ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT

Journal of Chromatography B, 784 (2003) 25–31

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simple and sensitive determination of the new antitumor drug CKD-602 in human plasma by liquid chromatography

Joo-Youn Cho*, Hyo-Bum Seo, Kyung-Sang Yu, Kyun-Seop Bae, So-Young Yi, In-Jin Jang, Sang-Goo Shin

Department of Pharmacology and Clinical Trial Center/Clinical Research Institute, Seoul National University College of Medicine and Hospital, 28 Yongun-dong, Chongro-gu, Seoul, 110-799, South Korea

Received 10 September 2001; received in revised form 20 September 2002; accepted 20 September 2002

Abstract

A simple and sensitive high-performance liquid chromatographic with fluorescence detection method has been developed and validated for the determination of the new antitumor agent CKD-602 in human plasma. Plasma proteins were precipitated with methanol and the samples were acidified with 7% (v/v) perchloric acid. The supernatants were analyzed by HPLC using a Capcell Pak C_{18} UG120 column and a mixture of methanol–0.1 *M* hexane-1-sulfonic acid in methanol–0.01 *M* TEMED in water at pH 6.0 (40:1:59, v/v) as the mobile phase. The lower limit of quantification was 0.2 ng/ml using 200 μl plasma samples. Mean within-run precision and between-run precision at six tested concentrations (0.2–400 ng/ml) were ≤10% and mean accuracy was ≤15%. Stability studies showed that CKD-602 is stable in both plasma and methanol extracts for at least 3 months at −30 °C. The described method was used for the pharmacokinetic analysis of CKD-602 during clinical phase I studies, in patients with solid tumors.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: CKD-602

1. Introduction

CKD-602 (7-[2-(*N*-isopropylamino)ethyl]-(20*S*)-camptothecin; Fig. 1) is a semi-synthetic derivative of the cytotoxic plant alkaloid camptothecin, an anticancer drug, which inhibits the mammalian intranuclear enzyme topoisomerase I [1,2]. Owing to camptothecin's serious and unpredictable gastrointestinal, urothelial and myelosuppressive toxicities [3], interest in new structure development was accelerated. New water-soluble camptothecins, topotecan and irinotecan, have been synthesized to reduce camptothecin's toxicities and registered for treatment of ovarian and colorectal cancer in various countries [4]. CKD-602 was also developed by Chong Kun Dang Pharmaceuticals (Seoul, Korea), as a new water-soluble camptothecin derivative. Preclinical studies of CKD-602 demonstrated broad antitumor activities against various animal and human tumor cell lines, and the results were equal or superior to those of camptothecins [5]. Phase I clinical trials of CKD-602 have been completed and phase II clinical trials are underway [6].

Camptothecins are unstable in aqueous solutions

*Corresponding author. Tel.: +82-2-740-8286; fax: +82-2-745-7996.
E-mail address: jycho@cpt.snu.ac.kr (J.-Y. Cho).

1570-0232/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1570-0232(02)00750-X

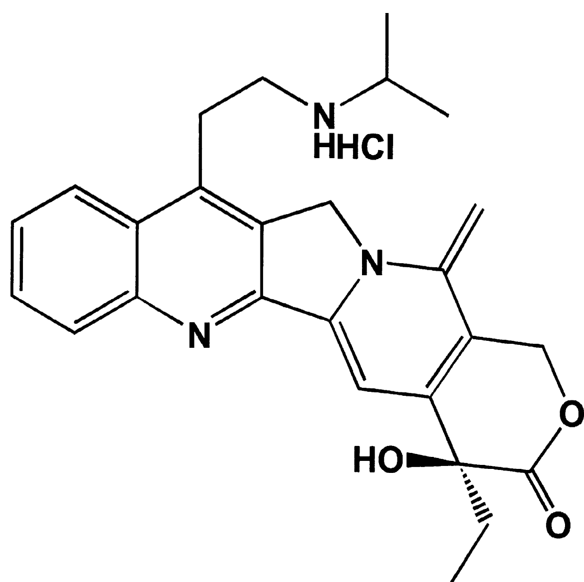

Fig. 1. Chemical structure of CKD-602.

and undergo a rapid, pH-dependent, nonenzymatic and reversible hydrolysis of the terminal lactone ring to form the more water-soluble, ring-opened carboxylate form [7]. The closed lactone ring predominates at acidic pH, whereas at physiological pH the equilibrium between these two species greatly favors the formation of the carboxylate form [8,9]. The presence of the intact terminal lactone ring is thought to be an essential feature for the topoisomerase I inhibition [10]. In recent clinical studies, more selective assays, which can discriminate between the lactone and carboxylate forms, has been used to investigate the relationship between pharmacokinetics and pharmacodynamics of camptothecins [11–13]. However, in several initial pharmacokinetic studies for new synthetic camptothecins, only total camptothecin levels, the lactone plus the carboxylate forms were measured [14]. Since the relationship between the plasma concentration of the lactone form and pharmacodynamic outcome of CKD-602 has not been established, we developed a method for determining total CKD-602, i.e., the sum of the lactone and carboxylate forms.

