ELSEVIER

Journal of Chromatography B, 772 (2002) 197–204

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of the carboxylate and lactone forms of 10-hydroxycamptothecin in human serum by restricted-access media high-performance liquid chromatography

Jun Ma[a], Chun-Li Liu[a], Peng-Ling Zhu[a,*], Zheng-Ping Jia[b], Li-Ting Xu[b], Rong Wang[b]

[a]*Department of Chemistry, Lanzhou University, Lanzhou, 730000 China*
[b]*Department of Pharmacy, General Hospital of Lanzhou Command of PLA, Lanzhou, 730050 China*

Received 28 August 2001; received in revised form 29 January 2002; accepted 5 February 2002

Abstract

A simple restricted-access media (RAM) HPLC method for simultaneous determination of the lactone and carboxylate forms of 10-hydroxycamptothecin (HCPT) in human serum was established. Using a RAM Hisep analytical column, serum samples were directly injected into the HPLC system. The eluted peaks of two forms of HCPT were monitored with a fluorescence detector. The separation was completed in 17 min. The linear range was 20–1000 ng/ml, intra-day and inter-day variations being less than 5%. The kinetic equation was introduced according to the analytical results. The equation shows that the course of the HCPT lactone form converting to carboxylate form in human serum at 4 °C is a first-order kinetic course. The concentration of each form at the moment of sampling was calculated by extrapolation.

Keywords: Restricted access media; 10-Hydroxycamptothecin

1. Introduction

Camptothecin (CPT) is a natural alkaloid extracted from *Camptotheca Acuminata Decne* in the 1960s [1]. Displaying promising activity of anti-tumor, the drug and its semi-synthetic analogues have been paid more attention to [2]. In the 1990s, several CPT analogues were semi-synthesized, including water-soluble irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11) and fat-soluble 10-hydroxycamptothecin (HCPT) [2,3]. The anti-tumor mechanism of these compounds is based on the inhibition of DNA replication and RNA transcription by stabilizing the cleavable complexes formed between topoisomerase I and DNA [4]. All CPT analogues can exist in two forms: carboxylate and lactone. At pHs less than 4, α-hydroxy-γ-lactone structure predominates. At more alkaline pHs, lactone form can transfer to carboxylate form by the reversible hydrolysis (Fig. 1). Compared with each other, the lactone form is the effective inhibitor of topoisomerase I while carboxylate form has much lower anti-tumor activity [5]. Thus, it is necessary to develop the analytical methods used for determining their own concen-

*Corresponding author. Tel.: +86-931-266-2432; fax: +86-931-891-2582.
E-mail address: mb1642@public.lz.gs.cn (P.-L. Zhu).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00085-5

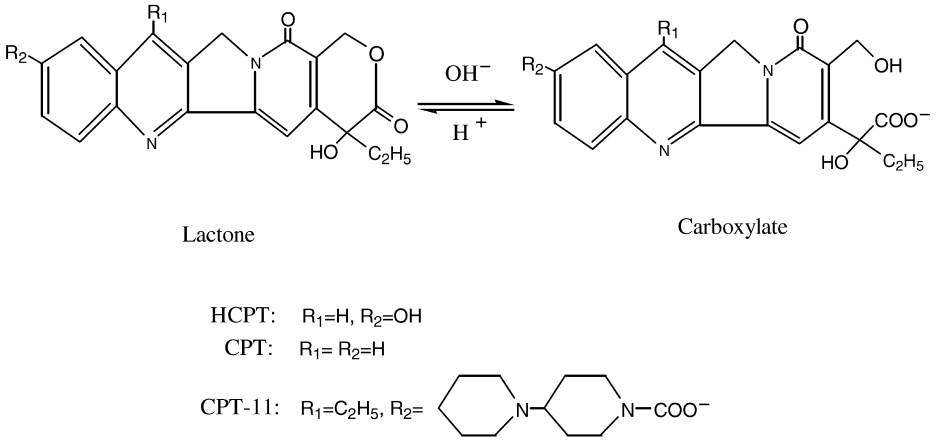

Fig. 1. Structures of HCPT, CPT and CPT-11.

trations of two forms in blood fully to understand the pharmacology of CPT analogues.

