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Determination by liquid chromatography with fluorescence detection of total 7-ethyl-10-hydroxy-camptothecin (SN-38) in beagle dog plasma after intravenous administration of liposome-based SN-38 (LE–SN38)

W. Guo, A. Ahmad, S. Khan, F. Dahhani, Y.F. Wang, I. Ahmad*

Pharmacokinetics, Metabolism and Bioanalytical Department, Research and Development, NeoPharm, 1850 Lakeside Drive, Waukegan, IL 60085, USA

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Abstract

An HPLC– fluorescence method to quantitate total 7-ethyl-10-hydroxy-camptothecin (SN-38) in beagle dog plasma spiked with liposome based formulation of SN-38 (LE–SN38) and using camptothecin (CPT) as the internal standard (I.S.) was developed and validated to support pharmacokinetics/toxicokinetics studies. Sample preparation was done by protein precipitation using acetonitrile with 0.5% acetic acid. The supernatant was evaporated, and reconstituted in acetonitrile–20 m*M* ammonium acetate, pH 3.5 (20:80, v/v). When injected onto a Zorbax SB-C_{18} HPLC column SN-38 as well as I.S. were detected by fluorescence using an excitation at 368 nm and emission at 515 nm. The SN-38 concentrations in samples were calculated from a standard curve of peak area ratios of SN-38 to the I.S. using weighted linear regression. The sensitivity limit for SN-38 was 1.00 ng/ml in beagle dog plasma with a precision (expressed as relative standard deviation) of 12.4% and an accuracy (expressed as analytical recovery) of 104%. The assay was linear within the standard curve range of 1–750 ng/ml. Acceptable precision and accuracy were also obtained for concentrations over the balance of the standard curve range from between-run and within-run calculations.

Keywords: SN-38; 7-Ethyl-10-hydroxy-camptothecin; Liposome-based SN-38

1. Introduction

Irinotecan, {7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin} known as CPT-11 is a topoisomerase I inhibitor used as an antineoplastic agent with a broad range antitumor activity [1]. SN-38 (7-ethyl-10-hydroxy camptothecin), the active metabolite of CPT-11, is approximately 100–1000-fold more cytotoxic than CPT-11. Despite its promising anti-cancer potential, SN-38 has not been developed as an anti-cancer drug due to its poor solubility in acceptable pharmaceutical solvents.

Liposomes are well recognized drug delivery vehicles that have been shown to enhance the therapeutic activity of a number of anticancer drugs

*Corresponding author. Tel.: +1-847-887-0800; fax: +1-847-887-9281.
E-mail address: imran@neophrm.com (I. Ahmad).

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[2–4]. Appropriately designed liposomes have the ability to passively accumulate in tumor tissues that exhibit poorly defined or leaky vasculature. This process, termed ''passive targeting'', can result in the accumulation of significantly greater amounts of cytotoxic drug in tumor than can be achieved by the administration of free drug [5,6]. We have currently developed a liposome-based SN-38 (LE–SN38) formulation, which shows promising results in terms of efficacy and toxicity in preclinical studies.

Several methods have been reported for measurement of SN-38 generated from CPT-11 in biological fluid using HPLC separation and fluorescence detection to support preclinical and clinical studies of CPT-11. Reported literature approaches for sample preparation include liquid–liquid extraction [7–9], solid-phase extraction [10,11] and protein precipitation [12,13]. Most of them involve long and tedious procedures. In this study, we describe the development and validation of a novel bioanalytical method using simple one step protein precipitation and HPLC–fluorescence for the quantitative determination of SN-38 in beagle dog plasma in the presence of LE–SN38. Validation parameters such as between-run and within-run precision and accuracy of the assay, the effect of freeze–thaw cycles, and dilution were evaluated. Assay specificity, short term room temperature stability, long term storage stability, matrix effect, and extraction recovery were also studied.

