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Quantification of topotecan and its metabolite *N*-desmethyltopotecan in human plasma, urine and faeces by high-performance liquid chromatographic methods

H. Rosing[a,*], D.M. van Zomeren[a], E. Doyle[b], W.W. ten Bokkel Huinink[c], J.H.M. Schellens[c], A. Bult[d], J.H. Beijnen[a,c,d]

[a]*Department of Pharmacy and Pharmacology, Slotervaart Hospital/The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

[b]*Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, The Frythe, Welwyn, Hertfordshire, UK*

[c]*Department of Medical Oncology, Antoni van Leeuwenhoek Hospital/The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands*

[d]*Department of Pharmaceutical Analysis and Toxicology, Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands*

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Abstract

Sensitive high-performance liquid chromatographic (HPLC) methods have been developed and validated for the simultaneous determination of the antitumor drug topotecan and its metabolite *N*-desmethyltopotecan in human plasma, urine and faeces. Both compounds are reversibly hydrolysed to their hydroxycarboxylate forms at physiologic pH. Separate HPLC systems have been developed for the determination of lactone and total (lactone plus hydroxycarboxylate forms) concentrations in plasma. The instability of the analytes in plasma requires immediate protein precipitation with ice-cold methanol. The lactone forms of the analytes were stable in the methanol extracts for at least 15 months when stored at −70°C. For the determination of the total levels, the plasma extracts were acidified with 25 m*M* phosphoric acid to convert the compounds into their lactone forms quantitatively. The sample pretreatment procedure for urine included dilution in methanol while the faecal samples were homogenized in distilled water and then extracted twice with an acetonitrile–ammonium acetate mixture. Separation was achieved on reversed-phase columns (Zorbax SB-C18) and detection was performed fluorimetrically at 380/527 nm. Within-run and between-run precisions were less than 10% and average accuracies were between 90 and 110%. The methods were used in a mass balance study in patients with malignant solid tumors to determine the disposition and routes of elimination of topotecan and *N*-desmethyltopotecan. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Urine; Plasma; Topotecan; *N*-Desmethyltopotecan

1. Introduction

Topotecan (Hycamtin™, Fig. 1) is a water-soluble anticancer analogue of camptothecin. The drug in-

*Corresponding author. Tel.: +31-20-512-4476; fax: +31-20-512-4753.
E-mail address: aphro@slz.nl (H. Rosing)

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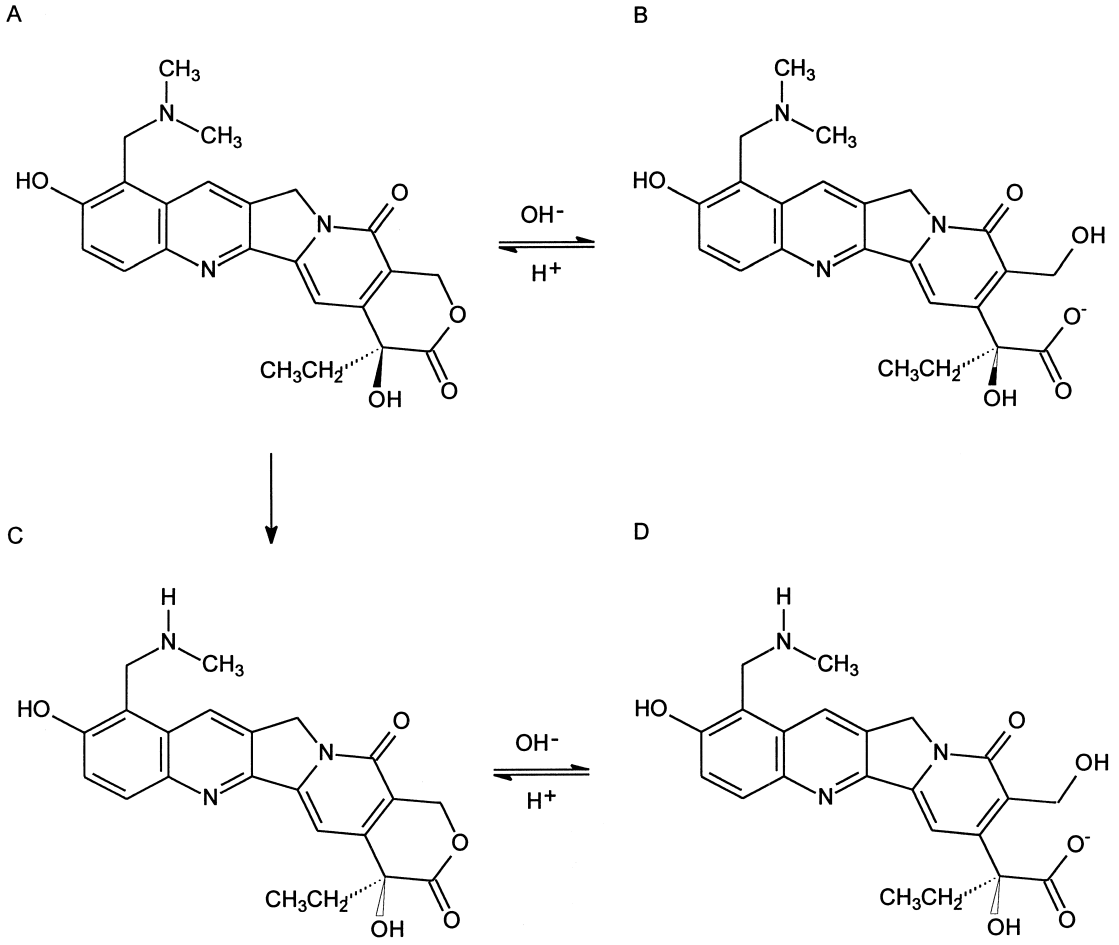

Fig. 1. Chemical structures of topotecan (A) and *N*-desmethyltopotecan (C) undergoing pH-dependent hydrolysis to their ring opened hydroxycarboxylate forms (B and D, respectively).