We now report simple and sensitive method for the analysis of CKD-602 in human plasma, using a high-performance liquid chromatography (HPLC) system with fluorescence detection. The method involves a protein precipitation step using cold methanol and perchloric acid. Assay sensitivity, as determined by its lower limit of quantification, was 0.2 ng/ml in human plasma. This assay has been successfully tested in clinical phase I studies in patients with solid tumors.

2. Experimental

2.1. Chemicals and reagents

CKD-602 (batch No. BA00200) was supplied by Chong Kun Dang Pharmaceuticals (Seoul, Korea). A purity of 98.5% for this compound was confirmed by analytical HPLC. N,N,N′,N′-tetramethylethylenediamine (TEMED), methanol, and hexane-1-sulfonic acid were purchased from Sigma (St Louis, MO, USA). The methanol used was of HPLC grade and all other reagents were of analytical quality. Milli-Q-purified water was used throughout. Drug-free human plasma from healthy donors was obtained from the Blood Bank of Seoul National University Hospital (Seoul, Korea).

2.2. Sample preparation

To quantify total CKD-602 levels, the sum of the lactone and carboxylate forms, 200 μl of plasma sample was mixed with 200 μl of cold methanol (−30 °C) to precipitate proteins. After vortex-mixing for 10 s and centrifuging at 3000 *g* for 10 min, 100 μl of the clear supernatant was transferred to a clean tube and 100 μl 7% (v/v) of perchloric acid added. After vortex-mixing for 10 s, 100 μl was injected into the HPLC system.

2.3. Chromatography

The chromatographic system consisted of a Model 304 solvent delivery system and an autosampling device Model 234 (both supplied by Gilson, Villiers-le-Bel, France). A FP920 Intelligent fluorescent detector (Jasco International, Tokyo, Japan) was used for the detection of CKD-602. Chromatographic separation was performed on a Capcell pak C_{18} column UG120 (5 μm particle size, 150×4.6 mm I.D.) from Shiseido (Tokyo, Japan). A Brownlee

guard cartridge column (15×3.2 mm) packed with reversed-phase material (C_{18}) (Perkin-Elmer, Norwalk, CT, USA) was used to protect the column. The mobile phase consisted of a mixture of methanol–0.1 *M* hexane-1-sulfonic acid in methanol–0.01 *M* TEMED in Milli-Q-purified water (the pH was adjusted with phosphoric acid to 6.0) (40:1:59, v/v). The mobile phase was delivered isocratically at a flow-rate set at 1.0 ml/min, and column eluant was monitored fluorimetrically at an excitation wavelength of 370 nm and an emission wavelength of 430 nm with a bandwidth of 40 nm. The capacity of the fluorescence detector flow-cell was 16 μl. The data were analyzed using the Unipoint data analysis system (Gilson, Villiers-le-Bel, France).

2.4. Calibration standards preparation

A stock solution containing 1.0 mg/ml was made by dissolving 10 mg of CKD-602 in 10 ml of dilution buffer, which was composed of 275 m*M* mannitol and 0.4 m*M* tartaric acid in water. The stock solution was stored at −30 °C. Working standard solutions were prepared daily by serial dilution with human blank plasma (2–5000 ng/ml). Nine calibration standards at 0.2–500 ng/ml were prepared by adding 20 μl of each calibration standard solution in human blank plasma (180 μl).

2.5. Validation

To validate the assays of CKD-602 as the sum of the lactone and carboxylate forms in human plasma, the following parameters were determined: within-run and between-run precision, accuracy, lower limit of quantification (LLQ), linearity, specificity and recovery.