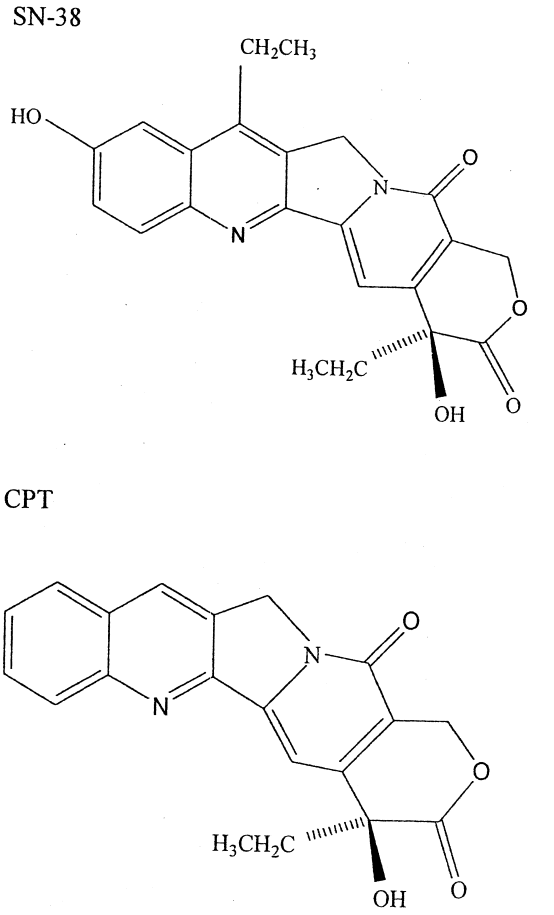

Fig. 1. Chemical structures of SN-38 and the internal standard, camptothecin (CPT).

2. Experimental

2.1. Chemicals and reagents

Camptothecin (CPT) (Fig. 1) was purchased from Sigma (St. Louis, MO, USA). SN-38 (Fig. 1) was obtained from Qventas (Newark, DE, USA). LE–SN38 was provided by Pharmaceutics Department at NeoPharm. HPLC grade solvents and all other analytical grade chemicals were obtained from commercial sources. Dog plasma was obtained from Bioreclaimation (Hicksville, NY, USA).

2.2. Equipment and chromatographic conditions

The HPLC 1100 apparatus (Agilent Technologies, Palo Alto, CA, USA) consisted of a binary pump, a degasser, an autosampler, a column oven and a fluorescence detector. Chromatographic separation was achieved by using a Zorbax SB-C_{18} (5 μm, 150×4.6 mm) analytical column (Agilent Technologies) maintained at approximately 35 °C and protected by a C_{18} guard cartridge (Agilent Technologies). Mobile phase A was 20 m*M* ammonium acetate, pH 3.5 and B was acetonitrile. The mobile phase at 1 ml/min was pumped in the isocratic mode with 24% B (v/v) for 2.7 min and then an elution gradient was applied. The gradient was achieved by increasing the proportion of B from 24 to 29% in 2.3 min, from 29 to 34% in 2 min, from 34 to 90% in 1 min and finally decreasing to 24% in 1 min. The fluorescence detector excitation wavelength was set at 368 nm and the emission wavelength at 515 nm.

Data collection and processing were performed using CHEMSTATION software (Agilent Technologies).

2.3. Standard and quality control solutions

CPT and SN-38 were dissolved in methanol–dimethylsulfoxide (50:50, v/v) at concentrations of 0.5 and 1 mg/ml, respectively. SN-38 was then further diluted with acetonitrile–20 m*M* ammonium acetate, pH 3.5 (20:80, v/v) to make a set of standards ranging from 1.00 to 750 ng/ml. These standards were used to determine the concentration of total SN-38 in LE–SN38 stock solution. Concentrations of two independent LE–SN38 stock solutions were determined and one LE–SN38 was spiked into dog plasma to make a 10 000 ng/ml dog plasma standard sample and the other was spiked into dog plasma to make a 60 000 ng/ml dog plasma quality control sample. Serial dilution was then employed to make a set of standards with concentrations ranging from 1.00 to 750 ng/ml and quality control samples at 1.00, 3.00, 6.00, 300, 600, 12 000 and 60 000 ng/ml. A CPT (I.S.) working solution of 25 ng/ml was prepared freshly for each run by dilution of the stock with acetonitrile containing 0.5% acetic acid.

2.4. Sample preparation

To 200 μl of thawed plasma with or without LE–SN38 were added 400 μl of I.S. (25 ng CPT per ml of acetonitrile) in a polypropylene microtube. The mixture was vortexed and centrifuged at 13 000 rpm for 10 min. The supernatant was transferred to a clean microtube and evaporated to dryness under vacuum at 50 °C. The dry extracts were redissolved in 100 μl of acetonitrile–20 m*M* ammonium acetate, pH 3.5 (20:80, v/v), of which 45 μl was injected onto the column of the HPLC system.