hibits DNA replication and RNA transcription by stabilizing the cleavable complexes formed between the nuclear enzyme topoisomerase I and DNA [1,2]. The lactone ring undergoes reversible hydrolysis which is pH-dependent (Fig. 1) [3]. It has been demonstrated that stabilization of the DNA–topoisomerase I complex, which is considered to mediate the antitumor activity of topotecan, requires the drug to be present in its lactone form [4]. Topotecan has been registered for the treatment of patients with metastatic carcinoma of the ovary after failure of first line chemotherapy [5]. Additionally, topotecan has shown clinical antitumor activity in the treatment of small cell lung cancer [6,7]. Demethylation to form *N*-desmethyltopotecan has been identified as a metabolic pathway for topotecan in humans [8]. This metabolite was found in plasma and urine of patients treated with topotecan. Although various papers have been published on the quantification of topotecan [9–12], these methodologies are not able to quantify *N*-desmethyltopotecan. Furthermore, no bio-analytical assays have been described for the determination of topotecan in faeces. We have developed and validated high-performance liquid chromatographic (HPLC) methods for the determination of topotecan and *N*-desmethyltopotecan in human plasma, urine and faeces in support of clinical studies to determine the pharmacokinetics and mass balance of topotecan.

Camptothecin derivatives are reversibly hydrolysed to their hydroxycarboxylate forms at physiologic pH. In vitro conversion is rapid and it is

important that blood samples are collected in an appropriate fashion (rapid cooling, centrifuged under cooled conditions and subsequent protein precipitation) to fix the equilibrium for adequate quantitation of the lactone forms [9]. The plasma samples can then be acidified for the determination of the total levels of topotecan and *N*-desmethyltopotecan. The hydroxycarboxylate concentrations are determined by calculating the difference between these concentrations. Separate measurements of lactone and total levels in urine and faeces were considered irrelevant due to rapid interconversion reactions of the compounds during collection of these matrices.

2. Experimental

2.1. Chemicals and control matrices

Topotecan (hydrochloride salt, SKF 104864-A, lot MM-15906-194, purity 89.2%) and *N*-desmethyltopotecan reference standard (hydrochloride salt, SB 209780-A, lot JW-19178-221A1, purity 86.5%) originated from SmithKline Beecham Pharmaceuticals (King of Prussia, PA). Acetonitrile and methanol (HPLC gradient grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Potassium dihydrogen phosphate, disodium hydrogen phosphate di-hydrate, triethylamine, 37% (w/v) hydrochloric acid, citric acid mono hydrate, ammonium acetate and hexane sulfonic acid (all analytical grade) were from Merck (Darmstadt, Germany). Phosphoric acid 85% (w/v) was purchased from BDH Chemicals (Poole, England). Tetrabutylammonium phosphate was from Waters Corporation (Milford, MA, USA). Double-distilled water was used throughout. Drug-free human plasma originated from Sanquin Blood Supply Foundation (Amsterdam, The Netherlands). Blank urine and faecal samples were obtained from healthy volunteers.

2.2. HPLC instrumentation and conditions

The chromatographic system consisted of a type P100 solvent delivery system, a Model AS300 automatic sample injection device (both Thermo Quest, Fremont, CA, USA) and a Model 7980 thermo regulator (Jones Chromatography Inc., Lakewood, CO, USA). HPLC System I consisted of a Zorbax SB-C18 column (75×4.6 mm I.D., particle size 3.5 μm; Rockland Technologies Inc., Newport, DE, USA) protected with a guard column (10×3 mm I.D.) packed with reversed-phase material (Chrompack, Middelburg, The Netherlands). The eluent was pumped through the column with a flow-rate of 1.0 ml/min and consisted of a mixture of 75 m*M* potassium dihydrogen phosphate in water containing 0.2% (v/v) triethylamine (adjusted to pH 6.0 with 4 *M* hydrochloric acid) and methanol (72.5:27.5, v/v). The autosampler tray was thermostated at approximately 4°C and the column was kept at ca. 50°C.

The same chromatographic equipment was used for HPLC System II, albeit with a longer Zorbax SB-C18 column (150×4.6 mm I.D., particle size 3.5 μm; Rockland Technologies Inc.) and a mobile phase comprising 10 m*M* citric acid–20 m*M* phosphate buffer pH 3.0–methanol (75:25, v/v). The analytical column was thermostated at ca. 34°C. Samples were injected at ambient temperatures.

In both systems, detection was performed fluorimetrically with an FP920 Intelligent Fluorescence Detector (Jasco International Co. Ltd., Tokyo, Japan); the excitation wavelength was 380 nm and the emission wavelength was 527 nm with a 40 nm bandwidth and a digital filter set at 10 s. The capacity of the flow cell of the fluorescence detector was 16 μl. A Data Jet integrator was coupled to a WINner/PC 1000 data system (both from Thermo Quest).

2.3. Preparation of stock and working solutions

Stock solutions of topotecan and *N*-desmethyltopotecan were prepared in acidified methanol (1 m*M* hydrochloric acid) at concentrations of 1.0 mg as free base per ml. Working solutions were prepared by successive 10-fold dilutions in acidified methanol to obtain analyte concentrations of 100 μg/ml to 0.1 ng/ml. All solutions were stored at approximately −30°C and were stable for at least 6 months.

2.4. Sample pretreatment of calibration standards

2.4.1. Plasma

For the determination of the analytes as their lactone forms, sample preparation was performed on an ice-water bath. In a polypropylene container of 1.5 ml, appropriate volumes (25–100 μl) of

topotecan and *N*-desmethyltopotecan working solutions were diluted with methanol to a final volume of 200 μl. Control human plasma (100 μl) was added and the plasma proteins were precipitated. The sample was homogenized briefly for 10 s and centrifuged at 4°C for approximately 3 min (23,000 *g*). The clear supernatant (75 μl) was diluted with distilled water (1:1, v/v) and immediately stored at −30°C for at most 8 h until analysis. A volume of 100 μl was injected onto HPLC System I. The analytes were stable in the autosampler (thermostated at 4°C) for maximal 30 min.

