ELSEVIER

Journal of Chromatography B, 766 (2001) 99–105

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of topotecan in human whole blood and unwashed erythrocytes by high-performance liquid chromatography

Walter J. Loos*, Desirée M. van Zomeren, Hans Gelderblom, Jaap Verweij, Kees Nooter, Gerrit Stoter, Alex Sparreboom

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, P.O. Box 5201, 3008 AE Rotterdam, The Netherlands

Received 19 July 2001; received in revised form 6 September 2001; accepted 25 September 2001

Abstract

A reversed-phase HPLC method for the quantitative determination of total topotecan in human whole blood and unwashed erythrocytes has been developed and validated in terms of sensitivity, specificity, precision and accuracy. Linear calibration curves were constructed in the range of 0.20 to 50.0 ng/ml. The sample pre-treatment for whole blood involved a two-step extraction with methanol and perchloric acid. Prior to extraction, erythrocytes were separated from other blood components by centrifugation in MESED instruments. Separations were achieved on an Inertsil ODS-80A analytical column (150×4.6 mm, 5 μm particle size), eluted at 50°C and a flow-rate of 1.00 ml/min, with a mixture of 100 m*M* ammonium acetate (pH 6.0)–tetrahydrofuran (94.6:5.4, v/v). Fluorescence detection was performed using excitation and emission wavelengths of 381 and 525 nm, respectively. With the applied method, 80% of topotecan was extracted out of whole blood. The lower limit of quantitation in whole blood was established at 0.20 ng/ml with within-run and between-run precisions, respectively, ranging from 1.7 to 9.3% and 1.5–6.1%, while the accuracy ranged from 100 to 113%. The described method will be used in clinical studies to explore the role of erythrocytes in the overall kinetic behavior of topotecan.

Keywords: Topotecan

1. Introduction

We have recently initiated a clinical trial with intravenous (i.v.) topotecan in which besides drug monitoring in plasma, red blood cells and whole blood will be analyzed to fully explore the influence of cellular partitioning and/or binding on overall drug disposition.

Within blood, drugs can bind to many components including blood cells and plasma proteins. As a consequence of this binding, the concentration of drug can differ greatly between whole blood, plasma and unbound drug in plasma water. For ease of chemical analysis, plasma is the most common fluid analyzed although in many respects this choice is unfortunate [1]. One of the primary goals of measuring drug concentrations is to relate the measurement to pharmacologic response and toxicity. However,

*Corresponding author. Department of Medical Oncology, Rotterdam Cancer Institute, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands. Tel.: +31-10-4391-252; fax: +31-10-4391-053.

E-mail address: loos@pcnh.azr.nl (W.J. Loos).

1570-0232/02/$ – see front matter
PII: S0378-4347(01)00432-7

only unbound drug can pass through cellular membranes, because protein-bound drugs are mostly too large. Accordingly, the unbound drug concentration and/or the cellular drug levels are more closely related to the activity of the drug than the total plasma concentration is. Yet unbound drug concentrations and whole blood or red blood cell concentrations are only occasionally measured [1], primarily because the methods for doing so are often tedious and lack accuracy and precision.

For several classes of anti-cancer drugs, including anti-metabolites, oxazaphosphorines and camptothecin analogues [2–5], measurement of whole blood concentration and/or red blood cell distribution is particularly important. The camptothecin analogues, including 9-aminocamptothecin (9-AC) and topotecan, share a pH- and protein-dependent reversible conversion between the pharmacologically active lactone form and its lactone-ring opened carboxylate form. Since only the pharmacologically active lactone forms of these analogues are able to diffuse across cell membranes, red blood cells play an important role in their pharmacokinetic behavior. We have shown, for example, that a major rebound peak in the plasma profile of 9-AC arises as the result of a balance between extensive initial red blood cell uptake of the lactone form and extensive protein binding of the carboxylate form, resulting in a shift in equilibrium between the two forms over time and thus accumulation of the total 9-AC concentrations in the plasma compartment [4]. Red blood cells also appear to play a significant role in the gender-dependent plasma pharmacokinetics of topotecan in adults. Indeed, we found that after oral drug administration, the gender-dependent plasma pharmacokinetics of topotecan was the result of a physiologic difference in hematocrit values between males and females [5]. In addition, gender was one of the predictors of total topotecan plasma clearance in a population model after i.v. administration [6]. It is also well known that the hematocrit values in a normal population of healthy adults are different in males and females, with values of 0.44 ± 0.02 and 0.39 ± 0.02 l/l, respectively [7].

