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H. Rosing · V.M.M. Herben · D.M. van Gortel-van Zomeren
E. Hop · J.J. Kettenes-van den Bosch
W.W. ten Bokkel Huinink · J.H. Beijnen

Isolation and structural confirmation of *N*-desmethyl topotecan, a metabolite of topotecan

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Abstract A sensitive high-performance liquid chromatography (HPLC) method for the determination of topotecan and total levels of topotecan (lactone plus its ring-opened hydroxycarboxylate form) was developed by the authors and used in several pharmacokinetics studies. During the analysis of plasma and urine samples collected in those studies, an additional peak eluting just after topotecan was observed. Approximately 100 ng of this potential metabolite was isolated from human urine using a solid-phase extraction procedure and purification by HPLC. Analysis of the isolated material by HPLC showed it to be approximately 95% pure. Mass spectrometry data along with the HPLC retention data and fluorescence data (in comparison with synthetic reference standard) are consistent with the metabolite's being *N*-desmethyl topotecan. The maximal concentrations of metabolite deteced in human plasma and urine were relatively low. When topotecan was given as a 30-min infusion at 1.0 mg/m^2 daily for 5 days every 3 weeks, the maximal plasma metabolite concentration (lactone plus the ring- opened hydroxycarboxylate form) was about 0.7% ($n = 4$) of the maximal total topotecan concentration. The average amount of metabolite excreted in urine during the treatment was 1–4% ($n = 20$) of the delivered dose.

H. Rosing (✉) · V.M.M. Herben
D.M. van Gortel-van Zomeren · J.H. Beijnen
Department of Pharmacy and Pharmacology, Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands

E. Hop · J.J. Kettenes-van den Bosch · J.H. Beijnen
Department of Pharmaceutical Analysis and Toxicology, Faculty of Pharmacy, University of Utrecht, Utrecht, The Netherlands

W.W. ten Bokkel Huinink · J.H. Beijnen
Department of Medical Oncology, Antoni van Leeuwenhoek Hospital/Netherlands Cancer Institute, Amsterdam, The Netherlands

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Introduction

Topotecan (SK&F 104864, [S]-9-dimethylaminomethyl-10-hydroxycamptothecin, NSC 609669; Fig. 1) [11], is a semisynthetic derivative of camptothecin. The mechanism of action of the camptothecin analogues has been explained by their ability to inhibit the intranuclear enzyme topoisomerase I [6, 19]. The lactone structure, which is in pH-dependent equilibrium with the open-ring hydroxycarboxylate form (SK&F 105992; Fig. 1) [1, 7, 21] is essential for this function. The drug is reaching an established place in oncology; activity has been shown in phase I studies [2, 5, 8, 9, 13, 18, 22, 24, 25], and it is currently undergoing evaluation in phase II and III clinical trials [4, 11, 23].

Thus far, no evidence has been published for metabolism of topotecan. Although Recondo and colleagues [15] obtained preliminary data using thermospray mass spectrometry that suggested demethylation as a possible metabolic pathway for topotecan, no conclusive evidence was presented. In a study by Stewart et al. [20] it has been found that maximal and minimal topotecan lactone and total drug clearance in a patient population might be explained by concomitant therapy with drugs that are inducers and inhibitors of human cytochrome P450 3A, respectively. It has thus been suggested by these investigators that topotecan may undergo metabolism catalyzed by cytochrome P450 3A [20].

In 1995 we developed a high-performance liquid chromatography (HPLC) technique for the determination of topotecan and its ring-opened hydroxycarboxylate form (SK&F 105992) in plasma [16]. The method involves a protein precipitation step with cold