The several HPLC methods were reported to analyze CPT drugs in human plasma [6–21], but only a few of them dealt with the simultaneous determination of carboxylate and lactone forms [6–11] and analytical method for two forms of HCPT reported only by Yu-Feng Li and Ruiwen Zhang [10]. The sample pre-treatment was required to remove proteins before injection. The means included precipitation by organic solvent, liquid–liquid extraction, solid-phase extraction and so on. In the process, the hydrolysis of lactone form was still going on [22,23] so that it is difficult to determine accurately the original concentration of the two forms. In Ref. [11], in order to restrict the transformation of lactone form to carboxylate form, blood samples, centrifuge and autosampler were kept at 1–4 °C and the organic solvent for protein precipitation was cooled at −20°C.

Different kinds of restricted-access media (RAM) were developed for the direct injection analysis of biological fluids. Their features can be described as follows [24,25]: (1) There is a hydrophilic external surface to avoid denaturing proteins. (2) The pore of packing is small enough for proteins to be eluted without any retention by size exclusion mechanism. (3) The internal surface of packing is of hydrophobility to a certain extent by means of which small molecules are retained and separated.

The purpose of this work is to develop a HPLC method for the simultaneous determination of HCPT carboxylate and lactone forms in human blood, which can be used in pharmacodynamics and pharmacokinetics. HCPT having the wide clinical application in China is selected as an object of study. A RAM analytical column packed with shielded hydrophobic phase (SHP) [26] is used to make serum samples injected directly without removing proteins. Thus, the procedure is simplified and the transformation of lactone form to carboxylate form during sample pretreatment is avoided. In addition, based on the principle of kinetic analysis, the kinetic equation is introduced according to the analytical results obtained at the different times after sampling to describe the transformation of lactone form to carboxylate form in given conditions. The concentration of each form at the moment of sampling can be calculated by extrapolation.

2. Experimental

2.1. Materials

HCPT and CPT were provided by Feiyun Pharmaceutical (Huangshi, Hubei, China). HPLC grade acetonitrile (ACN) was obtained from Siyou (Tianjin, China). All other chemicals were of analytical grade from a variety of suppliers. The aqueous solutions were prepared by using double distilled water.

HCPT and CPT stock solution: HCPT or CPT was dissolved in an appropriate amount of dimethylsul-

foxide (DMSO). The solutions were stored at −20°C. HCPT standard solutions: The stock solution was diluted with DMSO 0.01 *M* sodium borate, pH9 (50/50, v/v) and DMSO 0.01 *M* phosphate buffer, pH2.5 (50/50, v/v) for carboxylate and lactone forms standard solution, respectively. The solutions were equilibrated at ambient for 30 min and stored at 4°C. The standard solutions were used in the same day when they were prepared. CPT standard solution: the procedure is the same as that for HCPT.

2.2. Equipment

A HPLC system consisted of LC-6A pump (Shimadzu, Kyoto, Japan), Rheodyne 7125 injection valve with 20 μl loop (Catati, CA, USA), CTO-6A column oven and RF-530 fluorescence detector, the output of which was connected to C-R3A chromatopac integrator (Shimadzu, Kyoto, Japan). Hisep SHP analytical column, 15×0.46 cm, 5 μm; Hisep SHP guard column, 2×0.46 cm, 5 μm (Supelco, Bellefonte, PA, USA); solvent A, 0.05 *M* phosphate buffer, pH 7.0; solvent B, ACN; stepwise gradient, 10/10/18/18% B at 0/3/3.01/18 min; flow-rate, 1.5 ml/min; fluorescence detection, excitation (EX) 380 nm, emission (EM) 556 nm; temperature, 30°C.

2.3. Standard serum samples

Blank human serums without drugs were provided by healthy volunteers, stored at −20 °C and kept at 4 °C before use. The blood samples were kept at 4 °C in all experiments. Standard serum samples of HCPT: 30 μl of each in HCPT standard solution series (carboxylate or lactone form) was added into 270 μl of blank serum. The mixture was immediately injected after vortex for 30 s. The final concentrations of standard serum samples were 20, 40, 100, 200, 500 and 1000 ng/ml, respectively.