2.5. Assay validation

The analytical methodology has been validated in terms of specificity, extraction recovery, matrix effect, linearity, limit of quantitation, stability, between-run and within-run precision and accuracy for SN-38 in dog plasma spiked with LE–SN38. A total of six runs were conducted including five between-runs and one within-run, which served as a combination of between-run and within-run. Each validation run included nine-point calibration curve and quality control samples in duplicates, except within run, where quality control samples were analyzed in six replicates.

2.5.1. Between-run and within-run precision and accuracy

Between-run accuracy and precision were assessed by analyzing quality control samples at four concentrations (3, 6, 300, 600 ng/ml) of SN-38 in duplicate in six different runs. Within-run accuracy and precision were determined by assaying six replicates of four concentrations in a single run. Assay precision was calculated as the relative standard deviation (RSD) (coefficient of variation) expressed as a percentage of the mean observed concentrations. Accuracy was determined as the agreement between the interpolated concentration and the nominal concentration and was reported as a percent analytical recovery. Between-run and within-run experiments should have precision of $\leq$15% and accuracy of 100$\pm$15% except for the limit of quantitation (LOQ) where $\leq$20% is acceptable for both parameters.

2.5.2. Limit of quantitation (LOQ, sensitivity)

LOQ is defined as the lowest concentration of the standards that can be measured with acceptable accuracy and precision ($\leq$20% for both parameters). Six replicates of LOQ were prepared independently of standards used in the standard curve.

2.5.3. Specificity

Potential interference from endogenous compounds was investigated in dog plasma. Six different lots of dog plasma (three males and three females) with 1 ng/ml SN-38 were analyzed to determine the precision and accuracy. The responses were also compared with the corresponding lots without SN-38.

2.5.4. Dilution effect

Dilution effect was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. LE–SN38 spiked dog plasma samples prepared at three concentrations (300, 12 000, 60 000 ng/ml) of SN-38 were diluted with pooled dog plasma at dilution factors of 2, 20,

100 respectively in six replicates and analyzed. The six replicates should have precision of ≤15% and accuracy of 100±15%.

2.5.5. Matrix effect

The matrix effect was studied to evaluate the suppression or enhancement of the analyte and internal standard response by the matrix. The matrix effect was evaluated at three concentrations (6, 300, 600 ng/ml) of SN-38 in dog plasma spiked with LE–SN38 and at one concentration (100 ng/ml) of CPT in triplicates. The peak areas of the unextracted sample (prepared by spiking an equivalent amount of analyte into an extract of blank plasma) and the peak areas of the neat solutions at the same concentration were determined. The matrix effect was calculated by the following equation:

$$\%\ \text{matrix effect} = \frac{\text{mean peak area of unextracted sample} - \text{mean peak area of neat solution}}{\text{mean peak area of neat solution}} \cdot 100\%$$

2.5.6. Extraction recovery

The efficiency of the extraction process was determined at three concentrations (6, 300, 600 ng/ml) of SN-38 in triplicates and at one concentration (100 ng/ml) of CPT in triplicates. The recovery was determined from the percentage ratio of the mean peak area of extracted samples to the mean peak area of unextracted samples at the respective concentrations.

2.5.7. Stability

The freeze–thaw stability of SN-38 was investigated by using quality control samples at 6, 300 and 600 ng/ml. These samples were stored at −70±10 °C for >12 h and then thawed at room temperature. This procedure was repeated three times. Samples, which did not undergo freeze–thaw cycles, served as reference. Each concentration was analyzed in six replicates. The relative recovery between three freeze–thaw cycles and reference samples was compared.

Short term room temperature and long term storage stability at −70±10 °C were also studied.

3. Results and discussion

3.1. Assay development

HPLC conditions were optimized with pure standards of CPT and SN-38. Different gradients were tested to obtain better separation and peak shape. The retention times for SN-38 and CPT were approximately 6.8 and 7.9 min, respectively, and the overall run time was 11 min. Assays of drug-free dog plasma showed no interference from endogenous peaks at or near the retention times of SN-38 and CPT (Fig. 2a). Fig. 2b shows a representative chromatogram at the upper limit of quantitation (750 ng/ml) of SN-38 in dog plasma, indicating a good and acceptable separation between SN-38 and CPT.

