ELSEVIER

Journal of Chromatography B, 668 (1995) 107–115

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic determination of the novel antitumour drug topotecan and topotecan as the total of the lactone plus carboxylate forms, in human plasma

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

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

First received 5 August 1994; revised manuscript received 17 January 1995; accepted 30 January 1995

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 the total of its lactone and carboxylate forms in human plasma. Linear response in analyte standard peak area were observed over the concentration range 0.05–10 ng/ml using 100-μl plasma samples. The instability of the drug in the biological matrix necessitated that the plasma fraction was obtained within 5 min after blood sampling by centrifugation, immediately followed by protein precipitation with cold methanol (−30°C). Stability studies have indicated that topotecan is stable in these methanolic extracts for at least 4.5 months at −30°C and 2 months at −70°C. For the total determination of the lactone plus lactone ring-opened forms of the drug as topotecan, plasma samples were deproteinated with methanol and, subsequently, acidified with 7% (v/v) perchloric acid. Plasma samples for the measurement of total levels of the lactone and the ring-opened forms of topotecan were stable for at least 4.5 months when stored at −30°C. After centrifugation, the supernatants were analysed by HPLC using a Zorbax SB-C_{18} Stable Bond column and methanol–0.1 *M* hexane-1-sulfonic acid in methanol–0.01 *M* N,N,N′,N′-tetramethylethylenediamine (TEMED) in distilled water pH 6.0 (25:10:65, v/v) as the mobile phase. Detection was performed fluorimetrically. Within-run and between-run precision was always less than 12.1% in the concentration range of interest (0.05–10.0 ng/ml). The limit of quantitation is 0.05 ng/ml. Accuracy measurements ranged between 87.6 and 113.5%.

1. Introduction

Topotecan (SK&F 104864, (*S*)-9-dimethylaminomethyl-10-hydroxy-camptothecin, NSC609669; Fig. 1) [1], is a semisynthetic analog of the plant alkaloid camptothecin which inhibits specifically the intranuclear enzyme topoisomerase I [2,3]. Topotecan has shown 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-depen-

---

[*] Corresponding author.

SSDI 0378-4347(95)00054-2

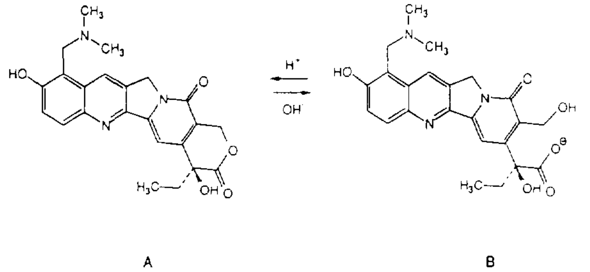

Fig. 1. Equilibrium reaction between topotecan (A) and its lactone ring-opened form (SK&F 105992) (B) [16].

dent, 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 important 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]. In 1990 we have reported an HPLC technique for the determination of topotecan and its lactone ring-opened form in plasma [14]. This assay has successfully been used by other investigators [5,7–9] and by ourselves [6] in clinical phase I studies. The method involves a protein precipitation step with cold methanol and subsequent injection of the methanol extract onto the HPLC system. The lower limit of quantitation the assay was 1.0 ng/ml. We have now improved the method and also adjusted it for the measurement of topotecan as the total of the lactone and carboxylate forms in human plasma since several pharmacokinetic studies have shown that these total levels are predictive for pharmacodynamic outcome [18,19]. Assay sensitivity has been improved by over 20-fold with a lower limit of quantitation of topotecan of 0.05 ng/ml using only 100 μl of human plasma. This improvement in sensitivity was necessary as the method is now implemented in a pharmacokinetic study with patients with extensive small-cell lung cancer receiving relatively low doses of topotecan as a continuous 21-day infusion every 28 days; the minimum infusion dose is 0.3 mg/m^2/day.