2.4. Calibration curve

The peak areas of carboxylate form were plotted against the concentrations to obtain the calibration curve for carboxylate form of HCPT. In view of the transformation of lactone form to carboxylate form during the chromatographic process, the part of the lactone should be deducted from the total concentration. The peak areas of lactone were plotted against the corrected concentrations. The detection limit was defined as the peak signal of HCPT equal to three times the average noise level.

2.5. Sample analysis

Samples were collected and immediately kept at 4 °C, the collecting time recorded. Then the sample was centrifuged for 2 min (5000 *g*, 4 °C) and serum was directly injected into HPLC system, meanwhile the time of eluted peak recorded. The HPLC system was run same as above again. When two points of the sample for times and corresponding concentrations were obtained, the rate constant was calculated for each individual based on kinetic analysis, and then the original concentration of HCPT in different time sampling was extrapolated.

3. Results and discussion

3.1. Mobile phase

In the reported HPLC methods for the analysis of CPT and its analogues, the mobile phase usually consists of organic modifier, buffer and ion-pair reagent. For example, in Ref. [11], 0.1 *M* ammonium acetate (pH6.4)/triethylamine/ACN (800:1:156, w/v/w) containing 5 m*M* tetrabutylammonium phosphate was used as the mobile phase. Here the presence of ion-pair reagent may restrain the ion-exchange process caused by silanol group on the bonded phase [8]. In this work, with SHP used as stationary phase, on the surface of packing there exists a layer of polymer which make the silanol effect ignored [26]. Thus, the adding ion-pair reagent in the mobile phase seems unnecessary. In addition, if it is close to physiologic pH, the mobile phase will influence little on the transformation between two forms of HCPT. Therefore, 0.05 *M* phosphate buffer (pH 7)/ACN combination was selected as the mobile phase. According to DryLarb computer simulation, a stepwise gradient with 10/10/18/18% at 0/3/3.01/18 min was set up, the chromatograms of HCPT standard solutions shown in Fig. 2.

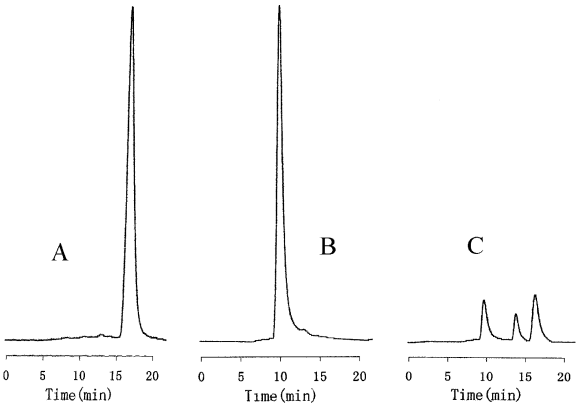

Fig. 2. Chromatograms of standard solutions: (A) HCPT lactone 1 μg/ml; (B) HCPT carbopxylate 1 μg/ml; (C) A mixture of HCPT carborxylate 100 ng/ml, CPT lactone 100 ng/ml and HCPT lactone 100 ng/ml in 0.05 *M* phosphate buffer, pH 6.4. Chromatographic conditions see Section 2.2.

As mentioned already, carboxylate and lactone forms can transfer to each other. The process also takes place during chromatographic separation. In order to observe the effect of mobile phase pH on the transformation, 1 μg/ml standard solutions of carboxylate and lactone forms were injected with the different pH values of the mobile phase (pH 6.4, 6.7, 7.0, 7.2). The retention of carboxylate form decreases with the increasing pH value, but the retention of lactone form increases a little bit. That the high pH favors the dissociation of carboxylic group in carboxylate form results in the decrease of retention. The promoted dissociation of carboxylic group in carboxylate form and the restricted protonation of the alkaline group in lactone form might result in the variation of retention with pH value of the mobile phase. The peak high shows a reverse change with pH value of the mobile phase, compared with the retention. The peak area for lactone form has reduction to a small extent when the pH value of mobile phase increases from 6.5 to 7.2, but the peak area of carboxylate form keeps constant. For both of two forms the peak area changes only little around pH 7. Based on the above-mentioned observation, phosphate buffer, pH 7 is selected as solvent A in the stepwise gradient and peak area is used for quantitation in HPLC.