We have now developed and validated a new high-performance liquid chromatography (HPLC) method for the determination of total topotecan concentrations in human heparinized whole blood and unwashed erythrocytes. Since only the lactone form is able to diffuse across cell membranes, and the fact that we are able to analyze the lactone and carboxylate form simultaneously in the plasma compartment [8,9], we here focused only on total topotecan concentrations in erythrocytes and whole blood.

2. Experimental

2.1. Chemicals

Topotecan, was obtained as the hydrochloric salt, from SmithKline Beecham (King of Prussia, PA, USA). Methanol (MeOH) was purchased from Biosolve (Valkenswaard, The Netherlands), tetrahydorfuran from Rathburn (Walkerburn, UK), while neat acetic acid, sodium hydroxide and perchloric acid (70–72%, w/v) were obtained from Baker (Deventer, The Netherlands). Ammonium acetate was purchased from Roth (Karlsruhe, Germany), orthophosphoric acid from Merck (Amsterdam, The Netherlands) and phosphate-buffered saline (PBS) from Oxoid (Basingstoke, UK). A Milli-Q-UF Plus system (Millipore, Bedford, MA, USA) supplied the purified deionized water, used for all solutions. Healthy volunteers kindly donated drug-free human heparinized whole blood, and blank human plasma was obtained from volunteers via the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Stock solutions

A number of three stock solutions, containing 0.50 mg/ml topotecan free base, was prepared by dissolving topotecan·HCl in two times the weighted mass times 921 μl of PBS–MeOH (1:1, v/v). The amounts of topotecan free base in the three stock solutions was determined by comparing, in triplicate, the peak-heights of diluted stock solutions and the difference between the stock solutions was found to be $<5\%$. Out of one of the stock solutions a working solution containing 10 μg/ml topotecan lactone was prepared in PBS–MeOH (1:1, v/v) with the pH adjusted to 3.0 with orthophosphoric acid. This solution was further used for the construction of the

calibration curves. A second working solution, containing 10 μg/ml topotecan carboxylate, was prepared in PBS–MeOH (1:1, v/v) with the pH adjusted to 11.0 with 1 *M* sodium hydroxide and was used for the preparation of the quality control samples.

2.3. Calibration standards and quality control samples

For each analytical run, calibration standards in human heparinized whole blood were prepared freshly in duplicate at topotecan concentrations of 0.20, 0.50, 1.00, 5.00, 10.0, 25.0 and 50.0 ng/ml.

Pools of quality control (QC) samples of topotecan in whole blood were prepared at concentrations of 0.80, 20.0, 40.0 and 200 ng/ml. The QC sample containing 200 ng/ml was used to demonstrate the accuracy and precision of analysis where dilution is required. Aliquots of 250 μl were stored at −80°C upon analysis.

QC samples of unwashed erythrocytes were prepared by incubation of ~35 ml whole blood for 15 min at 37°C simultaneously with topotecan lactone (5 ng/ml) and topotecan carboxylate (5 ng/ml). Subsequently, aliquots of 1 ml were transferred to 28 MESED (measuring off a sediment of pure blood cells) instruments, which were directly processed as described below for the isolation of unwashed erythrocytes and plasma compartment. The remaining blood sample was used for determination of the total topotecan concentration and for hematocrit measurement.

