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Bioanalysis of zosuquidar trihydrochloride (LY335979) in small volumes of human and murine plasma by ion−pairing reversed-phase high-performance liquid chromatography

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Abstract

We have developed and validated a sensitive and selective method for the quantitative determination of the P-glycoprotein inhibitor zosuquidar (LY335979) in human and murine plasma using only 50 μl sample volumes. Sample pretreatment involved liquid-liquid extraction with *tert*-butyl methyl ether. Zosuquidar and the internal standard chlorpromazine were separated using a narrow bore column (2.1 mm × 150 mm) packed with 3.5 μm symmetry C_{18} material. The mobile phase consisted of 38% (v/v) acetonitrile in 50 mM ammonium acetate buffer pH 3.8 containing 0.005 M 1-octyl sulfonic acid and was delivered at 0.2 ml/min. Detection was performed with a fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 460 nm. The calibration curve was prepared in blank human plasma and was linear over the dynamic range (10–1000 ng/ml). The lower limit of quantitation was 20 ng/ml. The validation results showed that the assay was selective and reproducible. Within the range of the calibration curve the accuracy was close to 100% and within-day and between-day precision were within the generally accepted 15% range. This method was applied to study the pharmacokinetics of i.v. administered zosuquidar in mice. The sensitivity of the assay was sufficient to determine the drug concentration in plasma samples obtained up to 24 h after administration.
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1. Introduction

The compound zosuquidar (Fig. 1) is a inhibitor of P-glycoprotein (Pgp) and currently tested in clinical trials [1]. Pgp is located in the cell membrane and is thought to play a role in multidrug resistance of tumor cells by acting as an outward directed transporter for many structurally unrelated anti-cancer drugs (e.g. taxanes, vinca-alkaloids, anthracyclines). Furthermore, Pgp is also expressed in normal tissues like the gut, the liver and kidneys, where it plays an important role in the elimination of substrate drugs and at barrier sites like the blood–brain barrier and placenta, where it has an important protective role [2].

To date, many inhibitors of Pgp have been tested in clinical trials. Lessons of these studies are that Pgp inhibitors should be potent in order to achieve a maximum inhibitory effect at non-toxic plasma concentrations and selective in order to have no or only minimal effects on the clearance of the anticancer drug. In contrast to several drugs with Pgp inhibitory properties (e.g. verapamil and cyclosporin A), zosuquidar has been developed specifically as a selective and potent Pgp inhibitor. Preclinical studies suggest that this compound is effective in murine leukemia models and non small cell lung cancer models of multidrug resistance [3,4],

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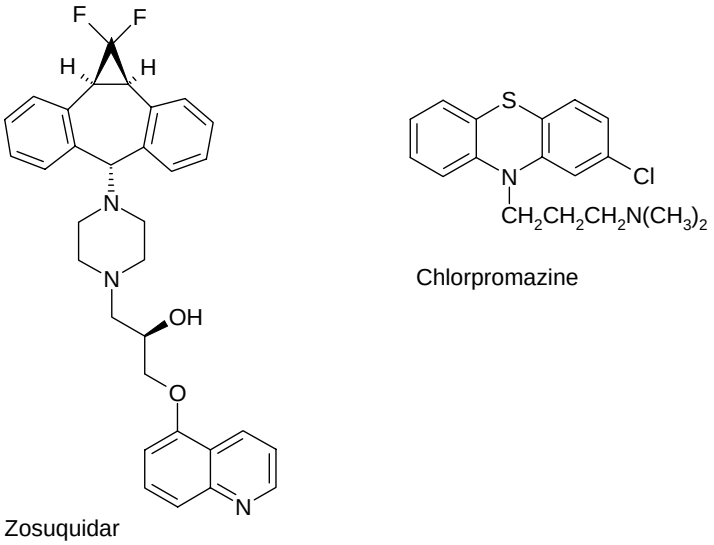

Fig. 1. Structures of zosuquidar and the internal standard chlorpromazine.

whereas it has no effect on the pharmacokinetics of paclitaxel or doxorubicin [1,4,5].

