ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 279–286

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

The impact of column temperature in the high performance liquid chromatographic analysis of topotecan in rat and dog plasma

H. Rosing[a,*], E. Doyle[b], J. H. Beijnen[a,c]

[a]*Department of Pharmacy, Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
[b]*Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Hertfordshire, UK*
[c]*Department of Pharmaceutical Analysis and Toxicology, Faculty of Pharmacy, University of Utrecht, Utrecht, The Netherlands*

Received for review 8 January 1996; revised manuscript received 21 March 1996

Abstract

A sensitive high performance liquid chromatographic (HPLC) assay has been developed and validated for the quantitation of the novel anticancer agent topotecan and topotecan as its lactone plus carboxylate forms in rat and dog plasma. Linear responses in analyte standard peak areas were observed over the concentration ranges 0.10–10 ng ml^{-1} using 100 μl of rat plasma and 0.2–100 ng ml^{-1} using 100 μl of dog plasma. Due to the instability of the drug in the biological matrix it was necessary to obtain the plasma fraction within 5 min after blood sampling by centrifugation, immediately followed by protein precipitation with cold methanol (−30°C). For the determination of total drug levels (lactone plus lactone ring-opened form), plasma samples were deproteinated with methanol and subsequently acidified with 2% (v/v) perchloric acid.

The samples were analysed by HPLC using a Zorbax SB-C18 Stable Bond column and methanol–0.1 M hexane-1-sulfonic acid in methanol–0.01 M *N,N,N′,N′*-tetramethylethylenediamine in distilled water pH 6.0 (25:10:65, v/v/v) as the mobile phase. The detection was performed fluorimetrically.

The analytical column was thermostated at 19–21°C to obtain baseline resolution between an interfering endogenous compound in rat and dog plasma and topotecan. This endogenous peak was absent in human plasma. Variation of chromatography temperature appeared to be a very useful tool in the bioanalysis of topotecan. It allowed optimization of the separation between the endogenous compound and the analyte; different mechanisms of solute interactions are apparently involved in this reversed-phase ion-pair chromatographic system.

Keywords: Bioanalysis; Dog; Rat; Temperature; Topotecan

* Corresponding author.

PII S0731-7085(96)01838-9

1. Introduction

Topotecan (SK&F 104864, [*S*]-9-dimethylaminomethyl-10-hydroxy-camptothecin, NSC 609669; Hycamtin®; Fig. 1) [1], is a semi-synthetic water-soluble analog of camptothecin and an inhibitor of the intranuclear enzyme topoisomerase-I [2,3]. Topotecan has exhibited antitumor activity in an array of experimental tumor models [1,4], has been tested recently in several phase I clinical trials [5–11] and is currently undergoing phase II clinical evaluation [12,13]. Topotecan is known to undergo a pH-dependent, reversible hydrolytic dissociation of its lactone moiety into the hydroxyl carboxylate form (SK&F 105992; Fig. 1) [14–16]. Knowledge of this hydrolytic process is considered essential in the evaluation of clinical studies, since it is assumed that the carboxylate form possesses less topoisomerase I inhibitive activity than the parent drug [17]. Several pharmacokinetic studies, however, have shown that the total levels (lactone plus carboxylate forms) of topotecan are predictive for pharmacodynamic outcome [18–20], which questions the clinical relevance of discriminating between the lactone and its ring-opened form. Recently, the present authors reported an improved high performance liquid chromatography (HPLC) technique for the determination of topotecan and total topotecan in human plasma with a lower limit of quantitation of 0.05 ng ml^{-1} [21]. This method was also intended to be used for topotecan determinations in rat and dog plasma to support 6 month oral toxicity studies. During validation with rat and dog plasma, however, interfering endogenous peaks were observed in these matrices which had not been seen in human plasma ($n > 4000$). An attempt was then made to improve the separation between the peaks of topotecan and the interfering endogenous compound by changing the composition of the eluent, the stationary phase and also the sample pretreatment procedure. Finally it was found that temperature had a very selective effect on the chromatography of topotecan and the interfering agent. In this paper the development and validation of an assay for topotecan and topotecan as its lactone plus carboxylate forms in rat and dog plasma is described exploiting the selective influences of temperature on the separation of the analytes.