3.2. Detection

Generally, fluorescence detector is used in HPLC analytical method of CPT analogues. High sensitive and specific fluorescence detector is suitable for the low level of CPT analogues in blood samples. CPT analogues being similar in structure, fluorescence spectra of HCPT and 7-ethyl-10-hydroxy-camptothecin (SN-38) do not have big difference. Based on the information about fluorescence detection of SN-38 [6,7,10,14] and the experimental results given by two forms of HCPT, 380 and 556 nm were selected as excitation and emission wavelength, respectively. In the circumstances, high sensitivity and little interference are obtained.

3.3. Serum sample

The chromatograms of serum samples are shown in Fig. 3. Proteins in the samples are eluted first at about 1.2 min with the mobile phase containing 10% ACN, and then carboxylate and lactone HCPT are eluted with phosphate buffer (pH 7)/ACN (82/18) mobile phase at 10 min and 16.5 min, respectively. The retentions are consistent with those obtained when aqueous standard samples are injected. The resolution is high enough for the quantitation of two forms of HCPT. Fig. 3D is a chromatogram of the blood sample taken from a patient at 15 min after intravenous administration of HCPT at a dose of 10 mg. A small peak of carboxylate form can be seen in Fig. 3B. It might result from the transformation of lactone form to carboxylate form in the period from sample preparation to the injection.

3.4. Calibration curve

Under the specified chromatographic conditions, the peak areas were plotted against the standard serum concentrations of HCPT to give the calibration curves: $Y=0.0072X+4.95$, $r=0.998$ for carboxylate form and $Y=0.0057X+7.81$, $r=0.999$ for lactone form. X and Y were the concentration and the peak area, respectively. The concentration ranges are 20–1000 ng/ml. According to the calibration curve of carboxylate form, the transformation of lactone form was deducted from the original concentration to obtain the realistic concentration of lactone form.

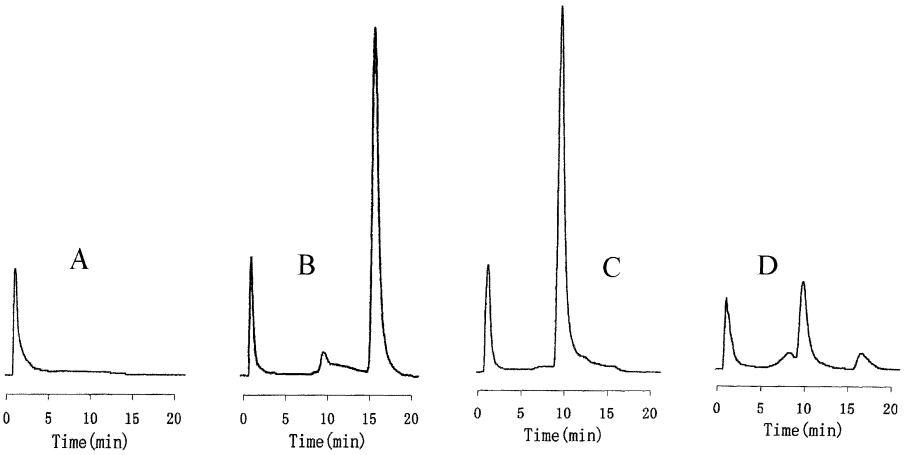

Fig. 3. Chromatograms of (A) the blank serum, (B) the serum spiked with 1 μg/ml of HCPT lactone, (C) the serum spiked with 1 μg/ml of HCPT carboxylate and (D) the sample obtained from a patient at 15 min after intravenous administration of HCPT at a dose of 10 mg. Chromatographic conditions: see Section 2.2.

The calibration curve was given by plotting peak areas against the corrected concentration.