In order to support our preclinical studies in mice, a bioanalytical assay for zosuquidar in mouse plasma was needed. Recently, two HPLC-fluorescence assays for zosuquidar have been reported. The first paper describes the determination of zosuquidar in liver microsomes [6]. However, a sample clean up procedure for the determination of zosuquidar in plasma samples was not reported. The second assay was developed to support a pharmacokinetic study of zosuquidar in patients. The paper, however, discloses only minimal information on the details of the methodology [1]. In contrast to the pharmacokinetic studies in patients, the maximum volume of the blood samples taken from a mouse is usually limited to a few hundred microliters. The aim of this study was, therefore, to develop and validate a sensitive assay for zosuquidar requiring only microvolumes of mouse plasma.

2. Experimental

2.1. Chemicals

Zosuquidar·3HCl (lot# 172SB9) was kindly provided by Eli Lilly and company (Indianapolis, USA). Chlorpromazine·HCl originated from BUFA (Uitgeest, The Netherlands). PIC B-8 (1-octane sulfonic acid) was purchased from Waters (Milford, MA, USA). Chloroform (HPLC-grade) originated from Biosolve BV (Valkenswaard, The Netherlands). All other chemicals were of analytical or Lichrosolv gradient grade and were purchased from E. Merck (Darmstadt, Germany). Water was purified by the Milli-Q Plus system (Millipore, Milford, MA, USA). Blank human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Stock solutions

Independent stock solutions (≅100 μg/ml; corresponding to 161 μM) were prepared by accurately weighing of approximately 10 mg of zosuquidar·3HCl, which was dissolved in 100 ml of purified water. Aliquots were stored at −20 °C.

A 1 mg/ml stock solution of the internal standard chlorpromazine·3HCl was prepared by dissolving 25 mg of accurately weighed chlorpromazine·3HCl in 25 ml of purified water. Aliquots were stored at −20 °C. With each run this stock solution was further diluted with purified water to a 250 ng/ml internal standard working solution.

2.3. HPLC instrumentation and conditions

The HPLC system consisted of a model 480 ternary gradient pump connected to a model GT-103 degasser (Gynkotek, Germering, Germany), a Model 360 autosampler (Kontron, Basel, Switzerland), provided with a 100-μl sample loop and a Model FP920 fluorescence detector (Jasco, Hachioju City, Japan). Separation was carried out with a narrow bore stainless steel column (150 mm × 2.1 mm i.d.) packed with 3.5 μm symmetry C_{18} material (Waters, Milford, MA, USA). The mobile phase consisted of 380 ml acetonitrile, 620 ml of 50 mM ammonium acetate buffer, pH 3.8, and one vial of Pic B-8 (resulting in a final concentration of 0.005 M 1-octane sulfonic acid). The flow rate was 0.2 ml/min. The fluorescence detector was set at excitation and emission wavelengths of 260 and 460 nm, respectively. The bandwidth of the emission monochromator was set at 40 nm and the gain at 100. Chromatographic data acquisition and reprocessing was performed using a Datajet 4600 integrator connected to a WINner on Windows Version 2.0 data station (ThermoSeparations Products, Fremont, CA, USA). Ratios of peak areas of zosuquidar and the internal standard were used for quantitative calculations.

2.4. Calibration standards and quality control samples

Independently prepared stock solutions were used for preparation of the calibration standards and the quality control samples. For each run, a set of calibration samples was prepared containing zosuquidar at concentrations of approximately 10, 20, 50, 100, 200, 500 and 1000 ng/ml by diluting the 100 μg/ml stock solution in blank human plasma.

Quality control samples in plasma were prepared by dilution of the stock solution in blank human plasma to final concentrations of 10, 20, 100 and 500 ng/ml and in mouse plasma to final concentrations of 10, 20, 50, 100 and 500 ng/ml.

2.5. Sample pretreatment

Volumes of 50 μl of human or mouse samples were pipetted into 2 ml eppendorf vials. Samples with concentrations of zosuquidar above the dynamic range of the calibration curve were diluted in blank human plasma prior to further sample pretreatment. Volumes of 50 μl of the internal standard working solution and 1 ml of *tert*-butyl methyl ether were added and the vials were mixed vigorously for 5 min. After centrifugation for 5 min at 2000 × *g*, the aqueous layer was frozen by placing the vials in a bath of ethanol-solid carbon dioxide. The upper organic layer was decanted into a 1.5 ml eppendorf vial. After evaporation in a Speed-Vac Plus SC210A (Savant, Farmingdale, NY, USA) at 43 °C, the residue was reconstituted in 200 μl acetonitrile-50 mM ammonium acetate buffer pH 3.8 (30:70, v/v) by sonication for 5 min and the vials were placed in the HPLC autosampler.