2. Experimental

2.1. Chemicals

Topotecan, as the hydrochloride salt (SKF 104864-A; lot. MM-15906-194), was supplied by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). All other reagents were of analytical grade and double-distilled water was used throughout.

2.2. Chromatography

The chromatographic system consisted of a solvent delivery system, type P1000, and an automatic sample injection device model AS300 (Thermo Separations Products, Fremont, CA) in which samples were cooled at 1°C for the determination of topotecan in non-acidified methanol extracts. Chromatographic separation was performed on a Zorbax SB-C18 column (internal diameter: 4.6 mm; length: 75 mm; particle size: 3.5 μm) (Rockland Technologies Inc., Newport, DE) which was thermostated at 19°C with a Haake D1 kryostate (Haake Mess-Technik, Karlsruhe, Germany). To protect the analytical column, a guard column (3 mm × 10 mm) packed with reversed-phase material (C-18) was used (Chrompack, Middelburg, The Netherlands). The mobile phase consisted of a mixture of methanol–0.1 M hexane-1-sulfonic acid in methanol-0.01 M *N,N,N′,N′*-tetramethylethylenediamine (TEMED) in distilled water (the pH was adjusted with phosphoric acid to 6.0) (25:10:65, v/v/v). The flow was 1.0 ml min^{-1} and the detection was performed fluorimetrically using a FP920 Intelligent Fluorescence Detector (Jasco International Co. Ltd.,

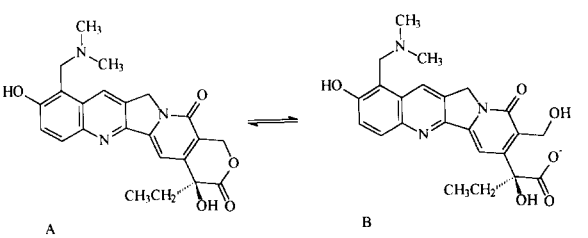

Fig. 1. Equilibrium reactions between (A) topotecan and (B) its lactone ring-opened form (SK&F 105992) [16].

Tokyo, Japan) with an excitation wavelength of 361 nm and an emission wavelength of 527 nm with a 40 nm bandwith. The volume of the flow-cell of the fluoresence detector was 16 μl.

2.3. Sample pretreatment

After centrifuging a blood sample at 2500 *g* for 5 min, a 100 μl plasma sample was taken and mixed with 200 μl of cold methanol (−30°C). After vortex mixing for 10 s and centrifugation at 9500 *g* for 3 min, the clear supernatant was transferred to a clean tube and stored, immediately, at −70°C.

For the determination of the lactone form the sample was diluted 1:1 (v/v) with distilled water prior to injection.

For the determination of total levels (the total of the lactone and carboxylate forms) the methanol extract was diluted 1:1 (v/v) with 2% (v/v) perchloric acid. After vortex mixing for 10 s and centrifugation at 9500 *g* for 10 min, the clear supernatant was injected into the HPLC system.

2.4 Calibration

Blank plasma samples (100 μl), in polypropylene Eppendorf tubes, were spiked with 25–100 μl of an appropriate amount of topotecan in methanol and stored at −30°C. Cold methanol (−30°C) was then added to achieve a total volume of 200 μl. After vortex mixing for 10 s and centrifugation at 9500 *g* for 3 min, the clear supernatant was transferred to a clean tube and stored, immediately, at −70°C. The samples were further treated as described in Section 2.3.

2.5. Validation

A single run validation was performed to determine topotecan and topotecan as its lactone plus carboxylate forms in rat and dog plasma. This method was an extension of a human plasma method that was validated over three runs and was previously published [21]. The following parameters were determined: linearity, within-run precision, accuracy, separation of endogenous plasma constituents and absolute recovery. Topotecan plasma calibration standards were prepared and analyzed, producing a calibration line ranging from 0.10–10 ng ml^{-1} in rat plasma and from 0.2–100 ng ml^{-1} in dog plasma. Based upon the analysis of residuals the linear regression of the peak area of topotecan versus the concentration was weighted by $1/x^2$, the reciprocal of the squared topotecan concentration. A test for lack of fit to a linear model was used to evaluate the linearity of the calibration lines and the presence of any translational and/or rotational bias in the assay was tested with a Student *t*-test [22].