2. Experimental

2.1. Chemicals

Topotecan, as the hydrochloride salt (SK&F 104864-A) (lot. MM-15906-194) originated from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). 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 AS100 (Thermo Separations Products, Fremont, CA, USA) which was cooled at 1°C for the determination of topotecan in non-acidified methanol extracts. A fixed sample loop of 50 μl was used. Chromatographic separation was performed on a Zorbax SB-C_{18} column (75 × 4.6 mm I.D.; particle size: 3.5 μm,) (Rockland Technologies, Newport, DE, USA). To protect the analytical column, a guard column (10 × 3 mm I.D.) packed with reversed-phase material (C_{18}) was used (Chrompack, Middelburg, 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). The flow-rate was 1.0 ml/min and the detection was performed fluorimetrically using a FP920 Intelligent Fluorescence Detector (Jasco International Co., Tokyo, Japan) with an excitation wavelength of 361 nm and an emission wavelength of 527 nm with an 40 nm bandwidth; the digital filter was set at 10 s. The capacity of the flow-cell of the fluorescence detector was 16 μl.

2.3. Sample pretreatment

Topotecan

For the determination of topotecan a 100-μl plasma sample is mixed with 200 μl cold methanol (−30°C). After vortex-mixing for 10 s and centrifugation at 9500 *g* for 3 min, the clear supernatant is transferred to a clean tube and stored, immediately, at −70°C. Prior to injection onto the HPLC system, the sample is diluted 1:1 (v/v) with distilled water.

Topotecan total levels

For the determination of topotecan as total of the lactone and carboxylate forms, a 100-μl sample is deproteinized with 100 μl methanol and 100 μl 7% (v/v) perchloric acid. After vortex-mixing for 10 s and centrifugation at 9500 *g* for 3 min, the clear supernatant is injected onto the HPLC system.

2.4. Calibration

Topotecan

Blank human plasma samples (100 μl), in polypropylene Eppendorf tubes, are spiked with 25–100 μl of an appropriate amount of topotecan in methanol. Cold methanol (−30°C) is 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 is transferred to a clean tube and stored, immediately, at −70°C. Prior to injection onto the HPLC system, the sample is diluted 1:1 (v/v) with distilled water.

Topotecan total levels

Blank human plasma samples (100 μl), in polypropylene Eppendorf tubes, are spiked with 25–100 μl of an appropriate amount of topotecan in methanol. Cold methanol (−30°C) is then added to achieve a total volume of 100 μl, followed by the addition of 100 μl 7% (v/v) perchloric acid. After vortex-mixing for 10 s and centrifugation at 9500 *g* for 3 min, the clear supernatant is injected onto the HPLC system.

2.5. Validation

A three-run validation was completed for the determination in human plasma of both topotecan and topotecan as the total of lactone and carboxylate forms. The following parameters were determined: linearity, within-run and between-run precision, accuracy, separation of endogenous plasma constituents and absolute recovery.

Seven topotecan plasma calibration standards were prepared and analysed in singular, producing a curve ranging from 0.05 to 10 ng/ml. 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. The correlation coefficient was used to evaluate the linearity of the calibration curve.

Five quality control samples containing 0.05, 0.1, 0.25, 5.0 and 10.0 ng/ml were prepared in human plasma, for each run, for the determination of the accuracy and precision. Six replicates of each quality control sample were processed and analysed in three different runs with plasma calibration standards to determine the lactone concentration and the total of the lactone and carboxylate forms.

Six batches control human plasma were tested whether endogenous plasma constituents co-eluted with topotecan.

The extraction efficiencies 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 in triplicate at two concentrations (0.25 ng/ml and 10.0 ng/ml) in three different runs.

2.6. Stability

The stability of topotecan and topotecan as total of lactone and carboxylate forms, in different ratios, has been studied at a concentration of 10 ng/ml in human plasma during a period of 4.5 months at −30°C and at a concentration of 5 ng/ml during 2 months at −70°C. Five replicates were analysed at every time point.

The stability of the drug in the methanol extract originating from plasma spiked with topotecan at a concentration of 10 ng/ml was evaluated after 4.5 months at −30°C and at a concentration of 5 ng/ml after 2 months at −70°C.

The stability of topotecan during storage in the autosampler at 1°C, prior to analysis, has been studied at a concentration of 1 ng/ml. The stability of methanol extracts from acidified samples for the measurement of topotecan as total of the lactone and carboxylate forms (concentration: 1 ng/ml) were evaluated during storage at room temperature during 24 h in the autosampler.