3.2. Validation

The standard curve was established by plotting the ratio of the peak area of SN-38 to that of CPT. A linear regression with $1/x$ weighting (i.e. the relative weight of each standard in the least squares fit regression as the inverse of that standard's concentration) resulted in minimal deviation from nominal concentrations, with linear-regression correlation coefficients of ≥0.999 in all validation runs, and was used for all the calculations. Standard curves generated acceptable data over the concentration range of 1.00–750 ng/ml SN-38 in beagle dog plasma spiked with LE–SN38 (Table 1).

3.2.1. Between-run and within-run

A summary of the between-run and within-run precision and accuracy data generated for the assay validation is presented in Table 2. The method was found to be precise (RSD<7.09% for between-run and <3.22% for within-run) and accurate (analytical recovery 96.6–104% for between-run and 96.5–103% for within-run).

3.2.2. Limit of quantitation (LOQ, sensitivity)

The LOQ, determined as 1 ng/ml met the acceptance criteria with a precision of 12.4% and accuracy of 104%. Fig. 2c illustrates a representative chromatogram at the LOQ of 1 ng/ml for SN-38 in dog plasma spiked with LE–SN38.

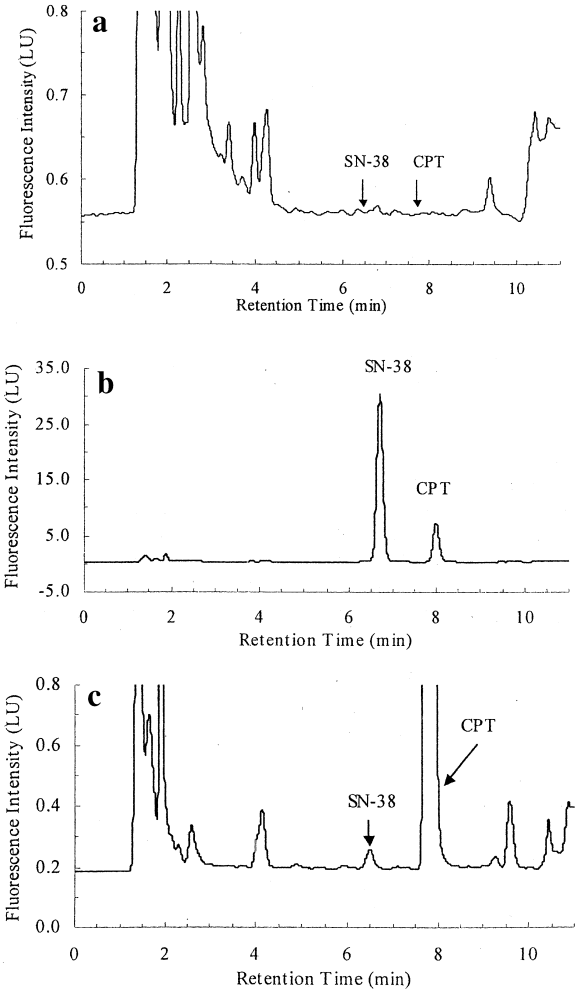

Fig. 2. Representative HPLC chromatograms of the extracts from (a) blank dog plasma sample. (b) SN-38 at upper limit of quantitation (750 ng/ml) level in dog plasma sample spiked with LE–SN38. (c) SN-38 at LOQ (1.00 ng/ml) level in dog plasma sample spiked with LE–SN38.

3.2.3. Specificity

No significant peak was observed at the retention of SN-38 and CPT in any lot of plasma used in the study, or in plasma samples obtained from beagle dogs who have not received LE–SN38. Fig. 2a showed a typical chromatogram obtained from the dog prior to the administration of LE–SN38. Quality control samples at 1.00 ng/ml, which were prepared using six different lots of beagle dog plasma, showed acceptable levels of precision and accuracy (Table 3). However, potential interference from endogenous compounds in plasma of other laboratory animals or humans should be evaluated before the use of the method to quantify SN-38.

Table 1
Validation standards of SN-38 in beagle dog plasma spiked with LE–SN38

Nominal concentration (ng/ml)	n[a]	Observed concentration (ng/ml)	RSD (%)	Accuracy (%)
1.00	9	1.03	10.4	103
2.00	12	2.03	8.95	102
5.00	12	4.86	6.72	97.2
10.0	12	10.6	4.70	106
50.0	12	50.6	4.30	101
100	12	96.3	3.47	96.3
200	12	198	2.74	99.2
500	12	508	2.46	102
750	12	746	2.40	99.4

[a] Number of replicate observations in six separate validation runs.