Four spiked plasma samples containing 0.1, 0.25, 5.0 and 10.0 ng / mL^{-1} topotecan in rat plasma and 0.2, 0.5, 10.0 and 100 mg / mL^{-1} topotecan in dog plasma were prepared for the determination of the accuracy and precision. Six replicates of each quality control sample were processed and analysed with plasma calibration standards to determine the lactone concentration and the total of the lactone and carboxylate forms. The within-run precision was calculated by the relative standard deviation, determined at each concentration. The average accuracy is the mean measured concentration divided by the nominal concentration multiplied by 100%, determined for each set of replicate spiked samples.

Six batches of control plasma were tested to determine whether or not endogenous plasma constituents co-eluted with topotecan.

The average extraction recoveries were determined by comparing the mean response of six processed quality control samples to the mean response of three processed samples of topotecan in distilled water. Recoveries were determined at two concentrations: 0.25 and 10.0 ng ml^{-1} in rat plasma and 0.5 and 100 ng ml^{-1} in dog plasma.

3. Results and discussion

3.1. Separation of the interfering endogenous compound and topotecan

The earlier reported method for the analysis of topotecan in human plasma [21] could not be used for the determination of the drug in rat and dog plasma: there was an interfering endogenous com-

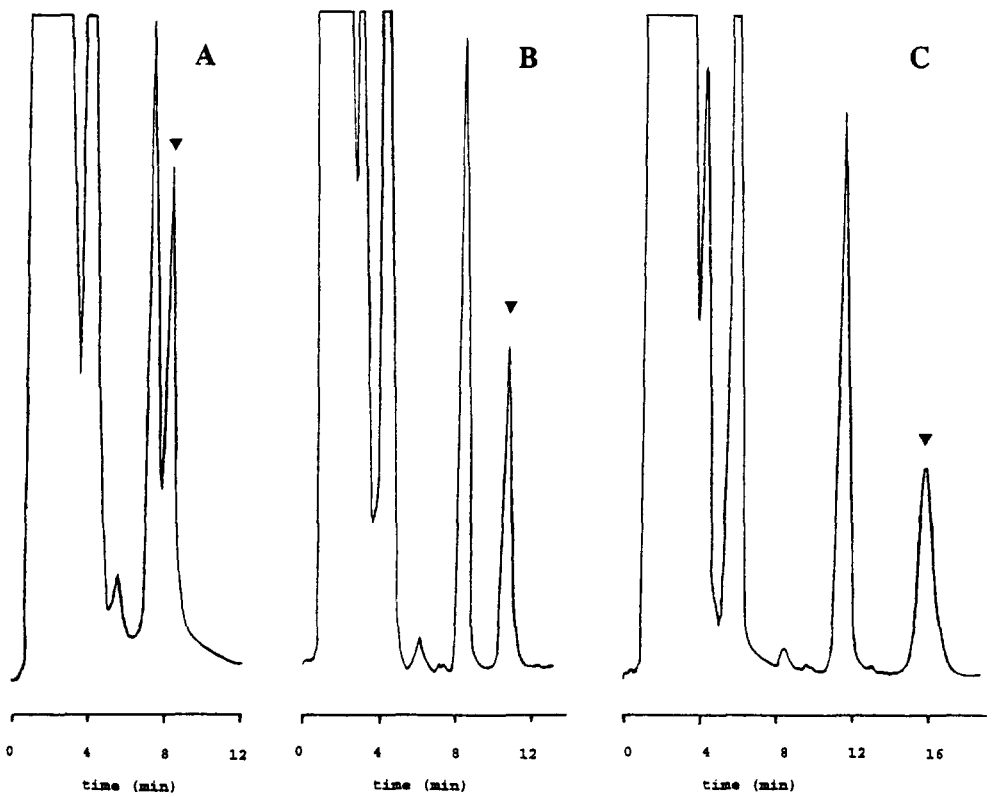

Fig. 2. HPLC chromatograms of total levels of topotecan (1 ng ml^{-1}) in rat plasma recorded at different temperatures: (A) 25, (B) 19 and (C) 12°C. The retention times of topotecan (indicated by ▼) in these systems are 8.5, 10.9 and 15.7 min respectively. Experimental conditions as described in the text.

