standard. Cells were lysed by addition of 1 ml of a chloroform–methanol mixture (4:1; v/v). After vigorous shaking and centrifugation, the organic phase was evaporated to dryness under nitrogen stream. The residue was reconstituted with 250 μl of mobile phase and filtered through a 0.45-μm filter. A 50-μl sample was injected onto the column.

2.6. Data analysis

Peak-area ratios of MEBCD to internal standard were used as the assay parameter and were plotted against theoretical concentrations.

Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data. The quality of the fit was evaluated by comparing back-calculated concentrations to the nominal ones.

The linearity of the method was confirmed by comparison of intercepts with zero and correlation coefficients with 1.

2.7. Recovery and specificity

The recovery of MEBCD was determined by comparing peak areas from drug-free plasma samples spiked with known amounts of drug (3, 30 and 80 μ*M*) processed according to the described method versus peak areas of the same concentrations prepared in purified water injected directly onto the analytical column. Each sample was determined in replicate ($n=3$).

Specificity was checked by comparing the retention times of endogenous compounds in plasma

and in cell lysate with those of MEBCD and internal standard.

2.8. Precision and accuracy

The intra-day precision and accuracy of the method were evaluated by analysing six replicates of spiked samples at each concentration (3, 30 and 80 μM) against a calibration curve the same day.

Inter-day precision and accuracy were assessed by performing analyses of spiked samples at 3, 30 and 80 μM, against a calibration curve on different days ($n=6$).

The accuracy was evaluated as percent error [(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.

2.9. Determination of the limit of quantitation and of the limit of detection

The limit of quantitation (LOQ) was defined as the lowest drug concentration which can be determined with a good accuracy and a precision ≤20% on a day-to-day basis. To determine inter-day accuracy and precision on the LOQ, spiked plasma and cell lysates were used ($n=6$). The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of 2 times the noise level.

2.10. Stability studies

For stability studies, drug-free plasma samples were spiked with 3, 30 and 80 μM of MEBCD. Each determination was performed in triplicate.

The short-term stability in plasma was assessed at 1, 2, 4 and 6 h at both ordinary laboratory conditions (20–22°C) and at 4°C. The long-term stability of MEBCD in frozen human plasma (−20°C) was determined by periodic analysis over 1 month. Samples were analyzed immediately after preparation (reference values) and after storage. Prior to their analyses, samples were brought to room temperature and Vortex-mixed well.

3. Results

3.1. Retention times and specificity

Observed retention times were 4.8 and 11.1 min for internal standard and MEBCD, respectively. Representative chromatograms are shown in Fig. 2. No peaks interfered at the retention times of MEBCD or internal standard either in plasma or in cell lysate (Fig. 2a and 2d, respectively).

3.2. Linearity

In plasma and cell lysate, the peak area ratio of MEBCD over the internal standard varied linearly with concentration over the range used which was 1 to 100 μM. The correlation coefficients for calibration curves were equal to or better than 0.996.

Plasma intra-assay reproducibility was determined for calibration curves prepared the same day in replicate ($n=6$) using the same stock solutions. The intra-day average slope of the fitted straight lines was $0.00753 \pm 7.02\ 10^{-4}\ \mu M^{-1}$ (C.V.=9.32%), the mean intercept of calibration curves was $-0.0022 \pm 7.78 \cdot 10^{-4}$. The corresponding mean (±S.D.) coefficient of the linear regression analysis was 0.9988±0.0012 (C.V.=1.2%).

For calibration curves prepared on different days ($n=6$) in plasma and cell lysate, the average results were as follows: correlation coefficients of the linear regression analysis $=0.9985 \pm 8.47 \cdot 10^{-4}$ (C.V.=0.084%) and $0.9992 \pm 5.7 \cdot 10^{-4}$ (C.V.=0.057%), slopes $=0.00745 \pm 6.07 \cdot 10^{-4}\ \mu M^{-1}$ (C.V.=8.13%) and $0.0143 \pm 1.51 \cdot 10^{-4}\ \mu M^{-1}$ (C.V.=10.6%), and intercepts $=-0.0020 \pm 2.20 \cdot 10^{-3}$ and $-0.0034 \pm 4.7 \cdot 10^{-3}$, respectively.