The same procedure was followed for the preparation of the calibration standards for the determination of the total levels of topotecan and *N*-desmethyltopotecan, except that the sample pretreatment was performed at ambient temperatures and the clear supernatant was acidified with 25 m*M* phosphoric acid (1:1, v/v). The sample was then centrifuged for 10 min at 23,000 *g* and HPLC System II was used to analyse a sample volume of 50 μl. The acidified samples for analysis of total levels were stable in the autosampler for at least 3 days at ambient temperatures.

2.4.2. Urine

A volume of 1000 μl control human urine was transferred to a volumetric flask of 25.0 ml. Appropriate volumes (25–100 μl) of topotecan and *N*-desmethyltopotecan working solutions were added. The spiked urine sample was then diluted with methanol to a final volume of 25.0 ml. After mixing, the diluted samples were acidified with 25 m*M* phosphoric acid (1:1, v/v). Volumes of 25 μl of these acidified samples were injected onto HPLC System II. Sample stability in the autosampler at ambient temperatures was assessed for at least 3 days.

2.4.3. Faeces

A drug-free faecal homogenate was prepared in distilled water (1+2, w/v) using a Ystral homogeniser (Type X1020, Dottingen, Germany). A 1.0 ml volume of the homogenate was transferred to a screw-capped test tube of 10 ml. The sample was spiked with 20–100 μl of topotecan and *N*-desmethyltopotecan working solutions. Methanol was then added to achieve a total (methanol) volume of 200 μl. Next, acetonitrile (4.0 ml) and 0.1 *M* ammonium acetate pH 4.0 (2.0 ml) were added and the sample was sonicated for at least 2 min and then shaken for at least 20 min. After extraction the sample was centrifuged (15 min at approximately 5000 *g*) and the supernatant was decanted into a clean tube. The procedure was repeated on the residue with a mixture of acetonitrile (3.0 ml) and 0.1 *M* ammonium acetate pH 4.0 (3.0 ml). The pooled supernatants were diluted with the eluent of HPLC System II (1:2, v/v) and 25 μl of the samples were analysed using HPLC System II. No significant changes in the total concentrations of the analytes were found in the diluted extracts when stored in the autosampler at ambient temperatures for at least 6 days.

2.5. Method validation

2.5.1. Linearity

Calibration standards in all tested biological matrices spiked with topotecan and *N*-desmethyltopotecan were analyzed in duplicate in three separate analytical runs. Every calibration curve consisted of seven non-blank calibration standards. The linear regressions of the area of both analytes versus the concentration were weighted by $1/x^2$ (reciprocal of the squared concentration). The *F* test for lack of fit ($\alpha=0.05$) was used to evaluate the linearity of the calibration curves [13].

2.5.2. Accuracy and precision

Quality control samples were independently prepared from the calibration standards, according to Section 2.4. The samples contained four concentration levels of topotecan and *N*-desmethyltopotecan: at the lower limit of quantitation (LLQ), 3–4 times the LLQ, mid-point of the concentration range and near the upper limit of quantitation (ULQ). Six replicates of each quality control sample were processed and analyzed in three different runs with plasma calibration standards. The accuracy and precisions were calculated at each test concentration using Analysis of Variance (software package Statistical Product and Service Solutions, version 6.1 for Windows, SPSS Inc., Chicago, IL, USA). The acceptance criteria for accuracy and precision are in accordance with the Washington conference report [14].

2.5.3. Selectivity

Six batches of control human plasma, 12 urine samples from healthy volunteers and five batches of control human faeces were processed and analyzed to determine whether endogenous constituents co-eluted with topotecan and/or *N*-desmethyltopotecan. To investigate the potential interference of co-medication with the analytical methods, several blank samples for all tested matrices taken from patients treated with topotecan were processed and then assayed according to the described methods. The following co-medication was given to these patients: acetaminophen, codeine, temazepam, oxazepam, metoclopramide, magnesium oxide, granisetron, lactulose and triamterene/hydrochlorothiazide.

2.5.4. Recovery

For the assays in plasma and urine, the overall extraction recoveries were determined by comparing the mean analyte responses of six processed quality control samples to the mean responses of three standards prepared in distilled water. Recoveries were determined in three different runs at two concentrations: at 3–4 times the LLQ and near the ULQ.

For the determination of the recoveries of topotecan and *N*-desmethyltopotecan from human faecal homogenate, five batches of blank human faeces were tested at two concentrations. After spiking, the homogenized samples were stored at ambient temperatures during 24 h before processing. Six replicates of each batch were processed and concentrations of topotecan and *N*-desmethyltopotecan in the spiked samples were calculated by means of a calibration curve which consisted of seven standard concentrations (containing topotecan and *N*-desmethyltopotecan) prepared in the eluent. The recoveries were determined at each test concentration by comparing the measured concentration with the nominal concentration.

2.5.5. Stability

Spiked blank samples were used to conduct stability tests. Stability data were acquired on 2–6-fold determinations and in most cases at two different (low and high) concentrations. If relevant, the following stability conditions were evaluated for each method: in process (during sample collection and sample pretreatment), processed (after sample pretreatment), freeze–thaw and long-term stability. An interval of ±10% around the nominal concentration was applied to assess analyte stability.