3.2.4. Dilution effect

Standard curve can be extended up to 60 000 ng/ml by dilution without affecting the final concentrations. The results in Table 4 show that the precision and accuracy for six replicates of diluted samples were within the acceptance range.

3.2.5. Matrix effect

The matrix effect on responses was determined at three concentration levels of SN-38 and one concentration level of CPT. The effect was evaluated by comparing the peak areas of unextracted samples with the peak areas of neat solutions at the same concentration. The neat solutions served as reference samples. The relative recovery ranged from 95.5 to 112% for SN-38 and 90.0% for CPT as displayed in Table 5.

3.2.6. Extraction recovery

Extraction recovery was determined from the ratio of the mean peak area of extracted samples to the mean peak area of the unextracted samples. The relative recovery was 78±4.5% for SN-38 and 86±7.2% for CPT. Extraction recoveries for SN-38 and I.S. were consistent, precise and reproducible

Table 2
Validation between- and within-run results of SN-38 in beagle dog plasma spiked with LE–SN38

	Nominal concentration (ng/ml)	n[a]	Observed concentration (ng/ml)	RSD (%)	Accuracy (%)
Between-run	3.00	11	2.90	7.09	96.6
	6.00	12	6.25	5.15	104
	300	12	297	2.09	99.1
	600	12	593	2.61	98.9
Within-run	3.00	6	2.97	3.22	98.9
	6.00	6	6.20	2.84	103
	300	6	290	0.936	96.5
	600	6	589	1.24	98.1

[a] Number of replicate observations.

Table 3
Specificity of SN-38 (1 ng/ml) in beagle dog plasma spiked with LE–SN38

Dog plasma lot no.	Observed concentration (ng/ml)
F1	1.08
	1.15
M1	0.955
	0.847
F2	0.848
	0.856
M2	0.832
	0.961
F3	0.840
	0.798
M3	1.04
	0.814
Mean	0.918
RSD (%)	12.8
Analytical recovery (%)	91.8

Table 5
Matrix effects on the responses of SN-38 (spiked as LE–SN38) and CPT in dog plasma

	Concentration (ng/ml)	n[a]	Matrix effect (%)
SN-38	6	3	12
	300	3	−4.5
	600	3	4.0
CPT	100	3	−8.7

[a] Number of replicate observations.

thoughout the validation experiments and were within the acceptance criteria [14].

3.2.7. Stability

SN-38 was shown to be stable in LE–SN38 spiked dog plasma through three freeze–thaw cycles. It was also found that SN-38 was stable in dog plasma for at least 4 h at room temperature, and at least 57 days

Table 4
Dilution effect of SN-38 in dog plasma spiked with LE–SN38

Nominal concentration (ng/ml)	Dilution factor	n[a]	Observed concentration (ng/ml)	RSD (%)	Accuracy (%AR)
300	2	6	310	14.0	103
12 000	20	6	12 462	1.60	104
60 000	100	6	67 008	6.29	112

[a] Number of replicate observations.

Table 6
Stabilities of SN-38 in dog plasma spiked with LE–SN38

	Concentration (ng/ml)	n^a	Relative recovery (%)
Three freeze–thaw cycles	6	6	101
	300	6	86.2
	600	6	104
4 h room temperature storage	6	6	101
	300	6	98.8
	600	6	98.3
57 days at −70±10 °C	6	6	92.1
	300	6	96.9
	600	6	99.4

[a] Number of replicate observations.

at −70±10 °C. The relative recoveries are detailed in Table 6.

3.3. Application

The method described here was successfully employed to quantify total SN-38 in plasma samples from preclinical pharmacokinetics and toxicokinetics studies of LE–SN38 in beagle dogs. Fig. 3 shows a typical chromatogram for a dog plasma sample obtained 1 h after intravenous administration of 1.2 mg/kg dose of SN-38 given as LE–SN38. A plasma concentration–time profile of SN-38 following LE–SN38 administration at this dosage is presented in Fig. 4.