2.4. Isolation of unwashed erythrocytes and plasma

Using MESED instruments (Fabre, Kelmis, Belgium), unwashed erythrocytes and plasma can both be obtained simultaneously from human heparinized whole blood samples, as has been described before [10–12]. In brief, aliquots of 1.5 ml of whole blood were transferred into the upper part of the MESED instrument (Fig. 1), which was fitted tightly into the lower-part, by fixing it in slid 2 of the lower part, sealed by the O-ring. After centrifugation for 10 min at 3600 *g*, in which the erythrocytes were forced in

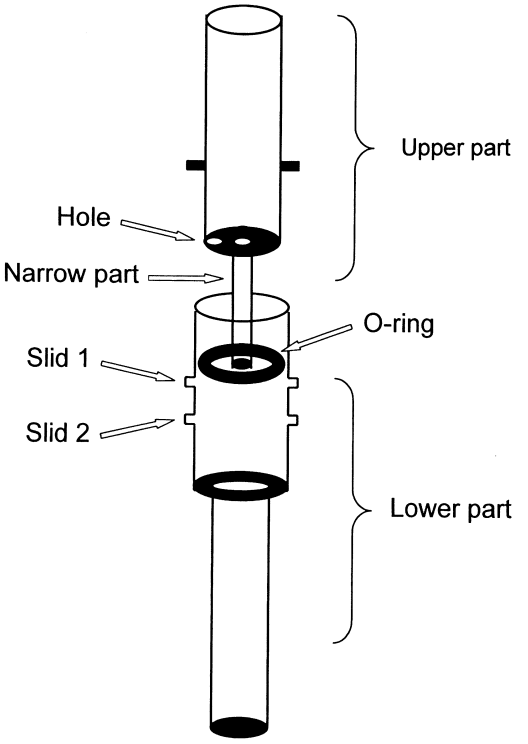

Fig. 1. Schematic plot of the MESED instrument.

the narrow part of the MESED instrument, a volume of 250 μl of the plasma-supernatant was transferred to a 2-ml polypropylene tube containing 750 μl of ice-cold methanol. After vortex mixing for 10 s, the methanolic plasma-extracts were stored at −80°C for simultaneous analysis of the carboxylate and lactone forms of topotecan, as has been described earlier [8,9]. The upper-part of the instrument was subsequently placed in slid 1 of the lower part, followed by a second centrifugation step of 10 min at 3600 *g*, in which the excess amount of red blood cells, plasma and the buffy-coat cells were forced out of the upper-part into the lower part of the MESED instrument by the small hole in the upper part, leaving an aliquot of 102 μl of packed unwashed red blood cells in the narrow part of the instrument [13]. The red blood cells were harvested by a third centrifugation step of 5 min at 200 *g*, in which part A is placed up-side-down in a centrifuge tube. The samples were stored at −80°C until analysis.

2.5. Sample treatment whole blood and erythrocytes

The first step in the extraction of topotecan from human whole blood was based on the methodology described for the determination of the lactone and carboxylate form of topotecan in human heparinized plasma [8,9]. Aliquots of 100 μl of human heparinized whole blood were processed by addition of 300 μl methanol in 2-ml polypropylene Eppendorf tubes. The samples were vigorously mixed on a multi-tube vortex mixer for 5 min, followed by incubation at −80°C for 10 min. Subsequently, the samples were again vigorously mixed on a multi-tube vortex mixer for 5 min, followed by centrifugation for 5 min at 23 000 *g*. A volume of 200 μl of the clear supernatant was subsequently mixed with 200 μl aqueous 5% (w/v) perchloric acid. The samples were subsequently vortex-mixed for 10 s, followed by centrifugation for 10 min at 23 000 *g*. An aliquot of 300 μl was transferred into a low-volume insert of glass, from which 100 μl were injected onto the HPLC column. Samples with a concentration above the highest calibration standard, i.e., 50.0 ng/ml, were 10-fold diluted prior to injection in PBS–MeOH–5% perchloric acid (1:3:4, v/v/v). Isolated erythrocytes were treated as whole blood samples after the addition and mixing with 150 μl of blank human plasma, resulting in samples roughly equal to whole blood.