2.6. Pharmacokinetic case study

A patient with a malignant solid tumor was treated with topotecan given as a 30 min intravenous infusion at a dose level of 1.5 mg/m^2 for five consecutive days. Whole blood samples (5 ml) were taken from the contra lateral arm receiving the infusion. Sampling times were prior to the start of the infusion, at 15, 30, 45, 60 and 90 min and 2, 4, 6, 8 and 12 h after the start of the infusion. The blood samples were collected in heparinized tubes and immediately immersed in ice-water. The tubes were centrifuged for 5 min under refrigerated conditions (4°C). A volume of 1.0 ml of the plasma layer was then added to 2.0 ml of cold methanol (−30°C) and the plasma proteins were precipitated. After brief mixing (10 s) the sample was centrifuged at 3000 *g* at 4°C for approximately 3 min. The clear supernatant was transferred to a polypropylene tube and immediately stored at −30°C. The remaining plasma in the heparinized tube was transferred to a poly-

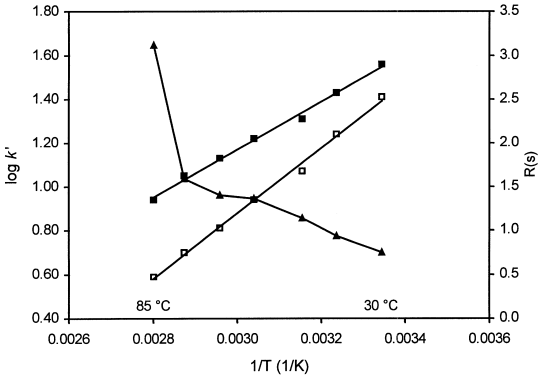

Fig. 2. Influence of temperature on the capacity factors (k') of topotecan (■) and *N*-desmethyltopotecan (□). The degree of separation (R_s; ▲) is calculated at each tested temperature. Experimental conditions of the HPLC system are described in the text.

propylene tube and stored at −30°C. Within 1 week, the methanol extracts were shipped to the laboratory and were then stored at −70°C until analysis. Urine and faeces were collected during 5 days of dosing and 4 days to follow. Urine was collected over 24-h intervals. The total volume was recorded and after mixing an aliquot of each 24-h sample was stored at −30°C. Stools were stored at 4–8°C for a maximum of 3 days. An homogenate was prepared in distilled water (1+2, w/v) and 1.0 ml volumes were transferred to six individual tubes and stored at −30°C until analysis. Analyses of study samples were performed singly, although faecal homogenates were assayed in duplicate.

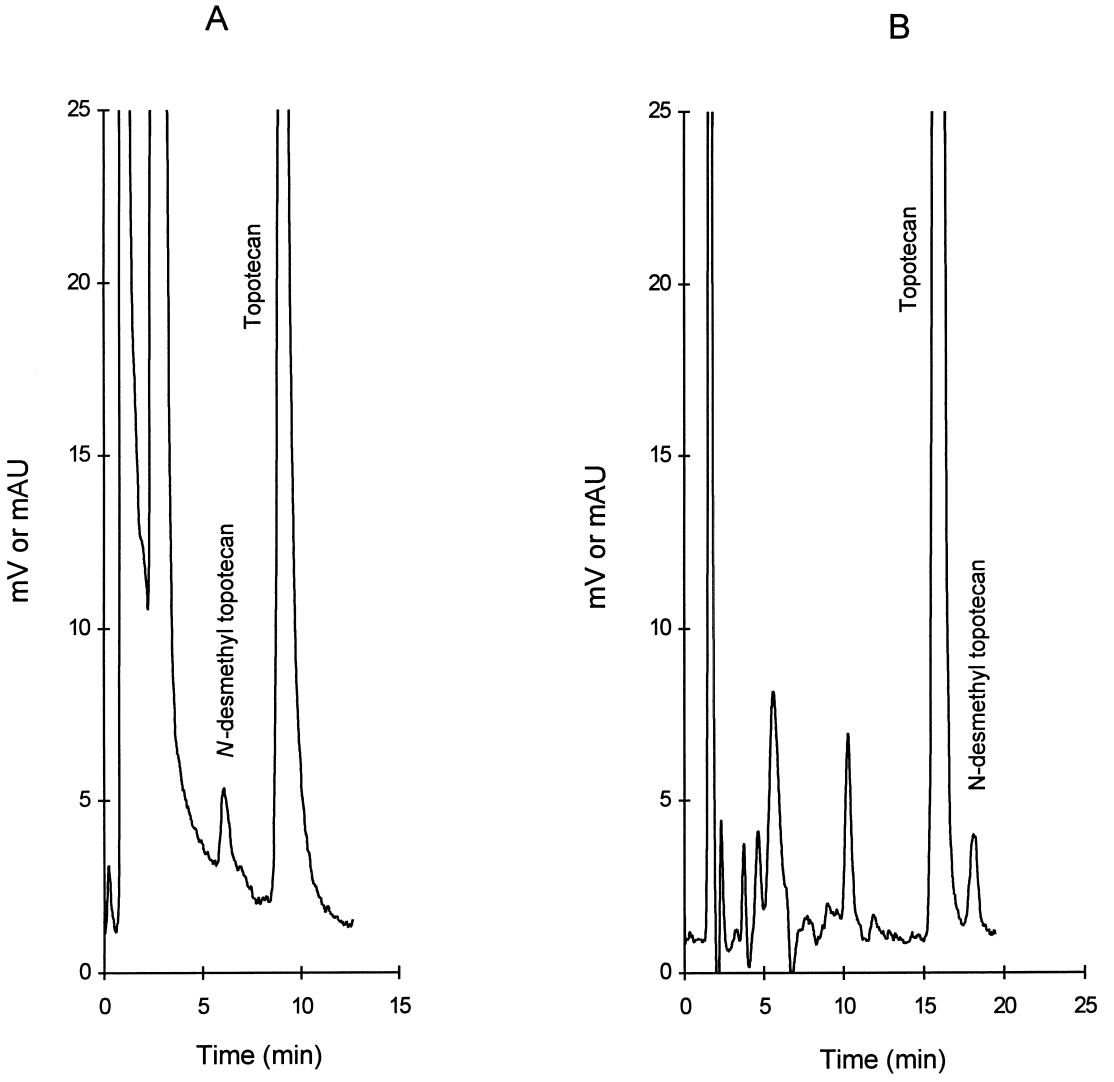

Fig. 3. Chromatograms of a plasma sample taken from a patient treated with topotecan at a dose of 1.5 mg/m^2 at day 1, 2 h after the start of a 30-min infusion: lactone levels of topotecan and *N*-desmethyltopotecan are 3.90 and 0.200 ng/ml, respectively (chromatogram A, HPLC System I) and total levels are 14.4 and 0.398 ng/ml, respectively (chromatogram B, HPLC System II).