pound present in these matrices (Fig. 2A). This observation was not an artifact as comparable chromatograms were recorded processing several batches of these matrices with the same result. The integration of topotecan concentrations lower than 0.5 ng ml^{-1} in rat and dog plasma was not

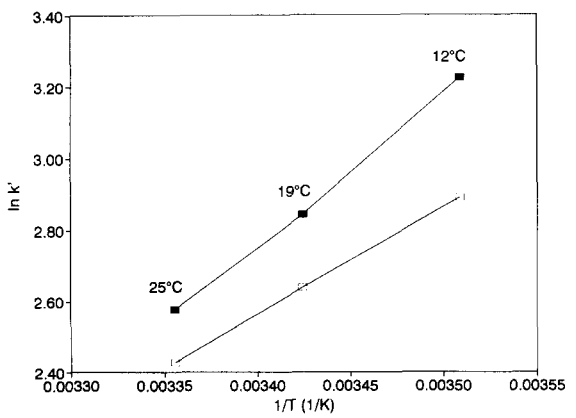

Fig. 3. Van't Hoff plots for topotecan (■) and the interfering endogenous compound (□) in rat and dog plasma.

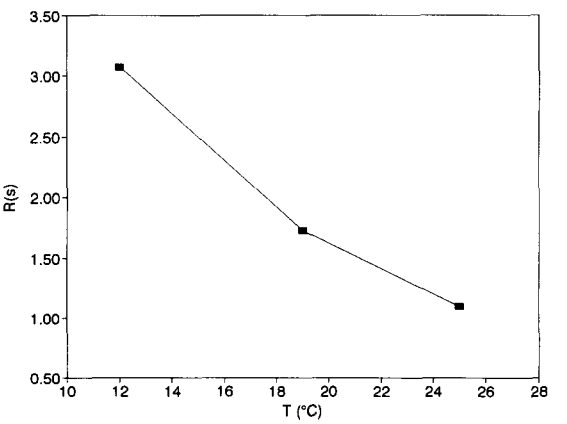

Fig. 4. Dependence of the resolution factor (R_s) on temperature for topotecan and the interfering endogenous compound.

Table 1
Assay performance data for the determination of topotecan and topotecan as lactone plus carboxylate forms in rat plasma ($n = 6$)

Nominal concentration (ng ml^{-1})	Average concentration found (ng ml^{-1})	Within-run precision (%)	Average accuracy (%)	Average recovery (%)
Determination of topotecan (lactone form)				
0.10	0.095	7.4	95.0	n.d.[a]
0.25	0.251	4.4	100.4	109
5.00	4.97	3.7	99.4	n.d.
10.00	10.08	2.3	100.8	86
Determination of total levels of topotecan				
0.10	0.111	13.5	111.0	n.d.
0.25	0.213	6.1	85.2	88
5.00	5.14	1.5	102.8	n.d.
10.00	10.35	1.7	103.5	103

[a] n.d. = not determined.

reproducible (deviations > 20%) under the reported chromatographic conditions. Changes were made to the composition of the eluent (decreasing the solvent strength, lowering the pH to 3.0, increasing the ion-pair concentration to 20 mM), the stationary phase (Inertsil®) and also the sample pretreatment procedure (solid-phase extractions on C18, C8, C2, phenyl, cyano, diol and SCX columns) to try to improve separation. Decreasing the solvent strength was the only parameter which affected the resolution, resulting in increased retention, but this was not sufficient to achieve the required lower limits of quantitation of 0.1 ng ml^{-1} in rat plasma and 0.2 ng ml^{-1} in dog plasma. Changing the stationary phase and sample pretreatment procedure did not improve the method either. Finally, the influence of temperature on the separation was investigated as there might be a difference in chromatographic interaction between the peaks.