2.6. Assay validation

Validation was performed in human and mouse plasma and included the determination of the linearity, accuracy, precision, selectivity, limit of detection, lower limit of quantification, recovery and stability. The statistical analyses were done with the computer program SPSS for Windows (version 9.0; SPSS, Chigago, IL, USA).

2.6.1. Linearity

The linearity of the calibration curves was determined using the *F*-test for lack of fit ($\alpha = 0.05$). A weight factor of $1/(\text{conc.})^2$ was most appropriate.

2.6.2. Selectivity

Drug-free human plasma from six different healthy donors and plasma from five different untreated FVB mice were processed and analyzed to determine whether endogenous plasma peaks co-elute with zosuquidar or the internal standard.

2.6.3. Accuracy and precision

Quality control samples in human plasma at a concentration of 20, 100 and 500 ng/ml and in mouse plasma at a concentration of 10, 20, 50, 100 and 500 ng/ml were processed and analyzed in five-fold in three runs. The accuracy was calculated by the ratio of the interpolated versus the nominal concentration (multiplied by 100%). The precision was calculated by one-way analysis of variance (ANOVA) for each control sample using the run day as the classification variable. The day mean square (DayMS), error mean square (ErrMS) and the grand mean (GM) of the observed concentrations across run days were obtained. The within-day precision (WPD, %) and the between-day precision (BDP, %) for each quality control sample were calculated using the formulas:

$$\text{WPD}(\%) = \frac{(\text{ErrMS})^{1/2}}{\text{GM} \times 100}$$

$$\text{BDP}(\%) = \frac{((\text{DayMS} - \text{ErrMS})/n)^{1/2}}{\text{GM} \times 100}$$

where n is the number of replicates in each run. When the DayMS is smaller than ErrMS it means that no additional variation was introduced by performing the analyses in different runs.

2.6.4. Lower limit of quantitation

To determine the lower limit of quantitation (LLQ) in human plasma, we spiked blank human plasma of seven different individuals with zosuquidar at a concentration of 10 ng/ml. The LLQ was established when the accuracy was within the 100 ±20% range and the WDP% was smaller than 20%.

2.6.5. Recovery

The extraction recovery was calculated from the ratio of the slopes of three independently processed calibration curves versus the slope of a set of standards prepared by dilution of the zosuquidar stock solution in acetonitrile 50 mM ammonium acetate buffer pH 3.8 (30:70, v/v).

2.6.6. Stability

The long term stability of zosuquidar under storage conditions of −20 °C was determined at concentrations of 20 and 500 ng/ml in human plasma for up to 12 months. Stability during three freeze–thaw cycles was determined in human and mouse plasma at concentrations of 20 and 500 ng/ml. Furthermore, to determine the in process stability, aliquots of 500 ng/ml of zosuquidar in human and mouse plasma were kept on melting ice (0–4 °C), at room temperature (22–24 °C) and at 37 °C for up to 4 h. The stability of zosuquidar in processed samples while standing at room temperature in the autosampler was investigated by analyzing processed quality control samples of 20 and 500 ng/ml after 0 and 72 h.

2.7. Applicability of the assay

To demonstrate the applicability of this method for pharmacokinetics purposes, we have analyzed a set of mouse

samples. The experiments were approved by the local committee for animal experiments. Zosuquidar was administered i.v. at a dose of 20 mg/kg. Sampling was performed at 10 and 30 min and 1, 2, 4, 8 and 24 h after administration, using four to five mice per time point. Blood was obtained by cardiac puncture under anesthesia with methoxyflurane (Medical Developments Australia, Melbourne, Australia) and immediately placed on ice. Plasma samples were obtained by centrifugation (10 min, 3000 × *g*) and stored at −20 °C until analysis.