Intra-day and inter-day variabilities at concentrations of calibration standards are given in Table 1. 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).

The linearity of this method was confirmed statistically. For each calibration curve, 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).

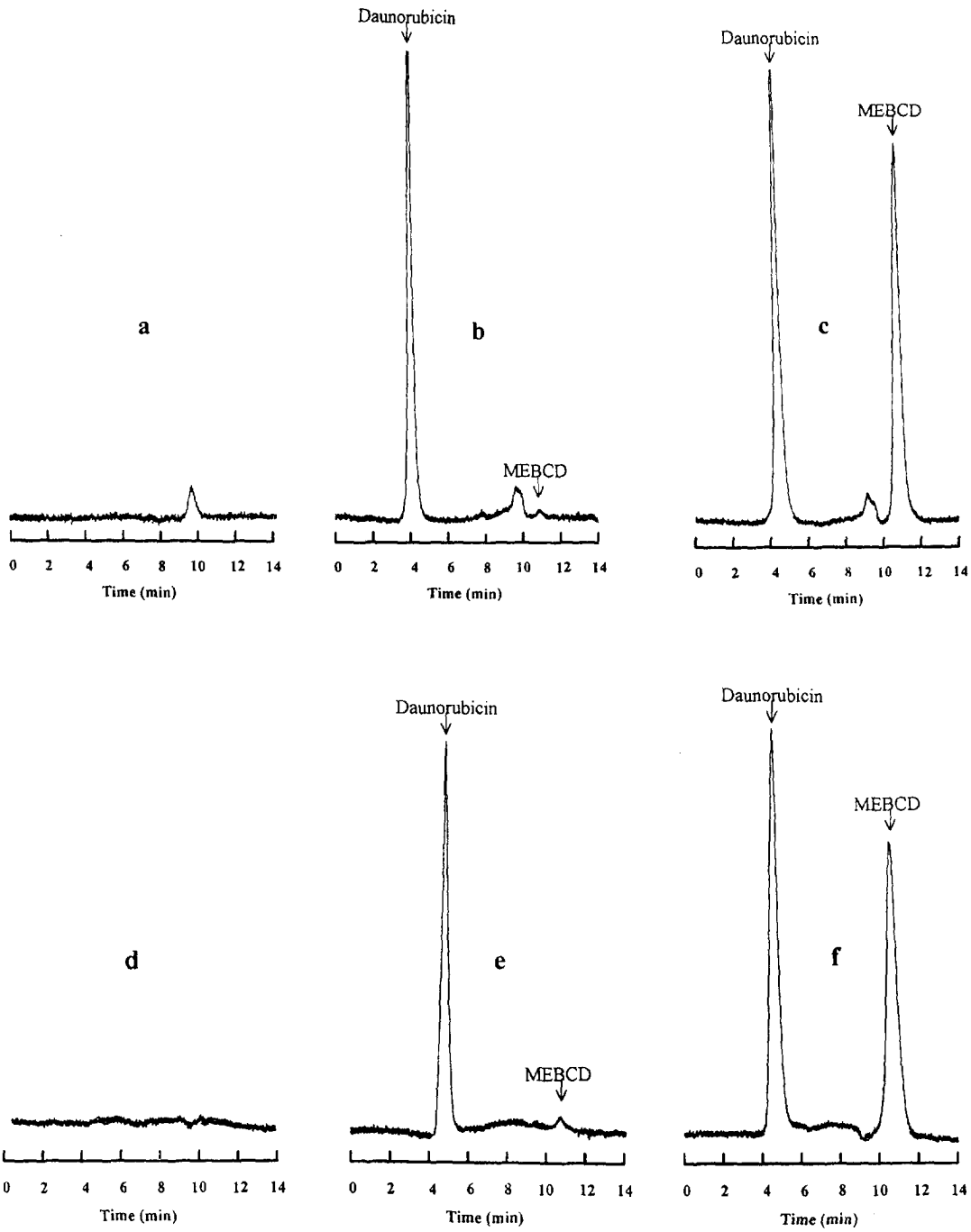

Fig. 2. Chromatograms of drug-free plasma or cell lysate (a and d) and of plasma or cell lysate spiked with 0.5 μ*M* (b and e) and 80 μ*M* (c and f) of MEBCD. Peak 1 is the internal standard and peak 2 is the MEBCD. For chromatographic conditions see Section 2.3.