3. Results and discussion

3.1. Optimization of the methods

The former assay for the analysis of topotecan in human plasma [10] could not be used for the quantitative determination of *N*-desmethyltopotecan due to insufficient resolution between both compounds. Like all known camptothecin derivatives, topotecan and *N*-desmethyltopotecan are reversibly hydrolysed to their carboxylate forms and it was the intention to develop a methodology enabling the quantification of both forms of the analytes in one analytical run. Using an end-capped Zorbax SB-C18 column (75×4.6 mm I.D.) in combination with methanol as modifier the peak widths were decreased significantly when compared to acetonitrile. Peak symmetry was improved when the silanol-blocking compound triethylamine (0.2%) was added to the mobile phase. The pH of the eluent was adjusted to 6.0 to prevent on-column conversion of the lactone analytes to their carboxylate forms and vice versa [9]. Under these conditions, the hydroxycarboxylate forms were eluting just after the solvent front. When the ion-pairing agent tetrabutylammonium phosphate (5 m*M*) was added to the eluent, the retention of the negatively charged carboxylate forms was increased. However, no adequate separation could be achieved between the lactone and carboxylate forms. It was then decided to develop two HPLC systems: one for the determination of the lactone concentrations of both analytes (System I) and the other for the total levels of the hydroxycarboxylate plus the lactone forms for each compound (System II). The hydroxycarboxylate concentrations were determined by calculating the difference between these concentrations.

By changing the temperature of the column (System I), separation between topotecan and *N*-desmethyltopotecan could be improved. The influence of temperature is shown in Fig. 2. To judge the degree of separation between the two peaks, the resolution factor (R_s) was calculated as follows:

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

where Δt is the retention time difference between the two peak maxima and W_1 and W_2 the base widths of the peaks. Sufficient baseline resolution was achieved at 50°C. Increasing temperatures resulted in lower fluorescence intensities; e.g., at 85 and 50°C the detected fluorescence signals of topotecan were 8 and 85%, respectively, compared with the response measured at 30°C. No peak broadening was obtained when an injection volume up to 100 μl was applied. A typical chromatogram for the analysis of topotecan and *N*-desmethyltopotecan as their lactone forms in human plasma (HPLC System I) is presented in Fig. 3A.

For the determination of the total levels of both compounds, an acidified eluent is to be preferred because (i) the peak symmetry of the basic compounds is improved, (ii) the acidity of the processed sample and the eluent is similar and (iii) on-column conversion of the lactone forms into their hydroxycarboxylate forms is prevented. To investigate the influence of pH, eluents were prepared with pH's of the aqueous part ranging from 3 to 7 comprising 10 m*M* citric acid–20 m*M* phosphate buffers and methanol (80:20, v/v). Analyses were performed at ca. 44°C on a 75 mm Zorbax SB-C18 column. Results are summarized in Fig. 4. At high pH values, resolution improves, however peak symmetry deteriorates. The chromatography at these pH values is probably mainly based upon hydrophobic interactions with the stationary phase. At pH 4, the order of retention changed, with a smaller capacity factor for topotecan. Peak symmetry was excellent for both

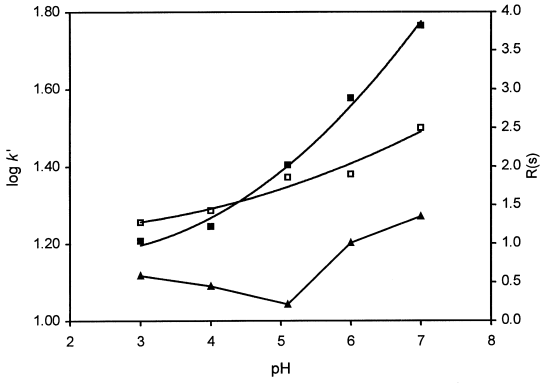

Fig. 4. Influence of pH on the resolution (▲) and chromatography of topotecan (■) and *N*-desmethyltopotecan (□). HPLC conditions (System II) are described in the text; a Zorbax SB-C18 (75×4.6 mm I.D.) was used to execute these experiments.

compounds and the resolution was further improved by the use of a 150 mm column operating at 34°C. Representative chromatograms for the determination of the total concentrations of topotecan and *N*-desmethyltopotecan in human plasma, urine and faeces are given in Figs. 3B, 5A and B, respectively.

SN38, irinotecan (CPT11) and camptothecin were tested as potential internal standards for the bioanalytical assays. The capacity factors of these compounds were too high in both HPLC systems compared to the analytes. It was then decided to execute the validation program without an internal standard.

3.2. Method validation

The assays were linear over the tested concentration ranges as determined by the *F* test for lack of fit. For every calibration curve the calibration concentrations were back-calculated from the peak areas of topotecan and *N*-desmethyltopotecan. The deviation from the nominal concentration for all tested concentrations was equal to or less than 6%. The performances of the assays are presented in Tables 1 and 2. The within-run and between-run precisions were all less than the required 15% [14]. Chromatograms of several batches of control matrices contained no endogenous peaks co-eluting with topotecan and/or *N*-desmethyltopotecan. From all tested potential interfering drugs used as co-medication by the patients, only triamterene interfered with topotecan. The mean overall extraction recoveries of topotecan and *N*-desmethyltopotecan from plasma, urine and faeces were all above 85% (Table 3). Comparable extraction recoveries were obtained when different batches of faecal homogenates were used. This resulted in small coefficients of variation ($\leq$3.1%, $N=30$) as shown in Table 3.