3. Results and discussion

3.1. Chromatography and detection

Topotecan is a basic compound with a highly polar character in its protonated form. To get acceptable chromatographic results we found that reversed-phase HPLC with a C_{18} column was most suitable. An eluent pH of 6.0 was chosen in view of the relatively low rate of the forward and backward conversion of the lactone form and the carboxylate form at this pH (Fig. 1) [16]. TEMED was used to buffer the eluent at pH 6.0 and also acted as tailing reducer. At pH 6.0 the basic side chain of topotecan is protonated (pK_a 10.50) [15] and will give ion pairs with hexane sulfonic acid in the eluent resulting in an increased retention and better peak shape. In the previously published method [14] we used a LiChrosorb RP-18 column with a methanol–water eluent to which sodium dioctylsulfosuccinate (DOSS) was added as ion-pairing agent. A major drawback of this system was the relatively rapid decrease of the number of theoretical plates of the column; this problem was not encountered any more with the presented assay using a Zorbax SB-18 Stable Bond column in combination with hexane sulfonic acid as ion-pairing agent. Tetrabutylammonium phosphate has been used as ion-pairing agent in the HPLC analysis of camptothecin [20] and the camptothecin derivative irinotecan [21].

The 35% (v/v) methanol concentration in the mobile phase was sufficient to get resolution of topotecan from endogenous substances in human plasma (Fig. 2). In a previously published method a LS 40 fluorescence detector (Perkin-Elmer, Beaconsfield, UK) equipped with a 7-μl flow-cell was used [14]. By using the FP920 detector with a 16-μl flow-cell in combination with an improved HPLC system, a 20-fold increase of sensitivity could be reached.

3.2. Sample pretreatment

A protein precipitation step with cold methanol or a mixture of methanol and acetonitrile is an adequate sample pretreatment procedure for the bio-analysis of camptothecins, fixing the lactone equilibrium reactions [14,20,21]. About 98.7% of the plasma proteins were precipitated when 200 μl methanol was added to 100 μl of plasma [22]. With the present assay there was no increase in the pressure drop over the analytical column and no decrease in column efficiency was observed after more than 1000 injections. In the earlier method [14] this was a substantial problem which necessitated changing the guard column every week.

It is our experience that the simple precipitation method can be easily learned by a research nurse and performed on the ward of the hospital.

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 [23].

The assay was linear over the concentration range 0.05–10 ng/ml in human plasma and correlation coefficients (r) of 0.995 or better were obtained. For every calibration curve the calibration concentrations were back-calculated from the peak areas. The results are presented in Tables 1 and 2.

The deviations of the nominal concentrations for all concentrations were less than 10%.

The assay performance data are presented in Tables 3 and 4. The average within-run precision and the between-run precision were less than 15% for all quality control samples. The average