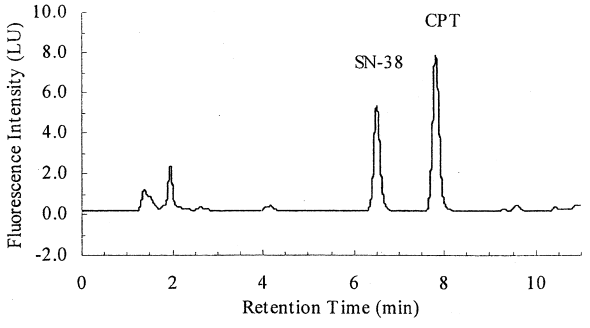

Fig. 3. HPLC chromatogram of a dog plasma sample collected 1 h after a single intravenous dose of liposome based SN-38 (LE–SN38) at a dose level of 1.2 mg/kg.

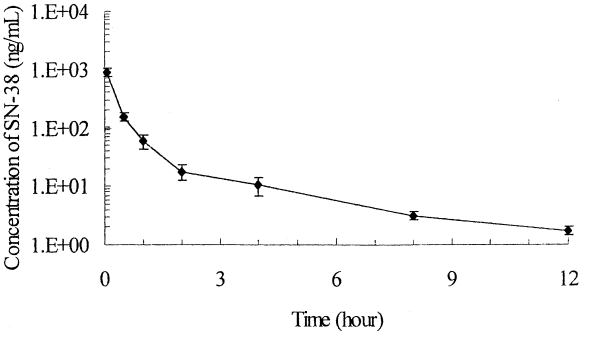

Fig. 4. Mean±SEM plasma concentration–time profile of SN-38 in dogs following a single intravenous dose of LE–SN38 at 1.2 mg/kg.

4. Conclusion

The method described here for SN-38 determination in dog plasma in the presence of LE–SN38 involves one-step sample preparation and therefore shortens the analysis time. It is a simple, sensitive, precise, accurate and specific HPLC–fluorescence method to quantitate SN-38 in dog plasma in the presence of LE–SN38. The method was successfully used to support preclinical pharmacokinetics and toxicokinetics studies of LE–SN38 in beagle dogs.

References

[1] G.J. Creemers, B. Lund, J. Verweij, Cancer Treat Rev. 20 (1994) 73.
[2] A. Gabizon, J. Liposome Res. 4 (1994) 445.
[3] L.D. Mayer, G. St. Onge, Anal. Biochem. 232 (1995) 149.
[4] R. Perez-Soler, I. Han, S. Al-Baker, A.R. Khokhar, Cancer Chemother. Pharmacol. 33 (1994) 378.
[5] S.K. Huang, K.D. Lee, K. Hong, D.S. Friend, D. Papahadjopolous, Cancer Res. 52 (1992) 5135.
[6] M.B. Bally, D. Masin, R. Nayar, P.R. Cullis, L.D. Mayer, Cancer Chemother. Pharmacol. 34 (1994) 137.
[7] S. Ragot, P. Marquet, F. Lachâtre, A. Rousseau, E. Lacassie, J.M. Gaulier, J.L. Dupuy, G. Lachâtre, J. Chromatogr. B 736 (1999) 175.
[8] P. de Bruijin, J. Verweij, W.J. Loos, K. Nooter, G. Stoter, A. Sparreboom, J. Chromatogr. B 698 (1997) 277.
[9] P. de Bruijin, M.J.A. de Jonge, J. Verweij, W.J. Loos, K. Nooter, G. Stoter, A. Sparreboom, Anal. Biochem. 269 (1999) 174.

[10] I. Barilero, D. Gandia, J.P. Armand, A. Mathieu-Boué, M. Ré, A. Gouyette, G.G. Chabot, J. Chromatogr. 575 (1992) 275.
[11] L.P. Rivory, M. Findlay, S. Clarke, J. Bishop, J. Chromatogr. B 714 (1998) 355.
[12] D.F. Chollet, L. Goumaz, A. Renard, G. Montay, L. Vernillet, V. Arnera, D.J. Mazzo, J. Chromatogr. B 718 (1998) 163.
[13] J. Eescoriaza, A. Aldaz, C. Castellanos, E. Calvo, J. Girâldez, J. Chromatogr. B 740 (2000) 159.
[14] US Department of Health and Human Services, in: Guidance for Industry, Bioanalytical Method Validation, Food and Drug Administration, Rockville, MD, 2001, p. 5.