Six quality control (QC) samples at concentrations of 0.2, 0.5, 1, 10, 100 and 400 ng/ml were prepared by spiking working standard solutions to human plasma. To determine the accuracy and precision of the developed analytical methods, six replicates of QC sample at each concentration were analyzed in five separate runs. The accuracy of the assay was expressed as the percentage deviation from the nominal concentrations (% DEV). The accuracy (% DEV) for each concentration, excluding the LLQ, should be ≤15% and should be ≤20% for the LLQ. The precision of the assay was assessed by mean within-run precision and between-run precision and was expressed as the percentage coefficient of variation (C.V.). The precision (% C.V.) for each concentration, excluding the LLQ, should be ≤15% and should be ≤20% for the LLQ. A calibration curve ranging from 0.2 to 500 ng/ml was prepared using weighted ($1/x$) least square linear regression analysis of the peak height versus the nominal concentration (x). The linearity of the calibration curve was evaluated by calculating the correlation coefficient (r). The accuracy and precision of calibration standards were determined from the back-calculated concentrations at each level. To determine the specificity, six batches of blank plasma were analyzed for the presence of interfering peaks with similar retention times to CKD-602 on the HPLC chromatograms. The extraction recovery of CKD-602 was assessed at three concentration levels (1, 10 and 100 ng/ml) by comparing the mean peak heights after extraction with the mean peak heights obtained from direct injection of equivalent quantities of the pure standards.

2.6. Stability

The stability of CKD-602 in human plasma and in methanol extracts was evaluated for 3 months at −30 °C. After the storage period, the samples were analyzed immediately. The room temperature stability of CKD-602 in human plasma was determined by incubating QC samples at room temperature (22 °C) for 24 h. The stability of the drug in human plasma to three additional freeze/thaw cycles was also tested. In addition, the stability of CKD-602 in acidified methanol extracts was investigated during storage in the autosampler at ambient temperature. The stability of CKD-602 under each condition was determined by comparing results obtained with the concentrations of the samples extracted in normal condition. Six replicates with concentrations of 1, 10 and 100 ng/ml were analyzed at every point.

2.7. Pharmacokinetic studies

In the phase I study, CKD-602 was administered daily by a 30-min infusion for 5 consecutive days of every 3 weeks to patients with advanced solid

malignancies at doses ranging from 0.5 to 0.9 mg/ m^2/day. On the 1st infusion day, blood samples were collected in heparinized tubes before infusion, 15 min into the infusion, and 0, 15, 30 and 60 min and 2, 3, 4, 6, 8, 12 and 24 h after infusion. On the 5th infusion day, blood samples were also collected during and after infusion for 48 h. After blood sampling, and without delay the cells were separated by centrifugation, and the plasma supernatant was mixed with the same volume of cold methanol (–30 °C). After vortex-mixing and centrifugation at 3000 *g* for 10 min, the clear supernatant was transferred to a clean tube and stored at −30 °C until analyzed.

3. Results and discussion

3.1. Chromatography and detection

CKD-602 is a semi-synthetic, water-soluble derivative of camptothecin and has chemical characteristics that are similar to topotecan, a representative semi-synthetic camptothecin. We developed a chromatographic and sample pretreatment method for the determination of CKD-602 based on the published method for topotecan [15]. Chromatography was initially performed using a Capcell pak C_{18} analytical column using a mixture of methanol–0.1 *M* hexane-1-sulfonic acid in methanol–0.01 *M* TEMED in water (pH 6.0) (25:10:65, v/v) as the mobile phase [15]. However, the total run time was too long. To optimize the total run time and the peak shapes, the concentration of hexane–sulfonic acid, added as an ion-pairing agent, was reduced to 1 m*M* in the mobile phase and the concentration of methanol was increased to 41% (v/v). The retention time of CKD-602 was found to be 6.9 min with overall chromatographic run time of 12 min (Fig. 2). To determine the level of CKD-602, the fluorescence wavelengths (excitation at 370 nm and emission at 430 nm) were selected by scanning the fluorescence of the analyte.

A protein precipitation step using cold methanol proved to be a suitable sample pretreatment procedure for the bio-analysis of camptothecins [15], and was adopted for the CKD-602 determination. The addition of 7% perchloric acid to the methanol extracts, allowed the total CKD-602 content, i.e. the sum of the lactone and the carboxylate form, to be analyzed.