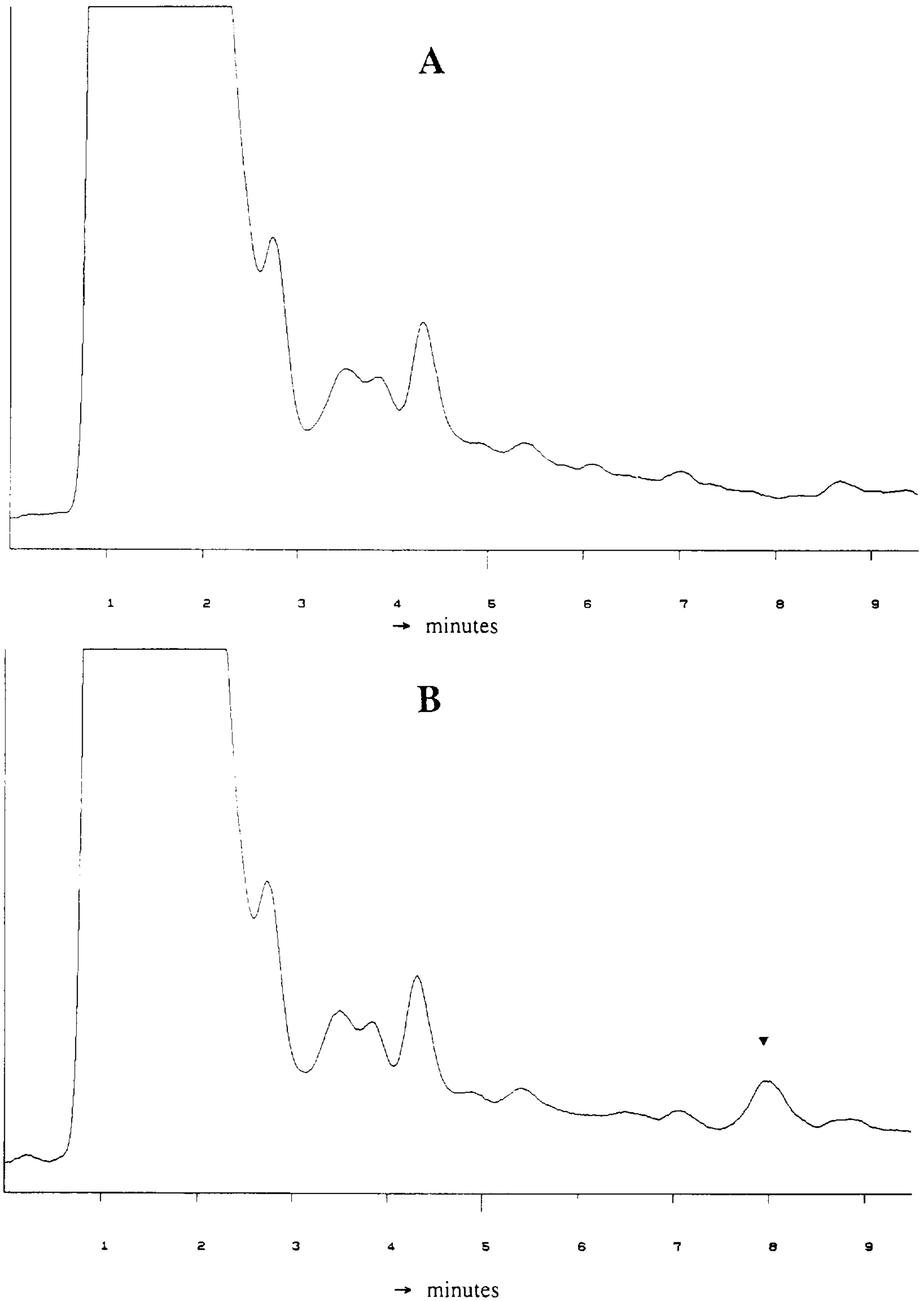

Fig. 2.

(Continued on p. 112)