3.2. Temperature effects

HPLC chromatograms recorded at 25, 19 and 12°C are depicted in Figs. 2A, 2B and 2C respectively. The effect of temperature on retention in reversed-phase chromatography is largely determined by the Gibbs free energy ($\Delta G°$) of the solute interaction with the stationary phase. The energy contribution is evaluated from the slope of plots of log k' versus $1/T$, called van't Hoff plots:

$$\ln k' = -\frac{\Delta G°}{RT} + \ln q$$

where k' is the capacity factor, R is the gas constant, T is the absolute temperature and q is the so-called phase ratio in the column [23–27]. The phase ratio is defined as the ratio of the volumes of the mobile and stationary phases. Van't Hoff plots were constructed for topotecan and the interfering endogenous compound in rat and dog plasma (Fig. 3).

In general, for components with identical stationary phase interactions, the van't Hoff plots are linear with slightly different slopes as a result of minor differences in $\Delta G°$ values. The separation will increase with a decrease in temperature. This suggests that the use of lower temperatures is advantageous to obtaining maximum resolution; however, this is usually a poor choice when separation is required between compounds which have the same stationary phase interaction. The reason is that decreased temperature reduces column efficiency and increases operating pressures. Therefore, temperature is often the last parameter to be optimized in reversed-phase chromatography and is used mainly for the fine tuning the separation [24].

If the van't Hoff plots deviate from linearity, the retention of the solute molecules in the stationary phase is affected by more than one mechanism, or results from very different types of

Table 2
Assay performance data for the determination of topotecan and topotecan as lactone plus carboxylate forms in dog plasma ($n = 6$)

Nominal concentration (ng ml^{-1})	Average concentration found (ng ml^{-1})	Within-run precision (%)	Average accuracy (%)	Average recovery (%)
Determination of topotecan (lactone form)				
0.20	0.198	2.6	99.0	n.d.[a]
0.50	0.582	2.4	116.4	112
10.0	10.37	0.8	103.7	n.d.
100.0	101.6	0.9	101.6	95
Determination of total levels of topotecan				
0.20	0.201	5.5	100.5	n.d.
0.50	0.496	2.2	99.2	98
10.0	10.77	4.3	107.7	n.d.
100.0	103.3	0.7	103.3	103

[a] n.d. = not determined.

interactions. Under these conditions the order of retention, the selectivity factor and the resolution may all vary with temperature. If ion-pair formation contributes significantly to the distribution, the effect of temperature on the selectivity is considerable [27]. In the chromatographic system used ion-pair formation is the predominant solute–stationary phase mechanism and the temperature had a great influence on the selectivity (Fig. 3). The van't Hoff plot for topotecan deviated slightly from linearity ($p = 0.05$) while that for the interference was linear ($p = 0.0049$), which indicated that differences in the retention mechanisms for both components may exist. The degree of separation between two peaks is defined by their resolution factor (R_s), the retention time difference between the two peak maxima (Δt) and the average base widths (W) of the two peaks:

$$R_s = \frac{\Delta t}{\frac{1}{2}(W_1 + W_2)}$$

Fig. 4 shows a plot of the resolution factor versus temperature. Baseline resolution corresponds to an R_s value of 1.5 or higher. At 19°C the value was about 1.7, sufficient to obtain resolution of topotecan from the endogenous substance in rat and dog plasma.

3.3. Validation

For the validation the report of the conference *Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies*, held in 1990, was used as a guideline [28].