In Tables 4–6 the stability data for topotecan and *N*-desmethyltopotecan are summarized. The conditions were chosen as relevant for the presented assays. The analytes were stable under the tested conditions, however the stabilities of topotecan and *N*-desmethyltopotecan as their lactone forms in plasma methanol extracts were limited when stored at −30°C. A lactone concentration-versus-time pro-

Table 1
Assay performance data for the determination of topotecan and *N*-desmethyltopotecan in human plasma

Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Accuracy (%)	Within-run precision (%)	Between-run precision (%)	No. of replicates
Lactone levels of topotecan in plasma					
0.1	0.102	102.0	7.3	3.2	18
0.4	0.378	94.5	5.4	2.8	18
5	5.16	103.2	1.9	5.5	18
50	54.1	108.2	1.8	6.4	18
Total levels of topotecan in plasma					
0.1	0.0950	95.0	9.5	6.6	18
0.4	0.401	100.3	2.6	4.9	18
25	24.8	99.2	4.1	1.6	18
50	52.9	105.8	1.1	2.3	18
Lactone levels of N-desmethyltopotecan in plasma					
0.1	0.106	106.0	4.2	6.1	18
0.4	0.414	103.5	3.3	6.0	18
1	0.987	98.7	3.0	3.8	18
2.5	2.62	104.8	2.5	3.4	18
Total levels of N-desmethyltopotecan in plasma					
0.1	0.0955	95.5	9.3	5.2	18
0.4	0.390	97.5	3.5	0.7	18
1	1.01	101.0	2.7	4.7	18
2.5	2.47	98.8	3.5	6.6	18

Table 2
Assay performance data for the determination of total levels of topotecan and *N*-desmethyltopotecan in human urine and faeces

Nominal conc. (ng/ml, μg/g)	Measured conc. (ng/ml, μg/g)	Accuracy (%)	Within-run Precision (%)	Between-run Precision (%)	No. of replicates
Total levels of topotecan in urine					
25	25.0	100.0	2.0	0.9	18
100	99.4	99.4	1.7	0.4	18
500	501	100.2	0.8	0.7	18
1000	1008	100.8	1.3	0.4	18
Total levels of N-desmethyltopotecan in urine					
2.5	2.61	104.4	6.1	3.9	18
10	10.3	103.0	2.9	2.9	18
25	49.2	98.4	2.5	6.2	18
100	101	101.0	2.2	5.5	18
Total levels of topotecan in faeces					
0.3	0.303	101.0	2.4	1.7	18
0.75	0.764	101.9	2.6	2.9	18
7.5	7.63	101.7	2.0	3.9	18
15	15.0	100.0	1.4	2.5	18
Total levels of N-desmethyltopotecan in faeces					
0.03	0.0309	103.0	4.9	2.6	18
0.075	0.0708	94.4	3.7	0.7	18
0.75	0.748	99.7	2.6	4.8	18
1.5	1.49	99.3	1.7	5.9	18

file for topotecan, stored under the described conditions, is presented in Fig. 6. Comparable stability data were obtained for *N*-desmethyltopotecan. As a result, methanol extracts from study samples can to be stored up to 1 week at the clinical site (−30°C) after which they must be transferred to the analytical

Table 3
Recovery data for topotecan and *N*-desmethyltopotecan[a]

	Initial conc.	Recovery (%)	CV[b] (%)	No. of replicates
Lactone levels of T in plasma	0.4 ng/ml	104	8.3	3
	50 ng/ml	102	4.0	3
Total levels of T in plasma	0.4 ng/ml	97.2	5.9	3
	50 ng/ml	97.6	2.5	3
Lactone levels of M in plasma	0.4 ng/ml	93.8	13.3	3
	2.5 ng/ml	90.9	8.4	3
Total levels of M in plasma	0.4 ng/ml	90.3	0.7	3
	2.5 ng/ml	93.4	6.4	3
Total levels of T in urine	100 ng/ml	103	1.1	3
	1000 ng/ml	103	1.6	3
Total levels of M in urine	10 ng/ml	99.2	2.6	3
	100 ng/ml	102	2.5	3
Total levels of T in faeces	0.4 μg/g	99.0	1.5	30
	10 μg/g	108	0.9	6
Total levels of M in faeces	0.04 μg/g	87.9	3.1	30
	1 μg/g	94.3	0.7	6

[a] T, topotecan; M, metabolite *N*-desmethyltopotecan.
[b] CV, coefficient of variation.

Table 4
Stability data of topotecan and *N*-desmethyltopotecan for the assays in human plasma

Storage conditions	Matrix	Initial conc. (ng/ml)	Recovery (%)	CV[a] (%)	No. of replicates
Lactone levels of topotecan					
Autosampler at 4°C for 75 min	Methanol extract–distilled water (1:1, v/v)	25	97.3		5[b]
−30°C for 15 months	Methanol extract	0.4	44.0	3.7	4
−30°C for 15 months	Methanol extract	50	43.6	1.9	4
−70°C for 15 months	Methanol extract	0.4	102.1	6.0	4
−70°C for 15 months	Methanol extract	50	106.7	1.2	4
Total levels of topotecan					
Autosampler for 2 days	Methanol extract–25 m*M* phosphoric acid (1:1, v/v)	25	98.2	0.1	3
−30°C, after three freeze–thaw cycles	Plasma	0.4	98.5	2.3	6
−30°C, after three freeze–thaw cycles	Plasma	50	97.4	0.4	6
−30°C for 12 months	Plasma	25	107.3	3.5	4
Lactone levels of N-desmethyltopotecan					
Autosampler at 4°C for 75 min	Methanol extract–distilled water (1:1, v/v)	2.5	95.4		5[b]
−30°C for 15 months	Methanol extract	0.4	44.0	6.1	4
−30°C for 15 months	Methanol extract	2.5	44.8	2.3	4
−70°C for 15 months	Methanol extract	0.4	101.9	1.3	4
−70°C for 15 months	Methanol extract	2.5	102.4	2.3	4
Total levels of N-desmethyltopotecan					
Autosampler for 2 days	Methanol extract–25 m*M* phosphoric acid (1:1, v/v)	1	97.8	0.5	3
−30°C, after three freeze–thaw cycles	Plasma	0.4	96.0	4.3	6
−30°C, after three freeze–thaw cycles	Plasma	2.5	96.7	0.7	6
−30°C for 12 months	Plasma	1	90.2	0.8	4

[a] CV, coefficient of variation.
[b] Based on regression of five time points.

laboratory on dry-ice and stored at −70°C for maximal 15 months until assay.