3.2. Validation

Blank human plasma samples from six different donors showed no significant interfering peaks around the retention time of CKD-602 (Fig. 2). The calibration curves of CKD-602 in human plasma were linear in the range 0.2–500 ng/ml and the regression correlation coefficients (*r*) were 0.995 or better by using 1/concentration weighted linear regression analysis to prevent bias by the highest concentrations. For every calibration curve, the calibration concentrations were back-calculated from the peak heights. The accuracy and precision for the back-calculated concentrations were less than 11% (Table 1). The mean values of accuracy (% DEV) for QC samples at the six tested concentrations (0.2–400 ng/ml) were less than 13.7% in five separate runs (Table 2). The mean within-run and between-run precisions (% C.V.) for all QC samples were less than 8.8% and 7.6%, respectively (Table 2). Therefore, 0.2 ng/ml was estimated to be the LLQ. The extraction efficiencies of CKD-602 (mean±S.D.) in the assay were 100.0±1.51% at 1 ng/ml, 98.8±4.24% at 10 ng/ml and 93.3±1.13% at 100 ng/ml.

3.3. Stability

CKD-602 was found to be stable both in human plasma and in methanol extracts for at least 3 months when stored at −30 °C (Table 3). No significant decrease of CKD-602 in plasma after 24 h incubation at room temperature or after exposing to three freeze–thaw cycles was observed. Extracted plasma samples were also found to be stable at ambient temperature upon standing in the autosampler tray for at least 20 h.

3.4. Application of the assay in a pharmacokinetic study

The described analytical methods were applied in our institute to a phase I and pharmacokinetic study

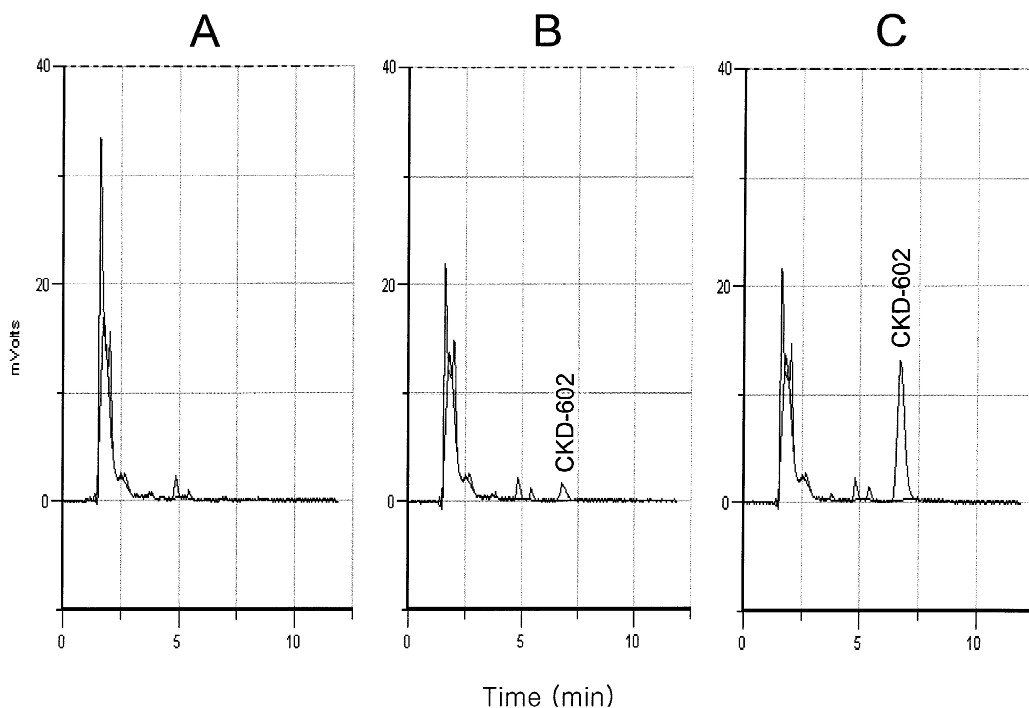

Fig. 2. HPLC chromatograms of CKD-602: (A) a human blank plasma sample; (B) a spiked human plasma sample containing 0.5 ng/ml; (C) a spiked human plasma sample containing 5 ng/ml. The mobile phase used was a mixture of methanol–0.1 *M* hexane-1-sulfonic acid in methanol–0.01 *M* TEMED in water (pH 6.0) (40:1:59, v/v). The retention time of CKD-602 was 6.9 min with overall chromatographic run time of 12 min.

of CKD-602 in patients with advanced solid cancer. Immediately after sampling, plasma proteins were precipitated with an equal volume of cold methanol. However, immediate protein precipitation might be unnecessary according to the above stability results, because CKD-602 as the sum of the lactone and

Table 1
The accuracy and precision of back-calculated concentrations from peak heights for determination of CKD-602 in human plasma