2.6. HPLC instrumentation

The HPLC system for the measurement of total topotecan concentrations in whole blood and erythrocytes consisted of a constaMetric 3200 solvent delivery systems (LDC Analytical, Riviera Beach, FL, USA), a Waters 717plus autosampling device (Milford, MA, USA) and a Jasco FP-920 fluorescence detector (Jasco Benelux, Maarssen, The Netherlands). Separations were achieved on a stainless steel analytical column (150×4.6 mm I.D.) packed with Inertsil ODS 80A (5 μm particle size), delivered by Alltech Applied Science (Breda, The Netherlands). The mobile phase was a mixture of 100 m*M* aqueous ammonium acetate with the pH adjusted to 6.0 with neat acetic acid–tetrahydrofuran (94.6:5.4, v/v) and was delivered at a flow-rate of 1.00 ml/min. The analytical column was maintained at 50°C using a Mistral column oven (Spark Holland, Meppel, The Netherlands). Integration of the topotecan peaks were performed by the Fisons ChromCard data analysis system (Milan, Italy). Calibration curves were generated and constructed by using $1/(\text{concentration})^2$ weighted least-squares linear-regression analysis.

2.7. Validation

Validation runs of topotecan in whole blood and erythrocytes included a set of calibration samples in heparinized whole blood assayed in duplicate, lower limit of quantitation (LLQ) and QC samples of whole blood and the QC sample in erythrocytes in quintuplicate, and was performed on four separate occasions. Between-run (BRP) and within-run precisions (WRP) were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable [14]. The accuracy of at least 80% of the samples assayed at each concentration should be in the range of 80 to 120%. The WRP and BRP should be <20% at the concentration of the LLQ and <15% at the concentrations of the QC samples and the average accuracy (ACC) should be within 85–115% for each concentration, including the LLQ. The specificity of the assay was tested by visual inspection of chromatograms of five different heparinized whole blood samples and isolated erythrocytes of volunteers for the presence of interference by endogenous compounds. The following potentially co-administered drugs were tested for chromatographic and extraction interference: alizapride, carboplatin, cisplatin, codeine, dexamethasone, domperidon, granisetron, lorazepam, metoclopramide, morphine, ondansetron, paracetamol, paroxetine, phenytoin, probenecid, ranitidine, rifampicin, triamterene and valproate. The extraction efficiency of the assay was measured by comparison of extracted whole blood and PBS samples at concentrations of 1.00 and 50.0 ng/ml. The stability of total topotecan in whole blood and erythrocytes was tested at the concentrations of the QC samples by three freeze–thaw cycles, by overnight incubation at ambient temperature and for 4 h at 37°C, while the stability in erythrocytes, also at the concentration of the QC sample, was investigated

in samples after three freeze–thaw cycles, followed by 4 h at 37°C. The stability of topotecan in the extracted samples in the autosampler was tested during 5 days, while the long term storage stability at −80°C of topotecan in whole blood and erythrocytes has been followed for 3 months up to now. Statistical computations were performed with the software package Number Cruncher Statistical Systems v5.0 (J.L. Hintze, East Kaysville, UT, USA, 1992).

2.8. Clinical applicability

The clinical applicability of the described analytical method was tested by measurement of whole blood, erythrocytes and plasma samples from a 60-year-old female patient with advanced ovarian cancer, receiving 1.2 mg (i.e., 0.75 mg/m^2) topotecan as a 30 min i.v. infusion. Whole blood samples were obtained in heparinized tubes at pre-dose and up to 12 h after the start of the infusion. All samples were directly cooled down on ice at the bedside of the patient and immediately processed as described above. The concentrations of topotecan lactone and carboxylate in the plasma sample were analyzed according to our previous described method [8], with minor modifications [9].