3. Results and discussion

3.1. Chromatography and detection

The fluorescence properties of zosuquidar were established by emission and excitation wavelength scans using the FP920 detector. Zosuquidar was infused in the flow cell at a concentration of 10 μg/ml in water. The excitation and emission maximum were found at 260 and 470 nm, respectively. Later, when appropriate HPLC conditions had been established, we determined the optimum signal-to-noise ratio at different emission and excitation wavelengths by repeated injections of a standard. The optimum was found at 260 nm for excitation and 460 nm for emission.

Initial experiments were performed with a standard HPLC column with an i.d. of 4.6 mm and packed with 3.5 μm symmetry C_{18} material (Waters, Milford, MA, USA). However, because the sensitivity was considered insufficient, we replaced it by a narrow bore column (2.1 mm i.d.) with the same packing material. This increased the intrinsic sensitivity of the system by five-fold due to a reduction in the peak dilution and reduced mobile phase consumption accordingly.

As in our previous assay for the Pgp inhibitor GF120918 (elacridar) [7], we used chlorpromazine as the internal standard. When using the same mobile phase consisting of acetonitrile–ammonium acetate buffer pH 4.2 (35:65, v/v) and 0.005 M of 1-octane sulfonic acid the retention time of zosuquidar and chlorpromazine were 29.7 and 18.0 min, respectively. By increasing the proportion of acetonitrile in the mobile phase to 38% the retention times shifted to respectively 16.8 and 12.0 min. However, when we tested blank plasma samples under these conditions, we observed several peaks from endogenous substances eluting between 17.2 and 73.1 min and interfering with the analysis of zosuquidar. By careful adjustment of the pH of the mobile phase to more acidic conditions (pH 3.8), we selectively decreased the retention time of zosuquidar to 15.2 min, without changing those of the interferences (Fig. 2). Chlorpromazine eluted at 12.5 min. To purge late-eluting peaks of endogenous substances, the column was flushed for 10 min with a mobile phase containing acetonitrile-water (80:20, v/v). Next, the mobile phase was returned to its original composition and maintained for 20 min prior to injection of the next sample. The total analysis time was 45 min per sample.

3.2. Sample pretreatment

We tested several organic solvents for sample pretreatment by liquid–liquid extraction. Extraction with *tert*-butylmethylether resulted in high extraction recoveries of 83%.

3.3. Validation

The chromatograms of blank plasma from six different individuals and five different FVB mice did not show peaks that interfered with the analysis of zosuquidar.

The LLQ in human plasma was established by spiking blank human plasma from seven different individuals. At a concentration of 10 ng/ml the accuracy and precision were respectively 115.5 and 7.4% and met the requirements.

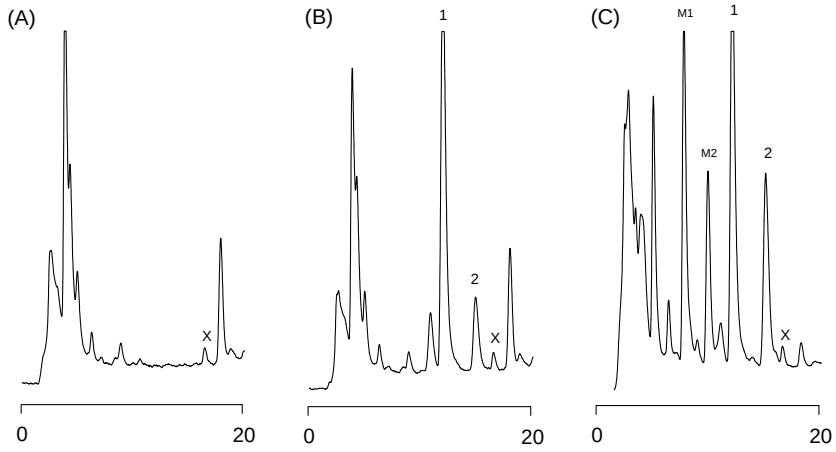

Fig. 2. Representative chromatograms of blank human plasma (A), human plasma spiked with 100 ng/ml zosuquidar (B) and plasma samples from mice obtained at 4 h following 20 mg/kg i.v. zosuquidar, resulting in a concentration of 390 ng/ml (C). (1) chlorpromazine (internal standard); (2) zosuquidar, X is an endogenous peak and the peaks indicated with M1 and M2 are putative metabolites of zosuquidar.