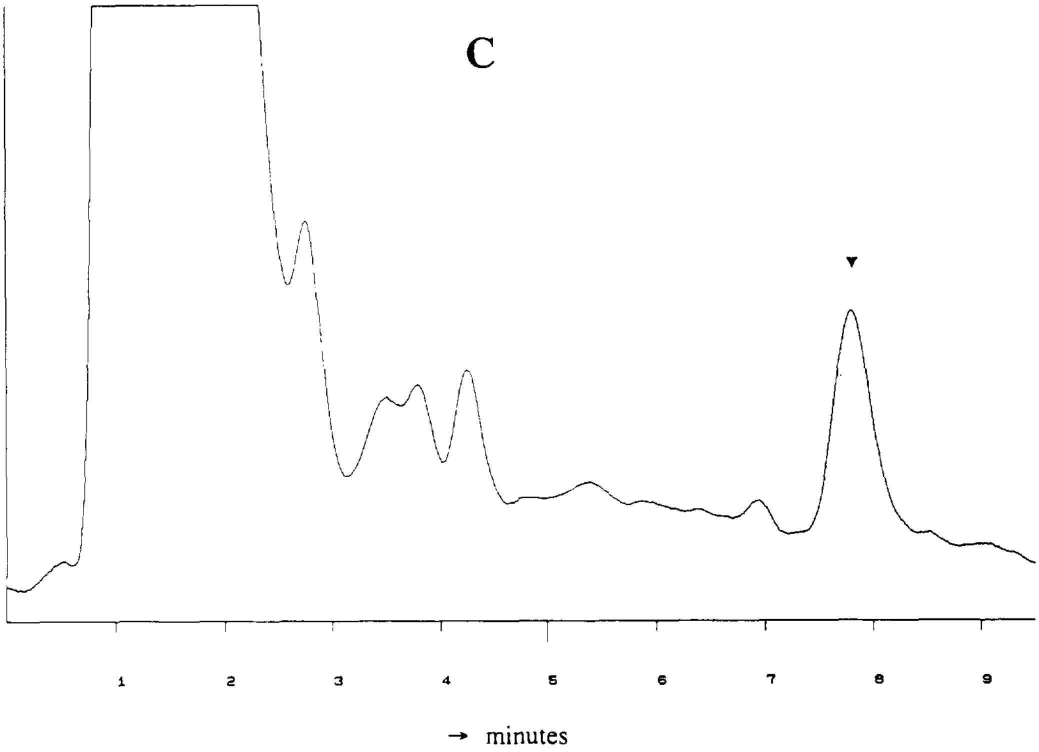

Fig. 2. HPLC chromatograms for the analysis of topotecan: human control plasma (A) and two spiked plasma calibration samples (0.05 and 0.25 ng/ml: B and C, respectively). The topotecan peak is indicated by ▼.

accuracies were within 85 and 115%. The chromatograms of six batches of control human plasma contained no endogenous plasma constituents co-eluting with topotecan.

The topotecan extraction efficiencies were 71.9 ± 5.0% at 0.25 ng/ml and 86.1 ± 3.4% at 10.0 ng/ml ($n = 9$). The recovery was slightly lower at the low end of the assay range. However, linearity was good and the performance of the assay was satisfactory across the range.

The extraction efficiencies of topotecan as total of lactone and carboxylate were 90.8 ±

Table 1
Calibration concentrations back-calculated from peak areas for the determination of topotecan

Run number	Concentration (ng/ml)						
	0.050	0.100	0.250	0.500	1.000	5.00	10.00
1	0.052	0.095	0.227	0.483	1.077	5.08	10.38
2	0.049	0.108	0.222	0.520	0.968	4.72	11.01
3	0.053	0.090	0.229	0.493	1.036	5.26	10.48
Mean	0.051	0.098	0.226	0.499	1.027	5.02	10.62
C.V.(%)	4.1	9.5	1.6	3.8	5.4	5.5	3.2
Dev.(%)	2.0	−2.0	−9.6	−0.2	2.7	0.4	6.2

C.V.: coefficient of variation; Dev.: deviation of the nominal concentration.

Table 2
Calibration concentrations back-calculated from peak areas for the determination of topotecan as total of lactone and carboxylate

Run number	Concentration (ng/ml)						
	0.050	0.100	0.250	0.500	1.000	5.00	10.00
1	0.053	0.088	0.241	0.506	1.009	5.19	10.33
2	0.050	0.097	0.251	0.504	1.037	4.97	9.75
3	0.051	0.096	0.258	0.507	0.977	4.94	10.11
Mean	0.051	0.094	0.250	0.506	1.008	5.03	10.06
C.V.(%)	3.0	5.2	3.4	0.3	3.0	2.7	2.9
Dev.(%)	2.0	−6.0	0.0	1.2	0.8	0.6	0.6

C.V.: coefficient of variation; Dev.: deviation of the nominal concentration.

4.5% at 0.25 ng/ml and 92.8 ± 2.4% at 10.0 ng/ml ($n = 9$).

3.4. Stability

Stability data of the analytes in plasma at −30°C are presented in Table 5. After 4.5 months of storage the topotecan concentration was about 40% of the original value.

There was no significant decrease of topotecan as total of lactone and carboxylate forms in plasma under these conditions.