Table 1
Reproducibilities of the HPLC analysis in plasma (intra- and inter-day assay) and in cell lysate (inter-day assay)

Theoretical concentration (μM)	Experimental concentration (mean±S.D.) (μM)	C.V. (%)
Intra-day reproducibility (n=6) in plasma		
1	0.99±0.10	9.93
5	5.23±0.39	7.38
10	10.6±0.95	8.91
50	49.0±2.78	5.67
100	100.4±1.24	1.24
Inter-day reproducibility (n=6) in plasma		
1	1.06±0.11	10.0
5	4.56±0.41	8.96
10	9.05±0.57	6.30
50	52.4±1.59	3.03
100	98.9±0.73	0.73
Inter-day reproducibility (n=6) in cell lysate		
1	0.95±0.12	12.3
5	4.89±0.18	3.70
10	9.13±0.41	4.50
50	51.9±0.76	1.50
100	99.2±0.34	0.30

3.3. Recovery, precision and accuracy

The mean recovery of MEBCD in plasma averaged 43±4.5% (n=5). It is not statistically different over the range of concentrations studied.

For concentrations of calibration standards ranging from 1 to 100 μM, the precision around the mean value did not exceed 10% (Table 1).

Intra-day and inter-day precision and accuracy of the method, assessed by analysing quality control samples, are presented in Table 2.

3.4. Limit of quantitation and limit of detection

The limit of quantitation in plasma was 0.80 μM. At this level, the mean concentration found was 0.73 μM (precision, 18.2%; accuracy, 91.3%). In cell lysate, LOQ was 0.50 μM; the mean back-calculated concentration was 0.45 μM (precision, 16.4%; accuracy, 90%).

The limits of detection were 0.50 (precision, 40%) and 0.30 μM (precision, 30%) in plasma and cell lysate, respectively.

3.5. Stability studies

Stock solutions of MEBCD (10^{-1} M) and internal standard (0.5 mg/ml) were stable for at least 3 months.

At 4 and 20°C, in plasma samples, MEBCD was stable for 6 h. For each of the three concentrations (3, 30 and 80 μM), percent recoveries averaged 96±5.8, 101±7.2 and 102±6.4%, respectively.

Table 2
Accuracy and precision of the HPLC method in plasma (intra- and inter-day assay) and cell lysate (inter-day assay)

Theoretical concentration (μM)	Experimental concentration (mean±S.D.) (μM)	C.V. (%)	Deviation from theoretical value, (%)	Recovery (%)
Intra-day (n=6) in plasma				
3	2.66±0.23	8.82	11.3	88.7
30	28.5±2.65	9.29	4.94	95.6
80	81.0±8.29	10.2	1.21	101.2
Inter-day (n=6) in plasma				
3	2.83±0.26	9.44	5.92	94.1
30	28.6±2.21	7.73	4.74	95.3
80	75.1±7.49	9.98	6.11	93.9
Inter-day (n=6) in cell lysate				
3	2.7±0.32	11.9	10.1	89.9
30	29.5±2.84	9.62	1.51	98.5
80	84.3±3.32	3.94	5.33	105.3

The long-term freezer stability indicated that MEBCD was stable for at least 1 month, the percent recovery averaged 98±8.7%. Compared to the reference values, no statistical difference appeared.