Table 1
Validation parameters of zosuquidar in human and mouse plasma

	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	WDP (%)	BDP (%)
Human	19.8	19.3	97.4	5.6	3.4
	99.2	93.7	94.4	3.6	6.1
	496	505	101.8	2.4	5.7
Mouse	10.6	9.8	93.1	11.4	24.9
	21.1	19.7	93.3	11.0	11.0
	52.8	50.0	94.8	6.6	4.2
	106	95.9	90.9	3.4	5.2
	528	519.4	98.4	6.3	4.1

Table 2
Stability of zosuquidar

Condition	Time	20 ng/ml		500 ng/ml	
		Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
−20 °C, human plasma[a]	0.5 years	105	3.1	–	–
−20 °C, human plasma[a]	1 year	83	1.5	110	2.3
−20 °C, human plasma[b] (three freeze–thaw cycli)	4 days	98	13	108	1.1
−20 °C, murine plasma[b] (three freeze–thaw cycli)	4 days	106	12	107	1.0
Processed sample in autosampler[b]	3 days	114	4.0	103	1.7

[a] Mean of three samples.
[b] Mean of five samples.

Thus, the LLQ in human plasma is 10 ng/ml. Since a similar procedure to establish the LLQ in mouse plasma is not possible due to the small sample size, we have chosen to include a set of quality control specimens in pooled mouse plasma including a sample containing 10 ng/ml of zosuquidar. However, the results for precision at this concentration did not meet the criteria for the LLQ and the LLQ for mouse plasma was set at 20 ng/ml (Table 1). The reason for this discrepancy between mouse and human plasma probably results from the presence of minor peaks in mouse plasma eluting in the vicinity of the zosuquidar peak. Accuracy and precision at the other levels were within the generally accepted ranges for bio-analytical assays [8]. However, overall the results for accuracy in mouse plasma ranged between 90 and 100% of the nominal concentration, suggesting that the extraction recovery of zosuquidar in mouse plasma is slightly less than in human plasma. The results show that it is possible to read the concentrations of zosuquidar in mouse samples from a calibration curve prepared in human plasma. This is advantageous, because of the limited availability of blank mouse plasma. Calibration curves in human plasma were linear over a concentration range of 10 to 1000 ng/ml, which was considered acceptable for pharmacokinetics studies. The optimum weighing factor for the calibration curve was $1/x^2$ (reciprocal of the squared concentration).

3.4. Stability

Overall, zosuquidar was found to be very stable. In human plasma kept at −20 °C, zosuquidar was stable for 1 year (Table 2). However, although the accuracy of the samples at the LLQ (20 ng/ml) met the requirements of 20%, the deviation was more than 10% of the initial value. Consequently, we would recommend storage for a maximum period of 6 months. Zosuquidar was stable in human and mouse plasma during three freeze–thaw cycles and when kept at 4 °C, room temperature and at 37 °C for at least 4 h. Processed samples were stable in the autosampler for up to 72 h, which is far sufficient for a batch of samples (Table 2).

Long term stability of zosuquidar in mouse plasma samples could not be determined due to unexpected problems in this matrix. The extraction recoveries of both zosuquidar and the internal standard were much lower in mouse plasma that had been stored at −20 °C for several months, than in mouse plasma stored for only a very short period of time. The recovery of zosuquidar and the internal standard did not decrease proportionally, resulting in an unacceptable accuracy. Although this problem is not due to the instability of zosuquidar in mouse plasma, it is obvious that is hampers the accurate analysis of zosuquidar stored at −20 °C for several months. Based on our experience a maximum storage period of 2 months should be advised.

3.5. Applicability

The applicability of the assay was tested in plasma samples of mice receiving 20 mg/kg zosuquidar by intravenous administration (Fig. 3). In most plasma samples of mice, two peaks are present at 7 and 9.5 min.