The assay was linear for the determination of lactone and total levels of topotecan over concentration ranges of 0.10–10 ng ml^{-1} in rat plasma and 0.2–100 ng ml^{-1} in dog plasma. No lack of fit was detected with the one-tailed test ($\alpha = 0.01$). Calculated values of the Student t-test were all less than their critical values ($\alpha = 0.05$), indicating

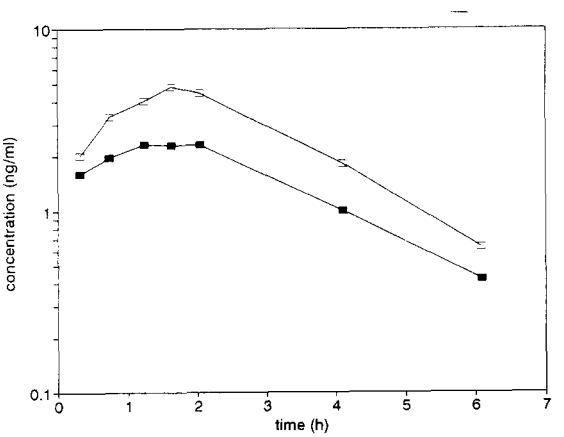

Fig. 5. Plasma concentrations of topotecan (■) and topotecan as lactone plus carboxylate forms (□) in the rat after oral administration of the drug (dose: 0.23 mg kg^{-1}).

that the analytical assay was not afflicted by any bias.

The assay performance data are presented in Tables 1 and 2. The within-run precision was less than 15% for all quality control samples. The average accuracy determined at each concentration was within 85% and 117%. Average recoveries, determined at two concentrations, were between 86% and 112%. When the column was thermostated at 19°C, the chromatograms of six batches of control rat and dog plasma contained no endogenous plasma constituents co-eluting with topotecan. Stability data have been reported earlier [21].

Plasma concentrations of topotecan and topotecan as its lactone plus carboxylate forms from a rat after oral administration of 230 μg kg^{-1} are depicted in Fig. 5 and demonstrate the applicability of the assay for pharmacokinetic toxicity studies at very low dosages of the drug.

4. Conclusion

In conclusion, a simple, sensitive and validated HPLC assay for the quantitative determination of topotecan and topotecan as its lactone plus ring-opened forms in rat and dog plasma is described. The assay quantifies topotecan concentrations of 0.1–10 ng ml^{-1} using 100 μl of rat plasma and 0.2–100 ng ml^{-1} dog plasma. The influence of temperature on the chromatographic behavior of topotecan and an interfering endogenous substance is considerable in this reversed-phase ion-pair chromatographic system. The analytical column must be thermostated at 19–21°C to obtain baseline resolution between the endogenous compound in rat and dog plasma and topotecan.

References

[1] W.D. Kingsbury, J.C. Boehm, D.R. Jakas, K.G. Holde, S.M. Hecht, G. Gallagher, M.H. Caranfa, F.L. McCabe, L.F. Faucette and R.K. Johnson, J. Med. Chem., 34 (1989) 98–107.

[2] J. Cummings and J.F. Smyth, Ann. Oncol., 4 (1993) 533–543.

[3] W.J. Slichenmeyer, E.K. Rowinsky, R.C. Donehower and S.H. Kaufmann, J. Natl. Cancer Inst., 85 (1993) 271–291.

[4] H.A. Burris, A.R. Hanauske, R.K. Johnson, M.H. Marshall, J.G. Kuhn, S.G. Hilsenbeck and D.D. Von Hoff, J. Natl,. Cancer Inst., 84 (1992) 1816–1820.

[5] E.K. Rowinsky, L.B. Grochow, C.B. Hendricks, D.S. Ettinger, A.A. Forastiere, L.A. Hurowitz, W.P. McGuire, S.E. Sartorius, B.G. Lubejko, S.H. Kaufmann and R.C. Donehower, J. Clin. Oncol., 10 (1992) 647–656.

[6] J. Verweij, B. Lund, J. Beijnen, A. Planting, M. de Boer-Dennert, I. Koier, H. Rosing and H. Hansen, Ann. Oncol., 4 (1993) 673–678.

[7] H. Hochster, L. Liebes, J. Speyer, J. Sorich, B. Taubes, R. Oratz, J. Wernz, A. Chachoua, B. Raphael, R.Z. Vinci and R.H. Blum, J. Clin. Oncol., 12 (1994) 553–559.