3. Results and discussion

The choice of the extraction procedure, the mobile phase and column is based on our experience during the development of this methodology using other extraction procedures in combination with several mobile phases, including methanol and acetonitrile as organic modifier, and different reversed-phase C_{18} columns. Since we only focused on the measurement of total (i.e., lactone plus carboxylate) topotecan concentrations in whole blood, we initially tried to use a mobile phase with a lower pH. However, a lower pH results in shorter retention times of topotecan, lacking baseline separations of the topotecan peak. Moreover, also the fluorescence signal of topotecan decreased with the pH of the mobile phase, which in turn deteriorated the LLQ. A mobile phase with a pH of 6.0 with methanol or acetonitrile as organic modifier, in combination with several analytical reversed-phase C_{18} HPLC columns did not result in a baseline separation of topotecan and endogenous compounds with an acceptable lower limit of quantitation. However, the change to tetrahydrofuran as organic solvent in the mobile phase, in combination with the two-step extraction procedure, resulted in a baseline separation of topotecan and endogenous compounds. The selectivity of the final method is shown in Fig. 2, by the sharp resolution of the peaks, while no interfering from endogenous compounds was detected in the tested drug-free human heparinized whole blood samples and erythrocytes. The retention time of topotecan was 8 min, with an overall chromatographic run time of 20 min.

Optimum results were obtained by using weighted [1/(concentration)^2] linear regression analysis, with regression correlation coefficients in nine validation runs in the range of 0.9961–0.9994. The LLQ for total topotecan in human whole blood was determined to be 0.20 ng/ml. Thus, after correction for the dilution factor, the LLQ in the isolated erythrocytes is 0.49 ng/ml. As shown in Table 1, 90% of the interpolated concentrations of the assayed LLQ samples were within the 80–120% range, with the WRP and BRP <10% and an average accuracy of 100.7%. The WRP and BRP of the tested QC samples were <5%, with an acceptable accuracy range of 100.0–113.0%. In Table 2, the validation characteristics of the erythrocyte QC samples are shown. Since we did not have a nominal value for these samples, we established the acceptable range of

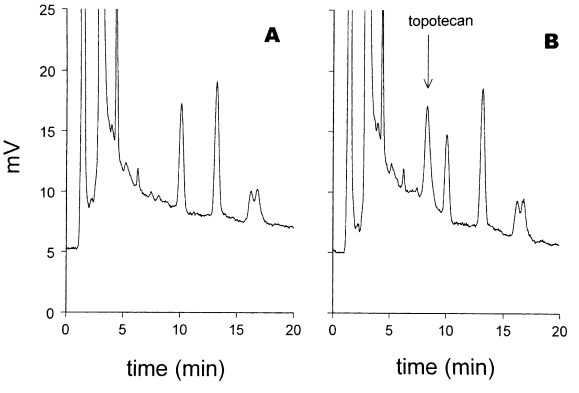

Fig. 2. HPLC chromatograms of a (A) blank human heparinized whole blood sample and (B) a sample spiked with topotecan at a concentration of 1.00 ng/ml.

Table 1
HPLC validation characteristics of QC samples in human whole blood

Nominal concentration (ng/ml)	*n*	Observed concentration (ng/ml)	ACC (%)	WRP (%)	BRP (%)
0.20[a]	18	0.201	100.7	9.3	1.5
0.80	19	0.904	113.0	2.9	3.5
20.0	20	20.42	102.1	1.7	3.3
40.0	20	40.01	100.0	2.9	3.6
200	20	209.8	104.9	2.0	4.4

Abbreviations: *n*, number of replicates of each concentration in four separate validation runs; ACC, average accuracy; WRP, within-run precision; BRP, between-run precision.
[a] Lower limit of quantitation.