3.5. Precision and accuracy

According to the calibration curve, the concentrations of the spiked serum samples were injected, then, the precision and accuracy were calculated and shown in Table 1. The average intra-day precision, inter-day precision and accuracy are 2.41, 4.33 and 4.03% for lactone form, 3.55, 3.97 and 3.96% for carboxylate form, respectively. The limit of detection and limit of quantitation for both forms is 5 and 16.5 ng/ml, respectively.

Table 1
Precision and accuracy of RAM-HPLC analysis of lactone and carboxylate forms of HCPT in standard serum sample

Added concentration (ng/ml)	Intra-day		Inter-day		
	Mean measured concentration (ng/ml)	RSD (%)	Mean measured concentration (ng/ml)	RSD (%)	Error (%)
Lactone					
40	39.94	1.85	37.38	7.52	7.78
100	91.89	6.06	93.01	6.19	4.66
200	183.7	0.77	190.0	2.72	2.25
500	474.8	0.95	475.0	0.88	1.42
Carboxylate					
40	42.02	5.57	42.05	7.67	7.84
100	101.7	2.72	98.62	1.02	1.38
200	195.7	3.14	194.3	4.69	4.44
500	502.6	2.75	504.1	2.50	2.17

$n=6$. RSD: Relative standard deviation.
Error = |measured value − true value| × 100/true value.

3.6. Correcting in terms of kinetic analysis

The standard serum samples of lactone form were prepared at three levels. With the time counted at the point of sample preparation, the sample at each level was injected at the different intervals. The concentrations of lactone form were given, based on the calibration curve. The plot of the logarithm of concentration vs. the time passed till the band of lactone form was eluted is shown in Fig. 4. The rate constants at 3 levels (500, 200, 100 ng/ml) are −0.0030, −0.0031 and −0.0029, with correlation coefficients are 0.999, 0.998 and 0.995, respectively. The average of rate constant is −0.0030. It is indicated from the linear relationship between log C and t that the transformation of lactone form to carboxylate form is a first order-kinetic process.

3.7. Recovery

The blood sample was taken from the patient administrated intravenously by HCPT and was centrifuged. The serum sample was divided into A and B parts where the standard solution of lactone form and the same volume of blank solvent, DMSO 0.01 M phosphate buffer, pH 2.5 (50/50, v/v) were added, respectively. The time was counted from the point of the standard and blank solvent addition. With the sequential injections in the order of B, A and B carried out, the concentration of lactone form was determined and the time when the lactone band was eluted was recorded. The rate constant and the content of lactone form at $t=0$ were calculated, based on the two points of sample B. Then the data of sample A and the obtained rate constant were used for the calculation of lactone concentration at $t=0$ for simple A. The results are shown in Table 2, with a average total HCPT recovery 99.3±3.1% and average lactone recovery 100.4±5.3%. The individuals gave the different rate constant, indicating that the transformation of lactone form to carboxylate form effected by blood conditions.

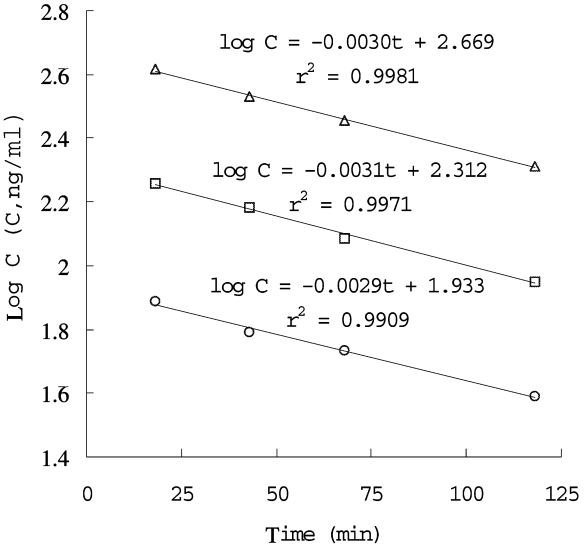

Fig. 4. The kinetic equations of the HCPT lactone converting to carboxylate form in human serum at 4 °C under the recommended experimental conditions. The intact concentration (at time=0 min) of HCPT lactone: ○=100 ng/ml, □=200 ng/ml and △=500 ng/ml.