Run number	Back-calculated concentrations (ng/ml)								
	0.2[a]	0.5[a]	1.0[a]	5.0[a]	10[a]	20[a]	50[a]	200[a]	500[a]
1	0.209	0.475	0.909	4.511	8.978	21.33	53.66	214.66	542.51
2	0.211	0.470	0.884	4.428	9.052	21.16	53.76	216.84	557.87
3	0.209	0.476	0.905	4.458	8.929	21.16	52.86	217.47	556.16
4	0.207	0.472	0.954	4.589	9.076	21.17	51.80	219.46	524.68
5	0.208	0.477	0.910	4.488	8.990	21.01	53.70	211.46	557.72
Mean	0.209	0.474	0.912	4.495	9.005	21.16	53.16	215.98	547.79
Accuracy (% DEV)[b]	4.3	−5.2	−8.8	−10.1	−10.0	5.8	6.3	8.0	9.6
Precision (% C.V.)[c]	0.6	0.6	2.8	1.4	0.7	0.5	1.6	1.4	2.6

[a] Nominal concentration.
[b] Deviation from the nominal concentration.
[c] Coefficient of variation.

Table 2
The mean accuracy, mean within-run precision and between-run precision of QC samples in five separate runs for determination of CKD-602 in human plasma (n=30)

QC sample (ng/ml)	Mean accuracy (% DEV)[b]	Precision (% C.V.)[c]	
		Mean within-run	Between-run
0.2[a]	1.3	8.8	7.6
0.5[a]	−8.5	2.1	6.9
1[a]	0.1	1.7	5.4
10[a]	−5.3	0.9	4.2
100[a]	11.6	1.5	4.0
400[a]	13.7	1.3	3.5

[a] Nominal concentration.
[b] Deviation from the nominal concentration.
[c] Coefficient of variation.

carboxylate forms was stable both in plasma and in methanol extracts during storage for 3 months.

The plasma concentration–time curves of CKD-602 were obtained from patients treated with daily 30-min infusion of 0.5–0.9 mg/m^2/day for 5 days (Fig. 3). In the first dose (0.5 mg/m^2/day) of our clinical phase I study, we could analyze the concentration of CKD-602 in samples taken using 200 μl of human plasma. The method described above with an LLQ of 0.2 ng/ml for CKD-602 proved to be suitable for monitoring drug levels in plasma samples obtained from patients treated at low doses (0.5 mg/m^2/day).

4. Conclusion

A simple, sensitive and validated isocratic reversed-phase HPLC method was developed for the analysis of a new antitumor drug, CKD-602 in human plasma. The assay allows the quantification of CKD-602 concentrations in the range 0.2–500

Table 3
Stability of CKD-602 in human plasma and in methanol extracts following storage at −30 °C for 3 months

Time (months)		Nominal concentration in plasma (ng/ml)			Nominal concentration in methanol extracts (ng/ml)		
		1	10	100	1	10	100
0	% change[a]	100.0	100.0	100.0	100.0	100.0	100.0
	% C.V.[b]	0.9	2.0	1.5	2.0	1.4	1.1
1	% change[a]	94.0	101.0	104.8	105.1	99.0	98.4
	% C.V.[b]	1.4	1.8	1.6	1.1	1.8	2.6
2	% change[a]	94.3	98.6	93.8	91.8	93.4	88.0
	% C.V.[b]	2.8	3.2	2.9	2.2	2.5	3.1
3	% change[a]	103.2	103.9	96.9	99.8	95.0	93.1
	% C.V.[b]	1.5	1.1	1.0	1.6	3.8	0.9

[a] Percentage of mean concentration of CKD-602 to initial mean concentration.
[b] Coefficient of variation; n=6.