accuracy from 80 to 120% on the basis of the average observed concentration of all the 20 analyzed samples, i.e., 4.47–6.71 ng/ml. This resulted in a WRP of 8.1% and a BRP of 3.9%, using 90% of the analyzed samples. Inclusion of the rejected data of the two replicates with a deviation of >20%, resulted in an observed concentration of 5.59 ng/ml, with still an acceptable WRP of 12.3%, without additional variation in the different runs. Calculation of the total blood concentration, using the mean measured hematocrit value of 0.48 l/l, the erythrocyte concentration of 5.58 ng/ml and the total topotecan plasma concentration of 12.97 ng/ml (Table 2), results in a hypothetical whole blood concentration of 9.42 ng/ml (i.e., 0.48×5.58 ng/ml+0.52×12.97 ng/ml). Since this value differs only 4.7% from the averaged observed concentration of 9.00 ng/ml in whole blood (Table 2), no accumulation of topotecan affecting the kinetics is expected in platelets or white blood cells, while the small contamination of plasma of approximately 2 to 4% [10,13] is negligible for the determination of total topotecan concentrations in erythrocytes.

The extraction recovery in human whole blood was found to be independent of the concentration ($P=0.15$, Student's *t*-test) with, respectively, values of 82.8±6.3 and 79.4±6.1% at the tested topotecan concentrations of 1.00 and 50.0 ng/ml.

Topotecan in human whole blood was found to be stable during three freeze–thaw cycles, during overnight incubation at ambient temperature and also no degradation of topotecan was observed after 4 h incubation at 37°C. In addition, topotecan was also found to be stable in isolated erythrocytes during three freeze–thaw cycles followed by a 4 h incubation at 37°C. Since also no degradation was observed of topotecan in the processed samples, when stored for 5 days in the autosampler, large numbers of samples can be analyzed in each analytical run. Topotecan has also found to be stable in whole blood

Table 2
HPLC validation characteristics of erythrocyte QC samples

Matrix	Topotecan (form)	*n*	Observed concentration (ng/ml)	WRP (%)	BRP (%)
Erythrocytes	Total	18	5.58	8.1	3.9
Blood	Total	20	9.00	3.8	6.1
Plasma	Carboxylate	20	10.10	3.3	0.81
Plasma	Lactone	19	2.90	4.1	3.0
Plasma	Total[a]	19	12.97	3.4	0.88

Abbreviations: *n*, number of replicates of each concentration in four separate validation runs; WRP, within-run precision; BRP, between-run precision.
[a] Lactone plus carboxylate concentration.

and erythrocytes during at least 3 months when stored at −80°C.

All tested potentially co-administered drugs, at concentrations ranging from 200 ng/ml up to 10 μg/ml, did not result in the presence of chromatographic peaks with the same retention time as topotecan, neither did they influence the extraction recovery of topotecan. Special attention was paid to triamterene, since this compound interfered with topotecan in the analytical method of Rosing et al. [15]. However, in the method described here, the retention time of triamterene was 9.6 min, while topotecan in this particular run had an retention time of 7.7 min.

In Fig. 3A, the pharmacokinetic profile of topotecan lactone and carboxylate in the plasma compartment of a cancer patient after the i.v. administration of 1.2 mg topotecan is shown, while Fig. 3B shows the profiles of total topotecan in plasma, whole blood and erythrocytes.

In conclusion, the presented method for the determination of total topotecan concentrations in human heparinized whole blood and in erythrocytes was shown to be specific, accurate and precise, and found to be sensitive enough to be implemented in clinical studies in which topotecan is administered i.v. at low doses. Combined with the simultaneous determination of topotecan lactone and carboxylate forms in the plasma compartment [8,9], this method is currently implemented in a clinical study to fully explore the role of erythrocytes in the plasma pharmacokinetics of topotecan.