The recommended method can be used for the study of clinical pharmacokinetics. As long as the rate constant of the transformation for individuals is determined, the original concentration of lactone form at the time of collected the blood sample can be obtained by exploration. The variation of ambient temperature and the difference between ambience and HPLC system in temperature might lead to the error of analytical results. However, if the analytical conditions keep constant, the error could be canceled out.

3.8. Application

An example for method application is given here. A patient was administrated intravenously with a dosage of 0.5 mg of HCPT/kg in 30 min. Then, the blood samples were taken from the patient at the different intervals. The concentrations of both forms were determined by means of the developed method. Fig. 5 is the plot of the concentration in serum vs. the time post infusion.

4. Conclusion

We have developed simple direct injecting HPLC methods for the simultaneous separation of lactone and carboxylate forms of HPLC in human serum, in

Table 2
Recovery

Injecting sample	Time (min)	Lactone (ng/ml)	Log C	Lactone+Carboxylate (ng/ml)
Patient 1				
B	18	48.13	1.682	871.4
A (added 100 ng lactone)	43	101.6	2.007	977.3
B	68	29.10	1.464	887.5
Rate constant=−0.00436, Lactone recovery=98.76%, Total recovery=97.85%				
Patient 2				
B	18	39.21	1.593	248.2
A (added 200 ng lactone)	43	157.3	2.197	452.1
B	68	24.11	1.382	243.7
Rate constant=−0.00422, Lactone recovery=96.07%, Total recovery=102.9%				
Patient 3				
B	18	43.16	1.636	695.2
A (added 100 ng lactone)	43	100.7	2.003	802.1
B	68	25.52	1.407	714.7
Rate constant=−0.00458, Lactone recovery=106.3%, Total recovery=97.15%				

Procedures see Section 3.7.

which the solid-phase is RAM and the mobile phase includes only phosphate buffer and ACN. In this application, using RAM is time-saving, with good reproducibility and less sample size because of eliminating the need for biological sample preparation. It is particularly that two forms of HCPT were determined during dynamic state so that the nature concentrations of lactone and carboxylate were

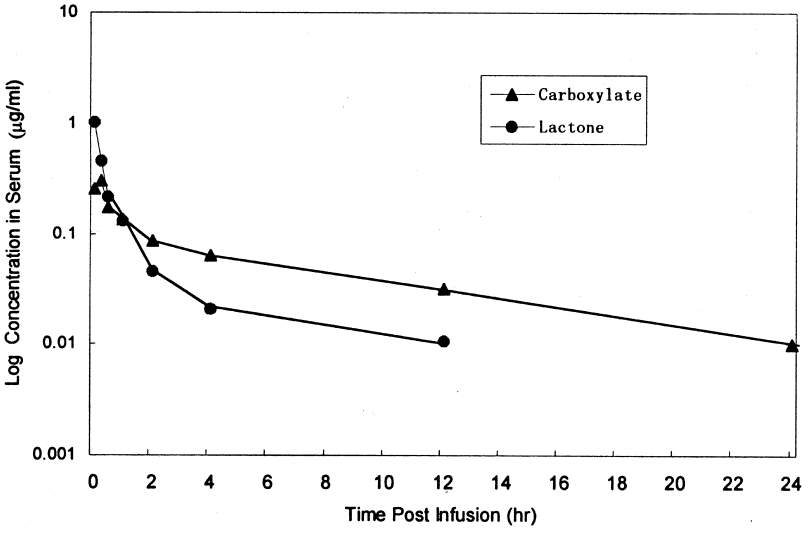

Fig. 5. Concentration-time course of the carboxylate and lactone forms of HCPT determined in the serum of a patient (dose 0.5 mg/kg, infusion time 30 min).

calculated by a chemical kinetic equation. This method will be useful in the future clinical pharmacokinetic and pharmacodynamic study of HCPT and its derivatives.