4. Discussion and conclusion

MEBCD has a very high solubility in water which results in a difficult purification from biological matrix. The developed method proved to be useful for the determination of plasma and cell lysate concentrations of MEBCD. Known chromatographic methods for determination of CDs with amperometric detection in serum [10,11] are useful, but require expensive equipment, and cannot be applied with a good sensitivity to methylated β-CDs. Other methods using inclusion complex formation with phenolphthalein [12,13] are very selective but detection limits obtained are still high (20 μ*M* for hydroxy-propyl-β-CD), and they require a post-column complexation. The present method is based on the principle of fluorescence enhancement of inclusion complexes formed by MEBCD with 1-naphthol [14]. Chromatographic conditions were optimized in order to be compatible with a determination in biological media like plasma or cell lysate. Other CDs were checked as potential internal standards, but they were not adequately separated from MEBCD. The choice of daunorubicin was based (i) on its sufficient molecular mass to be retained by the exclusion column with adequate retention time, (ii) its natural fluorescence at the wavelengths where MEBCD is detected, (iii) its solubility in organic and aqueous phases and (iv) the absence of complexation with both 1-naphthol and CDs [18] avoiding interference in MEBCD determination. The sample treatment procedure, involving a direct alkaline extraction with chloroform without deproteinization, is simple and rapid and does not require a solid-phase extraction as described elsewhere [10,11]. The alkaline pH allows an optimal MEBCD extraction by the organic phase, while water-soluble ionized plasma endogenous compounds are weakly soluble in chloroform. Assay performance of this method was assessed both on the basis of the statistical characteristics of individual calibration lines, and from the results of quality control samples. This method, validated for concentrations ranging from 1 to 100 μ*M*, has a good reproducibility and accuracy, and low limits of quantitation and detection compared to the most published HPLC methods for other CDs derivatives. The advantage of the present method is the absence of interfering endogenous compounds from the matrix. Stability studies carried out directly in plasma indicated that samples were stable for at least 1 month when stored at −20°C and 6 h when stored at 4 and 20°C.

Acknowledgments

We thank Mme F. Malosse for her excellent technical assistance.

References

[1] M. Allegre and A. Deratani, Agro-Food-Industry Hi-Tech, 1 (1994) 9.
[2] J. Szetlji, Med. Res. Rev., 14 (1994) 353.
[3] F. Bressolle, M. Audran, T.N. Pham and J.J. Vallon. J. Chromatogr. B, 687 (1996) 303.
[4] R. Krishnamoorthy, A.M. Wolka, S. Zezhi and A.K. Mitra, Eur. J. Biopharm., 41 (1995) 296.
[5] N.G.M. Schipper, J.C. Verhoef, S.G. Romeijn and W.H.M. Merkus, Calcified Tissue Int., 56 (1995) 280.
[6] L. Hovgaard and H. Brøndsted, Pharm. Res., 12 (1995) 1328.
[7] P.Y. Grosse, F. Bressolle and F. Pinguet, Cancer Chemother. Pharmacol., (1997) in press.
[8] E.P.C. Kilsdonk, P.G. Yancey, G.W. Stoudt, F. Wen Bangerter, W.J. Johnson, M.C. Phillips and G.H. Rothblat, J. Biol. Biochem., 270 (1995) 17 250.
[9] F. Giordano, Bolletino Chimico Farmaceutico, 130 (1991) 239.
[10] M. Fukuda, Y. Kubota, A. Ikuta, K. Hasegawa and K. Koizimi, Anal. Biochem., 212 (1993) 289.
[11] J. Haginaka, Y. Nishimura and H. Yasuda, J. Pharm. Biomed. Anal., 11 (1993) 1023.
[12] H.W. Frijlink, J. Visser and B.F.H. Drenth, J. Chromatogr., 415 (1987) 325.
[13] H.W. Frijlink, J. Visser, N.R. Hefting, R. Oosting, D.K.F. Meijer and C.F. Lerk, Pharm. Res., 7 (1990) 1248.
[14] H.J.E.M. Reeuwijk, H. Irth, U.R. Tjaden, F.W.M.H. Merkus and J. Van der Greff, J. Chromatogr., 614 (1993) 95.
[15] United States Pharmacopoeia XXXIII, The United States Pharmacopeial Convention, Rockville, MD, 1994, p. 1929.

[16] 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.

[17] F. Bressolle, M. Bromet-Petit and M. Audran, J. Chromatogr. B, 686 (1996) 3.

[18] O. Bekers, J.H. Beijnen, M. Otagiri, A. Bult and W.J.M. Underberg, J. Pharm. Biomed. Anal., 8 (1990) 671.