3.3. Pharmacokinetic case study

To show the applicability of the methods, the plasma concentration-versus-time profiles of topotecan and *N*-desmethyltopotecan as their lactone and total (lactone plus hydroxycarboxylate) concentrations after intravenous administration of topotecan at a dose level of 1.5 mg/m^2 (total dose 2.40 mg) daily times five are depicted in Fig. 7. Plasma pharmacokinetics are given in Table 7. The area under the plasma-concentration–time curve (AUC) was estimated by the trapezoidal rule up to the last measured time-point with extrapolation to infinity using the terminal rate constant *k*. The terminal half-life ($t_{1/2}$) was calculated as 0.693/*k*. The total amount of topotecan (8.56 mg, 71.3%) plus *N*-desmethyltopotecan (0.61 mg, 5.1%) excreted within 9 days was 76.4% of the cumulative administered intravenous dose (12 mg). Urinary excretion was 53.4%.

Table 5
Stability data of topotecan and *N*-desmethyltopotecan for the assay in urine

Storage conditions	Matrix	Initial conc. (ng/ml)	Recovery (%)	CV[a] (%)	No. of replicates
Total levels of topotecan					
Autosampler for 3 days	Methanol extract–25 m*M* phosphoric acid (1:1, v/v)	100	100.2	0.5	4
Autosampler for 3 days	Methanol extract–25 m*M* phosphoric acid (1:1, v/v)	1000	100.4	0.2	4
−30°C, after three freeze–thaw cycles	Urine	40	96.7	1.1	4
−30°C, after three freeze–thaw cycles	Urine	1000	97.3	1.4	4
Ambient temperatures for 24 h	Urine	40	96.7	2.2	4
Ambient temperatures for 24 h	Urine	1000	96.9	0.7	4
−30°C for 14 months	Urine	40	100.9	1.3	4
−30°C for 14 months	Urine	1000	104.6	1.3	4
Total levels of N-desmethyltopotecan					
Autosampler for 3 days	Methanol extract–25 m*M* phosphoric acid (1:1, v/v)	10	97.7	1.6	4
Autosampler for 3 days	Methanol extract–25 m*M* phosphoric acid (1:1, v/v)	100	100.8	0.5	4
−30°C, after three freeze–thaw cycles	Urine	4	98.9	5.4	4
−30°C, after three freeze–thaw cycles	Urine	100	97.1	1.3	4
Ambient temperatures for 24 h	Urine	4	106.3	4.1	4
Ambient temperatures for 24 h	Urine	100	96.0	1.1	4
−30°C for 14 months	Urine	4	97.4	9.7	3
−30°C for 14 months	Urine	100	105.0	1.4	4

[a] CV, coefficient of variation.

Table 6
Stability data of topotecan and *N*-desmethyltopotecan for the assay in faeces

Storage conditions	Matrix	Initial conc. (μg/g)	Recovery (%)	CV[a] (%)	No. of replicates
Total levels of topotecan					
Autosampler for 6 days	Extraction fluid–HPLC eluent II (1:2, v/v)	1.5	99.8	0.8	3
Autosampler for 6 days	Extraction fluid–HPLC eluent II (1:2, v/v)	12	98.8	0.5	4
Ambient temperatures for 24 h	Extraction fluid	1.5	99.7	0.4	4
Ambient temperatures for 24 h	Extraction fluid	12	102.4	6.4	4
4–8°C for 3 days	Faecal homogenate	10	98.0	1.5	6
−30°C for 7 months	Faecal homogenate	0.4	102.4	1.3	4
−30°C for 6 months	Faecal homogenate	10	110.7	1.0	4
Total levels of N-desmethyltopotecan					
Autosampler for 6 days	Extraction fluid–HPLC eluent II (1:2, v/v)	0.15	97.2	2.0	3
Autosampler for 6 days	Extraction fluid–HPLC eluent II (1:2, v/v)	1.2	102.3	0.7	4
Ambient temperatures for 24 h	Extraction fluid	0.15	99.1	2.3	4
Ambient temperatures for 24 h	Extraction fluid	1.2	99.2	2.2	4
4–8°C for 3 days	Faecal homogenate	1	96.2	0.8	6
−30°C for 7 months	Faecal homogenate	0.04	101.6	5.9	4
−30°C for 6 months	Faecal homogenate	1	111.8	2.0	4

[a] CV, coefficient of variation.