References

[1] M.E. Wall, M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPhail, G.A. Sim, J. Am. Chem. Soc. 88 (1966) 3888.
[2] B. Xu, J. Liu, Z.D. Zhang, Chin. J. Clin. Oncol. 27 (2000) 3.
[3] S. Sawada, S. Okajima, R. Aiyama, K.I. Nokata, T. Furuta, T. Yokokura, E. Sugino, K. Yamaguchi, T. Miyasaka, Chem. Pharm. Bull. 39 (1991) 1446.
[4] Y.H. Hsiang, R. Hertzberg, S. Hecht, L. Liu, J. Biol. Chem. 260 (1985) 14875.
[5] W.J. Slichemeyer, E.K. Rowinsky, R.C. Donehower, S.H. Kaufmann, J. Natl. Cancer Inst. 85 (1993) 271.
[6] L.P. Rivory, J. Robert, J. Chromatogr. B 661 (1994) 133.
[7] N. Kaneda, Y. Hosokawa, T. Yokokura, Biol. Pharm. Bull. 20 (1997) 815.
[8] D.L. Warner, T.G. Burke, J. Chromatogr. B 691 (1997) 161.
[9] D.F. Chollet, L. Goumaz, A. Goumaz, A. Renard, G. Montay, L. Verniller, V. Arnera, D.J. Mazzo, J. Chromatogr. B 718 (1998) 163.
[10] Y.F. Li, R. Zhang, J. Chromatogr. B 686 (1996) 257.
[11] V.M.M. Herben, D. Mazee, D.M. van Gortel–van Zomeren, S. Zeedijk, H. Rosing, J.H.M. Schellens, W.W. ten B. Huinink, J.H. Beijnen, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 1541.
[12] T. Oguma, Y. Ohshima, M. Nakaoka, J. Chromatogr. B 740 (2000) 237.
[13] P. de Bruijn, J. Verweij, W.J. Loos, K. Nooter, G. Stoter, A. Sparreboom, J. Chromatogr. B 698 (1997) 277.
[14] P. de Bruijin, M.J.A. de Jonge, J. Verweij, W.J. Loos, K. Nooter, G. Stoter, A. Sparreboom, Anal. Biochem. 269 (1999) 174.
[15] A. Sparreboom, P. de Bruijn, M.G.A. de Jonge, W.J. Loos, G. Stoter, J. Verweij, K. Nooter, J. Chromatogr. B 712 (1998) 225.
[16] S. Ragot, P. Marquet, F. Lachatre, A. Rousseau, E. Lacassie, J.M. Gaulier, J.L. Dupuy, G. Lachatre, J. Chromatogr. B 36 (1999) 175.
[17] A. Kurita, N. Kaneda, J. Chromatogr. B 724 (1999) 335.
[18] F. Ahmed, V. Vyas, A. Saleem, X.G. Li, R. Zamek, A. Cornfield, P. Haluska, N. Ibrahim, E.H. Rubin, E. Gupta, J. Chromatogr. B 707 (1998) 227.
[19] L.P. Rivory, M. Findlay, S. Clarke, J. Bishop, J. Chromatogr. B 714 (1998) 355.
[20] J. Escoriaza, A. Aldaz, C. Castellanos, E. Calvo, J. Giraldez, J. Chromatogr. B 740 (2000) 159.
[21] H. Rosing, D.M. van Zomeren, E. Doyle, W.W. ten B. Huinink, J.H.M. Schellens, A. Bult, J.H. Beijnen, J. Chromatogr. B 727 (1999) 191.
[22] J. Fassberg, V.J. Stella, J. Pharm. Sci. 81 (1992) 676.
[23] T.G. Burke, Z. Mi, J. Med. Chem. 37 (1994) 40.
[24] P.L. Zhu, D.J. Wang, Y. Qu, J. Chin. Pharm. 29 (1994) 129.
[25] A. Rudolpbi, K.-S. Boos, LC-GC 15 (1997) 614.
[26] D. Gisch, B.T. Hunter, B. Feibush, J. Chromatogr. B 433 (1988) 264.