[8] C.B. Pratt, C. Stewart, V.M. Santana, L. Bowman, W Furman, J. Ochs, N. Marina, J.F. Kuttesch, R. Heideman, J.T. Sandlund, L. Avery and W.H. Meyer, J. Clin. Oncol., 12 (1994) 539–543.

[9] S.M. Blaney, F.M. Balis, D.E. Cole, C. Craig, J.M. Reid, M.M. Ames, M. Krailo, G. Reaman, D. Hammond and D. Poplack, Cancer Res., 53 (1993) 1032–1036.

[10] J.G. Wall, H.A. Burris, D.D. Von Hoff, G. Rodriguez, R. Kneuper-Hall, D. Shaffer, T. O'Rourke, T. Brown, G. Weiss, G. Clark, S. McVea, J. Brown, R. Johnson, C. Friedman, B. Smith, W.S. Mann and J. Kuhn, Anti-Cancer Drugs, 3 (1992) 337–345.

[11] G.J. Creemers, B. Lund and J. Verweij, Cancer Treat. Rev., 20 (1994) 73–96.

[12] T.J. Lynch, L. Kalish, G. Strauss, A. Elias, A. Skarin, L.N. Shulman, M. Posner and E. Frei, J. Clin. Oncol., 12 (1994) 347–352.

[13] H.A. Burris, M.L. Rothenberg, J.G. Kuhn and D.D. Von Hoff, Semin. Oncol., 19 (1992) 663–669.

[14] J.H. Beijnen, B.R. Smith, W.J. Keijer, R. van Gijn, W.W. ten Bokkel Huinink, L.Th. Vlasveld, S. Rodenhuis and W.J.M. Underberg, J. Pharm. Biomed. Anal., 8 (1990) 789–794.

[15] J. Fassberg and V.J. Stella, J. Pharm. Sci., 81 (1992) 676–684.

[16] W.J.M. Underberg, R.M.J. Goossen, B.R. Smith and J.H. Beijnen, J. Pharm. Biomed. Anal., 8 (1990) 681–683.

[17] R.P. Hertzberg, M.J. Caranfa, K.G. Holden, D.R. Jakes, G. Gallagher, M.R. Mattern, S.M. Mong, J.O. Bartus, R.K. Johnson and W.D. Kingsbury, J. Med. Chem., 32 (1989) 715–720.

[18] L.B. Grochow, E.K. Rowinsky, R. Johnson, S. Ludeman, S.H. Kaufmann, F.L. McCabe, B.R. Smith, L. Hurowitz, A. DeLisa, R.C. Donehower and D.A. Noe, Drug Metab. Disp., 20 (1992) 706–713.

[19] L.J.C. van Warmerdam, J. Verweij, J.H.M. Schellens, H. Rosing, B.E. Davies, M. De Boer-Dennert, R.A.A. Maes and J.H. Beijnen, Cancer Chemother. Pharmacol., 35 (1995) 237–245.

[20] L.J.C. van Warmerdam, W.W. ten Bokkel Huinink, S. Rodenhuis, I. Koier, B.E. Davies, H. Rosing, R.A.A. Maes and J.H. Beijnen, J. Clin. Oncol., 13 (1995) 1768–1776.

[21] H. Rosing, E. Doyle, B.E. Davies and J.H. Beijnen, J. Chromatogr. B, 668 (1995) 107–115.
[22] M. Thompson, Anal. Proc., 27 (1990) 142–144.
[23] J.S. Kowalczyk and G. Herbut, J. Chromatogr., 196 (1980) 11–20.
[24] L.R. Snyder, J. Chromatogr., 179 (1979) 167–172.
[25] W.R. Melander, B.K. Chen and C. Horvath, J. Chromatogr., 185 (1979) 99–109.
[26] G. Vigh and Z. Varga-Puchony, J. Chromatogr., 196 (1980) 1–9.
[27] C.P. Terweij-Groen and J.C. Kraak, J. Chromatogr., 138 (1977) 245–266.
[28] 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) 309–312.