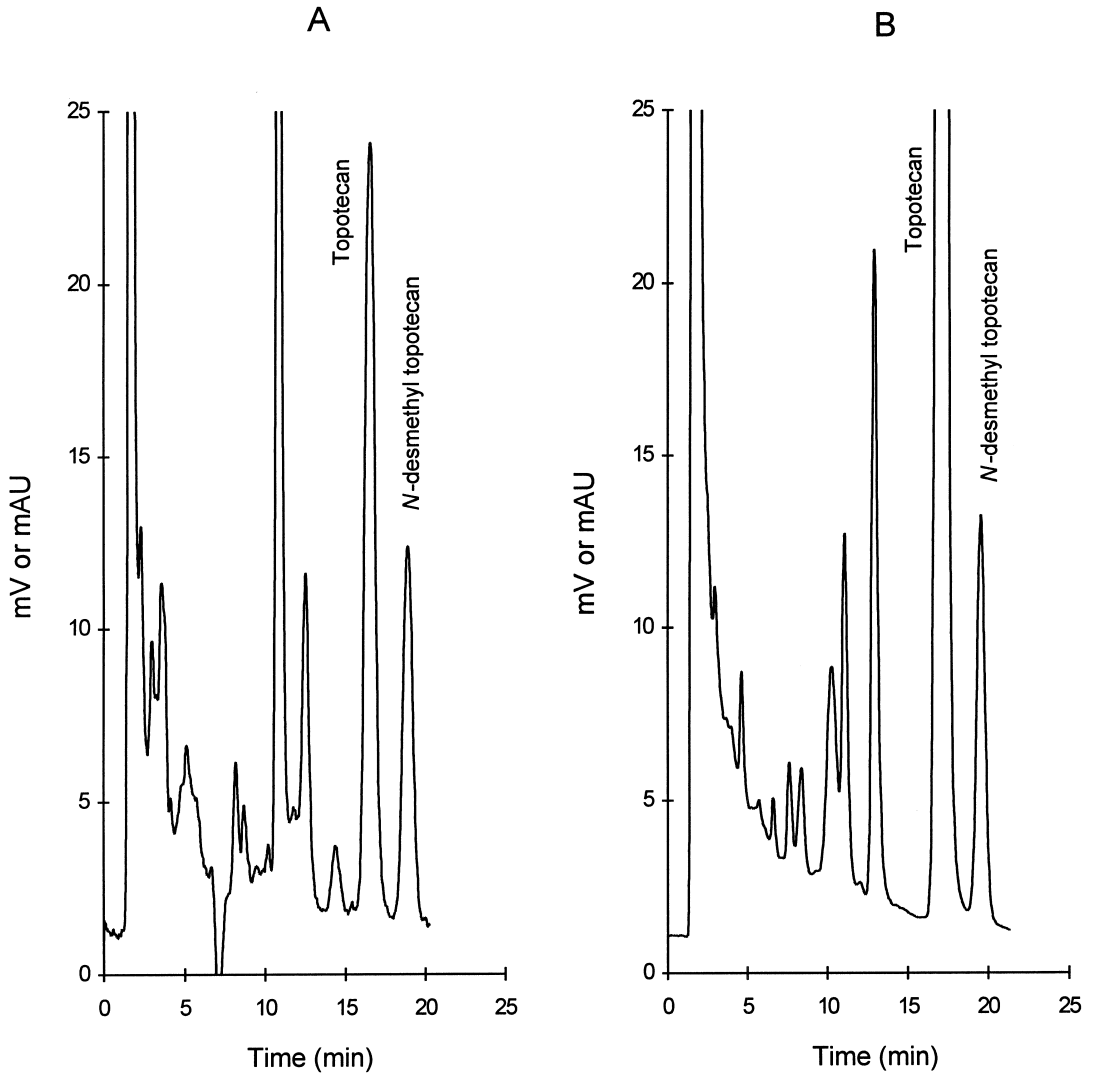

Fig. 5. Chromatograms of a 24-h urine (A) and a faecal sample (B) collected from a patient treated with intravenous topotecan at a dose of 1.5 mg/m^2 once daily for 5 days. The urine sample (day 5, 727 ml) contained total concentrations (lactone plus hydroxycarboxylate forms) of 1857 ng topotecan and 128 ng *N*-desmethyltopotecan per ml, total amounts in the stool (day 7, 62 g) were 9.16 and 0.786 μg/g for topotecan and *N*-desmethyltopotecan, respectively.

4. Conclusion

Accurate, precise and sensitive HPLC assays have been developed for the quantification of topotecan and *N*-desmethyltopotecan in several human matrices. These assays have been used to support a clinical study to determine the routes of elimination and disposition of topotecan in patients with malignant solid tumors. To minimize analyte degradation, blood samples taken from patients should be processed immediately and stored at −70°C. Under these conditions, stability data indicated that protein-free methanol extracts of plasma spiked with the analytes were stable for at least 15 months. For the determination of total concentrations of topotecan and *N*-desmethyltopotecan in plasma, urine and

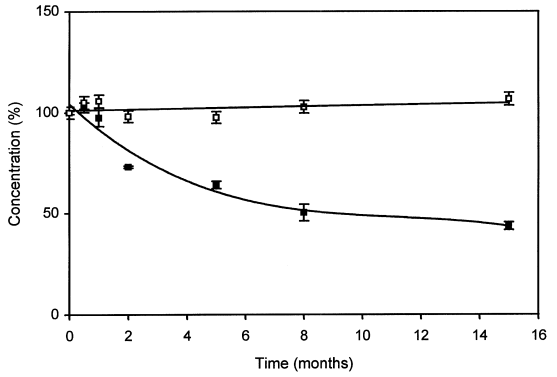

Fig. 6. Stability of topotecan in the methanol extract from plasma when stored at approximately −30°C (■) and −70°C (□). Comparable results were obtained for *N*-desmethyltopotecan.

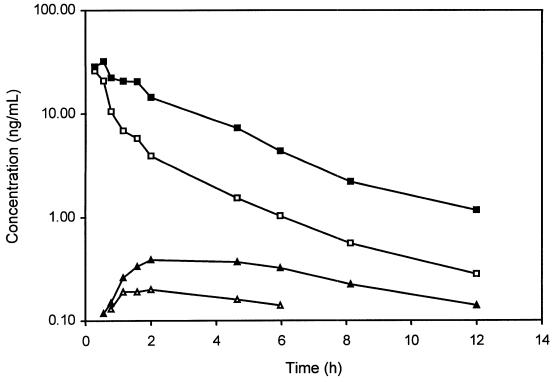

Fig. 7. Lactone and total concentrations of topotecan (□/■) and *N*-desmethyltopotecan (△/▲) in a patient treated with 1.5 mg/m^2 of topotecan administered as a 30-min infusion (day 1).

faeces, stability of the analytes was not critical. Processed samples were acidified before analysis to convert the analytes into their lactone forms.

Table 7
Plasma pharmacokinetics of topotecan and *N*-desmethyltopotecan after intravenous administration of 1.5 mg/m^2 topotecan

Pharmacokinetic parameter[a]	Topotecan	*N*-Desmethyl-topotecan
$t_{max,tot}$ (h)	0.5	2.0
$C_{max,tot}$ (ng/ml)	32.0	0.389
AUC_{tot} (h.μg/l)	93.4	3.17
$t_{1/2,tot}$ (h)	2.2	4.6
AUC_{lac}/AUC_{tot}	0.37	0.30

[a] lac, lactone form; tot, the total of the lactone and hydroxycarboxylate form.

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