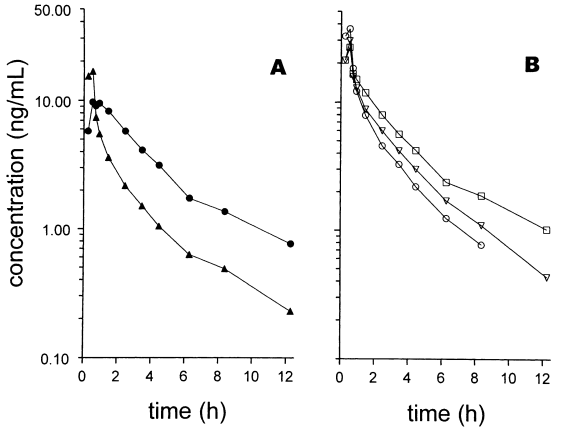

Fig. 3. Kinetic profiles of topotecan in a 60-year-old female patient receiving 1.2 mg topotecan as a 30 min i.v. infusion; (A) lactone (triangles) and carboxylate (circles) form of topotecan in the plasma compartment, (B) profile of total topotecan in the plasma compartment (squares), in whole blood (triangles) and in unwashed erythrocytes (circles).

References

[1] P.H. Hinderling, Pharmacol. Rev. 49 (1997) 279.
[2] D. Schrijvers, M.S. Higley, E.A. de Bruijn, A.T. van Oosterom, J.B. Vermoken, Anti-Cancer Drugs 10 (1999) 147.
[3] D.E. Cole, S.M. Blaney, F.M. Balis, in: Proceedings of the 86th Annual Meeting of the American Association for Cancer Research, Toronto, March 1995, 1995, p. 363, Abstract 2161.
[4] W.J. Loos, J. Verweij, A.J. Gelderblom, M.J.A. de Jonge, E. Brouwer, B.K. Dallaire, A. Sparreboom, Anti-Cancer Drugs 10 (1999) 705.
[5] W.J. Loos, H.J. Gelderblom, J. Verweij, E. Brouwer, M.J.A. de Jonge, A. Sparreboom, Anti-Cancer Drugs 11 (2000) 673.
[6] J.M. Gallo, P.B. Laub, E.K. Rowinsky, L.B. Grochow, S.D. Baker, J. Clin. Oncol. 18 (2000) 2549.
[7] S. Saxena, E.T. Wong, Arch. Pathol. Lab. Med. 114 (1990) 715.
[8] W.J. Loos, G. Stoter, J. Verweij, J.H.M. Schellens, J. Chromatogr. B 678 (1996) 309.
[9] M.J.A. de Jonge, W.J. Loos, H.J. Gelderblom, A.S.T. Planting, M.E.L. van der Burg, A. Sparreboom, E. Brouwer, V. van Beurden, M.A. Mantel, E. Doyle, S. Hearn, G. Ross, J. Verweij, J. Clin. Oncol. 18 (2000) 2104.
[10] O. Driessen, M.S. Highley, P.G. Harper, R.A.A. Maes, E.A. de Bruijn, Clin. Biochem. 27 (1994) 195.
[11] G. Momerency, K. van Cauwenberghe, M.S. Highley, P.G. Harper, A.T. van Oosterom, E.A. de Bruijn, J. Pharm. Sci. 85 (1996) 262.
[12] M.S. Higley, E.A. de Bruijn, Pharm. Res. 13 (1996) 186.
[13] Anonymous, MESED directions for use. October 2000.
[14] P. de Bruijn, M.J.A. de Jonge, J. Verweij, W.J. Loos, K. Nooter, G. Stoter, A. Sparreboom, Anal. Biochem. 269 (1999) 174.
[15] H. Rosing, D.M. van Zomeren, E. Doyle, W.W. ten Bokkel Huinink, J.H.M. Schellens, A. Bult, J.H. Beijnen, J. Chromatogr. B 727 (1999) 191.