After 2 months at −70°C, the topotecan concentration in plasma was about 71.9 ± 2.2% of the initial concentration. Topotecan, measured

Table 3
Assay performance data for the determination of topotecan

Nominal concentration (ng/ml)		Run 1	Run 2	Run 3	Average within-run precision	Between-run precision	Average accuracy
0.05	Mean	0.054	0.050	0.054	9.0	4.4	105
	C.V.	10.4	8.4	8.3			
	Acc.	108	100	108			
0.10	Mean	0.087	0.111	0.102	7.9	12.1	100
	C.V.	7.7	4.9	11.1			
	Acc.	87	111	102			
0.25	Mean	0.227	0.214	0.216	5.6	3.2	88
	C.V.	7.1	4.9	4.8			
	Acc.	91	86	86			
5.00	Mean	5.29	5.23	5.15	3.8	1.3	105
	C.V.	2.9	4.8	3.6			
	Acc.	106	105	103			
10.00	Mean	10.51	10.92	10.54	3.0	2.1	106
	C.V.	4.5	2.2	2.4			
	Acc.	105	109	105			

C.V.: coefficient of variation (%); Acc.: accuracy (%); $n = 6$.

Table 4
Assay performance data for the determination of topotecan as total of lactone and carboxylate

Nominal concentration (ng/ml)		Run 1	Run 2	Run 3	Average within-run precision	Between-run precision	Average accuracy
0.05	Mean	0.048	0.055	0.056	7.5	7.8	106
	C.V.	9.6	5.7	7.1			
	Acc.	96	110	112			
0.10	Mean	0.105	0.119	0.117	7.7	6.6	113
	C.V.	7.4	10.5	5.2			
	Acc.	105	119	116			
0.25	Mean	0.255	0.243	0.255	3.0	2.9	100
	C.V.	3.5	1.3	4.2			
	Acc.	102	97	102			
5.00	Mean	5.11	5.01	5.09	1.0	1.0	101
	C.V.	0.7	1.3	1.0			
	Acc.	102	100	102			
10.00	Mean	10.18	9.91	10.34	1.7	2.2	101
	C.V.	1.2	0.9	3.1			
	Acc.	102	99	103			

C.V.: coefficient of variation (%); Acc.: accuracy (%); $n = 6$.

as total of the lactone and carboxylate, was stable under these conditions and during this storage period.

Diluted methanol extracts from plasma spiked with topotecan were stable (concentration >95% of the initial concentration) in the autosampler at 1°C for only 30 min. After 120 min 85% of the initial topotecan concentration remained. Therefore, only a maximum of three samples can be placed in the autosampler and these must be injected within 30 min.

Topotecan as total of lactone and carboxylate

Table 5
Stability data in plasma at −30°C at 10 ng/ml during 4.5 months

Time (months)	Lactone form		Lactone form (acidified)		Carboxylate form (acidified)	
	x (%)	C.V. (%)	x (%)	C.V. (%)	x (%)	C.V. (%)
0.00	100	6.2	100	11.0	100	1.8
0.25	92	5.5	104	2.3	103	3.7
1.00	76	5.7	104	2.5	105	3.6
4.50	41	4.2	103	2.6	97	2.5

x = Concentration of topotecan as percentage of initial concentration; C.V.: coefficient of variation; $n = 5$.

was stable in acidified samples when stored in the autosampler at room temperature for at least 24 h.

Stability studies at −30 and −70°C in the methanolic plasma extracts indicated that topotecan was stable for at least 4.5 months and 2 months, respectively.

4. Conclusions

In conclusion, a simple, sensitive and validated HPLC assay for the quantitative determination of topotecan in human plasma is described. The assay quantifies topotecan concentrations of 0.050–10 ng/ml using a 100-μl sample volume.

Topotecan was unstable in plasma at −30 and −70°C. For the determination of topotecan, methanol extracts (100 μl plasma + 200 μl methanol) should be prepared immediately after sampling in the clinic and can then be stored at −30°C or −70°C for at least 4.5 months and 2 months, respectively. Plasma samples for total analysis of the lactone and carboxylate forms of topotecan were stable for at least 4.5 months at −30°C.

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.

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

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

[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. Canc. Inst.*, 84 (1992) 1816.

[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.

[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.

[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.

[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.

[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.

[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.

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

[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.

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

[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.

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

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

[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.

[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.

[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.*, in press.

[20] J.H. Beijnen, H. Rosing, W.W. ten Bokkel Huinink and H.M. Pinedo, *J. Chromatogr.*, 617 (1993) 111.

[21] L.P. Rivory and J. Robert, *J. Chromatogr.*, 661 (1994) 133.

[22] J. Blanchard, *J. Chromatogr.*, 226 (1981) 455.

[23] 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.