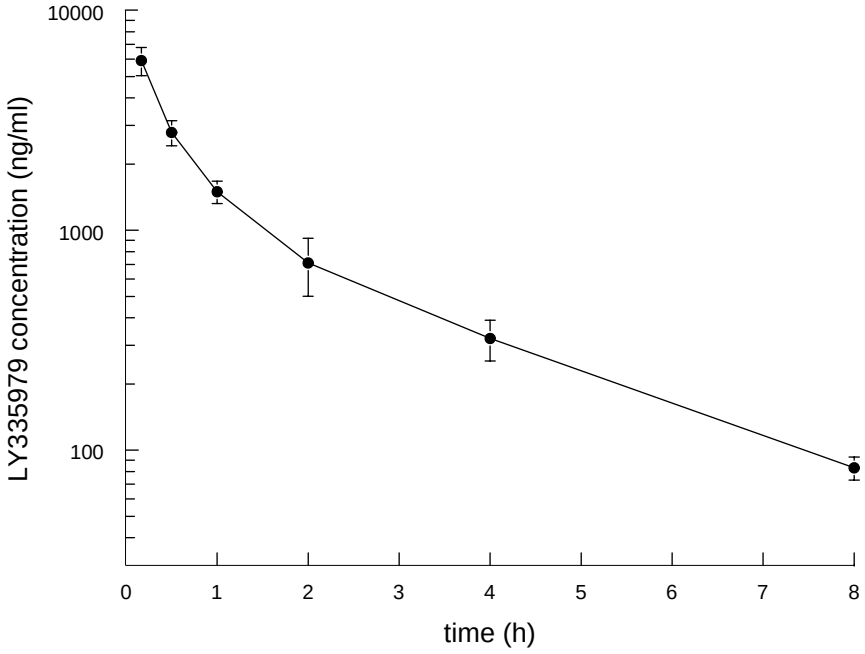

Fig. 3. Plasma concentration time curve of i.v administered zosuquidar (20 mg/kg). Data are shown as the mean of four mice ± S.D. At 24 h after drug administration the concentration of zosuquidar had fallen below the LLQ.

These peaks may represent putative metabolites of zosuquidar in mice, because they are absent in chromatograms of blank murine plasma. Both peaks do not interfere with the peaks of the internal standard and zosuquidar.

So far the applicability in human plasma could not be tested as no human study samples were available. However, given the results of the validation in human plasma, we expect that the assay will be suited for human pharmacokinetics studies as well. In a clinical study reported recently, the concentrations of zosuquidar in plasma ranged from 26 to 330 μg/l, which is within the range of the calibration curve of the presented assay.

Acknowledgements

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References

[1] E.H. Rubin, D.P. de Alwis, I. Pouliquen, L. Green, P. Marder, Y. Lin, R. Musanti, S.L. Grospe, S.L. Smith, D.L. Toppmeyer, J. Much, M. Kane, A. Chaudhary, C. Jordan, M. Burgess, C.A. Slapak, Clin. Cancer Res. 8 (2002) 3710.
[2] O. van Tellingen, Toxicol. Lett. 120 (2001) 31.
[3] D.L. Slate, N.A. Bruno, S.M. Casey, N. Zutshi, L.J. Garvin, H. Wu, J.R. Pfister, Anticancer Res. 15 (1995) 811.
[4] A.H. Dantzig, R.L. Shepard, J. Cao, K.L. Law, W.J. Ehlhardt, T.M. Baughman, T.F. Bumol, J.J. Starling, Cancer Res. 56 (1996) 4171.
[5] J.J. Starling, R.L. Shepard, J. Cao, K.L. Law, B.H. Norman, J.S. Kroin, W.J. Ehlhardt, T.M. Baughman, M.A. Winter, M.G. Bell, C. Shih, J. Gruber, W.F. Elmquist, A.H. Dantzig, Adv. Enzyme Regul. 37 (1997) 335.
[6] W.J. Ehlhardt, J.M. Woodland, T.M. Baughman, M. van den Branden, S.A. Wrighton, J.S. Kroin, B.H. Norman, S.R. Maple, Drug Metab. Dispos. 26 (1998) 42.
[7] E.M. Kemper, B. Jansen, K.R. Brouwer, J.H. Schellens, J.H. Beijnen, O. van Tellingen, J. Chromatogr. B Biomed. Sci. Appl. 759 (2001) 135.
[8] H. Rosing, W.Y. Man, E. Doyle, A. Bult, J.H. Beijen. J. Liq. Chrom. Rel. Technol. 23 (2000) 329.