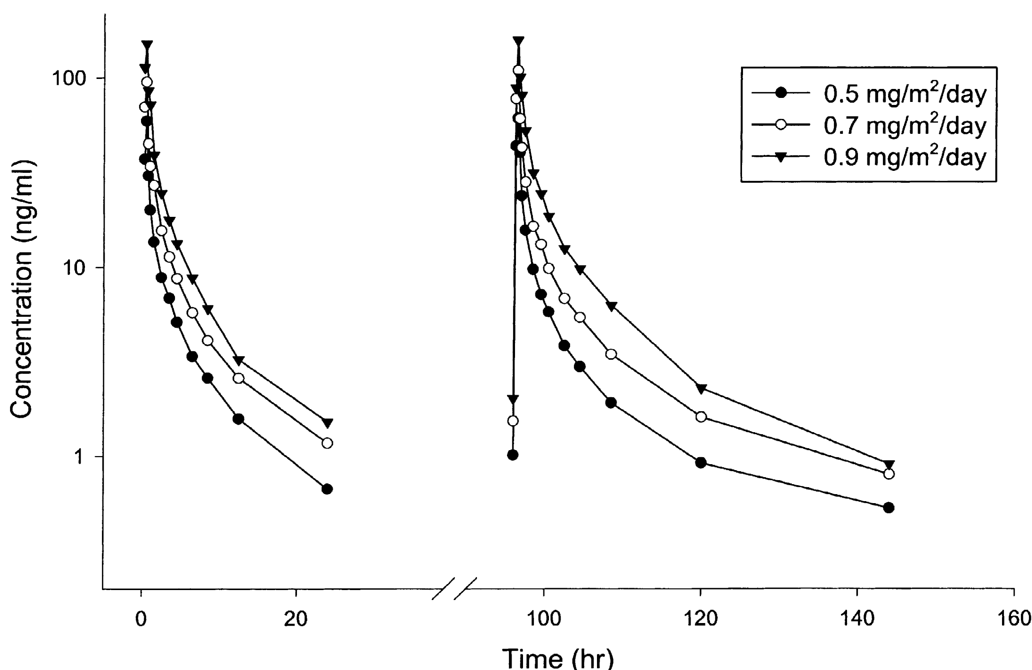

Fig. 3. Mean plasma concentration–time curves for CKD-602 in patients after a daily 30 min infusion of 0.5–0.9 $mg/m^2/day$ for 5 days.

ng/ml. CKD-602 was found to be stable both in human plasma and in methanol extracts at −30 °C for 3 months. The described assay was successfully applied to the analysis of plasma samples from pharmacokinetic studies of CKD-602.

Acknowledgements

This work was supported by the Chong Kun Dang Pharmaceutical Corporation.

References

[1] Y.H. Hsiang, L.F. Liu, Cancer Res. 48 (1988) 1722.
[2] W.J. Slichenmeyer, E.K. Rowinsky, R.C. Donehower, S.H. Kaufmann, J. Natl. Canc. Inst. 85 (1993) 271.
[3] C.G. Moertel, A.J. Schutt, R.J. Reitemeier, R.G. Hahn, Cancer Chemother. Rep. 56 (1972) 95.
[4] G.J. Creemers, B. Lund, J. Verweij, Cancer Treat. Rev. 20 (1994) 73.
[5] J.H. Lee, J.M. Lee, J.K. Kim, S.K. Ahn, S.J. Lee, M.Y. Kim, S.S. Jew, J.G. Park, C.I. Hong, Arch. Pharm. Res. 21 (1998) 581.
[6] J.H. Lee, J.M. Lee, K.H. Lim, J.K. Kim, S.K. Ahn, Y.J. Bang, C.I. Hong, Ann. N.Y. Acad. Sci. 922 (2000) 324.
[7] J. Fassberg, V.J. Stella, J. Pharm. Sci. 8 (1992) 676.
[8] C.H. Takimoto, R.W. Klecker, W. Dahut, L.K. Yee, J.M. Strong, C.J. Allegra et al., J. Chromatogr. 655 (1994) 97.
[9] T.G. Burke, Z. Mi, J. Med. Chem. 37 (1994) 40.
[10] R.P. Herzberg, M.J. Caranfa, S.M. Hecht, Biochemistry 28 (1989) 4629.
[11] J. Verweij, B. Lund, J. Beijnen, A. Planting, M. de Boer-Dennert, I. Koier, H. Rosing, H. Hansen, Ann. Oncol. 4 (1993) 673.
[12] L.J.C. Van Warmerdam, J. Verweij, J.H.M. Schellens, H. Rosing, B.E. Davies, M. de Boer-Dennert, R.A.A. Maes, J.H. Beijnen, Cancer Chemother. Pharmacol. 35 (1995) 237.
[13] L.R. Rivory, J. Robert, J. Chromatogr. 661 (1994) 133.
[14] H. Sumiyoshi, Y. Fujiwara, T. Ohune, N. Yamaoka, K. Tamura, M. Yamakido, J. Chromatogr. 670 (1995) 309.
[15] H. Rosing, E. Doyle, B.E. Davies, J.H. Beijnen, J. Chromatogr. B 668 (1995) 107.