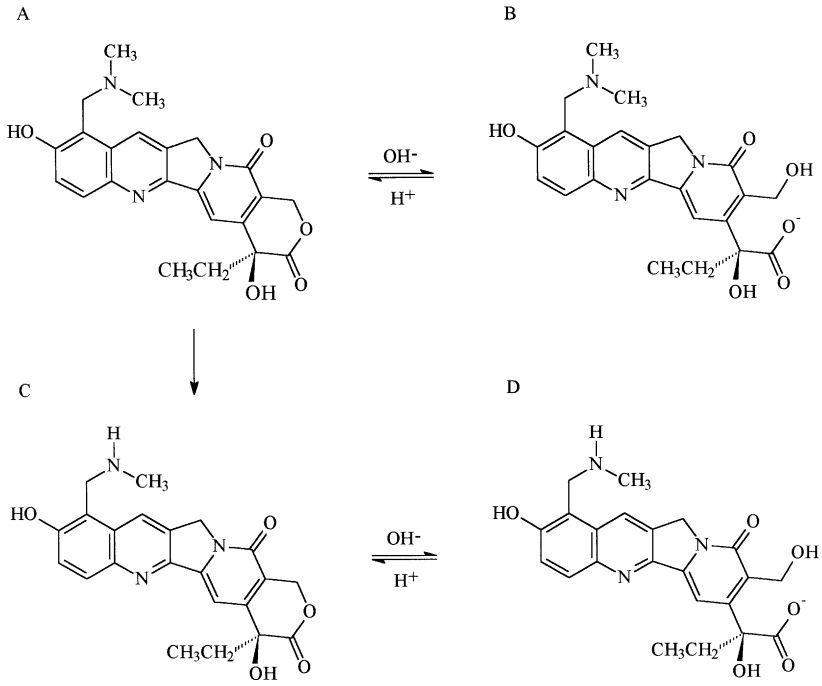

Fig. 1 Chemical structures of **A** topotecan undergoing pH-dependent hydrolysis to **B** its ring-opened hydroxycarboxylate form and the proton-catalyzed reverse reaction. The metabolite of topotecan, **C** *N*-desmethyl topotecan is in equilibrium with **D** its ring-opened hydroxycarboxylate form

methanol and subsequent injection of the extract into the HPLC system. The lower limit of quantitation of the assay is 0.05 ng/ml using only 100 µl of human plasma. This sensitive method has been implemented in several clinical pharmacokinetics studies [8, 22, 23]. During analysis of plasma and urine samples from these studies, another peak eluting just after topotecan was detected in the chromatograms that was not present in the chromatograms of blank calibration samples, quality controls, or samples originating from stability studies. A similar peak was also observed in the chromatograms of extracts of plasma from treated dogs in a toxicokinetics study. To investigate the physicochemical properties of the potential metabolite and to perform structural analysis we isolated the compound from human urine. This paper describes the isolation, purification, and mass spectrometry analysis of this potential metabolite.

Materials and methods

Chemicals

Topotecan (hydrochloride salt, SKF 104864-A, lot MM-15906-194, purity 89.2%) and *N*-desmethyl topotecan reference standard (hydrochloride salt, SB 209780-A, lot JW-19178-221A1, purity 86.5%) [3] originated from SmithKline Beecham Pharmaceuticals (King of Prussia, Pa., USA). All other reagents were of analytical grade and double-distilled water was used throughout.

HPLC instrumentation

The chromatography system consisted of a type P100 solvent-delivery system, and a model AS300 automatic sample-injection device (Thermo Separations Products, Fremont, Calif., USA). HPLC System I consisted of a Zorbax SB-C18 column (internal diameter 4.6 mm, length 75 mm, particle size 3.5 µm; Rockland Technologies Inc., Newport, Del., USA) protected with a guard column (3 × 10 mm) packed with reversed-phase material (Chrompack, Middelburg, The Netherlands). The eluent contained 0.02 *M* ammonium acetate-methanol (80:20, v/v). The flow rate was 1.0 ml/min, and volumes of 25 µl were injected on the column. The same chromatography equipment was used for HPLC System II, albeit with another mobile phase comprising methanol-0.1 *M* hexane-1-sulfonic acid in methanol-0.01 *M* *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) in distilled water (pH adjusted with phosphoric acid to 6.0; 25:10:65, by vol.). In both systems, detection was performed fluorimetrically with an FP920 Intelligent Fluorescence Detector (Jasco International Co. Ltd., Tokyo, Japan); the excitation wavelength was 361 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 data system (both from Thermo Separations Products).

For liquid chromatography-mass spectrometry (LC-MS; HPLC System III) a Novapak C18 column (internal diameter 2.0 mm, length 150 mm, particle size 4 µm; Waters Associates, Milford, Mass., USA) was used. The eluent contained 5% (v/v) acetic acid-methanol (70:30, v/v). Volumes of 5–25 µl were injected on the column. The flow rate was maintained at 0.2 ml/min. After 5 min the flow rate was reduced to 0.1 ml/min for optimal ionization of the analyte molecules in the mass spectrometer to improve the sensitivity of detection.

Clinical samples

Plasma and urine samples were obtained from patients with ovarian cancer who were participating in a phase I study in our clinic [8]. In this study, topotecan was given as a 30-min infusion at 1.0 mg/m^2 daily for 5 days every 3 weeks and in combination with oral etoposide. All patients gave informed consent.

Blood samples (5 ml) were collected in heparinized tubes before administration and at 5 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2.5 h,

3.5 h, 4.5 h, and 6.5 h after the start of drug infusion. Plasma was obtained by direct centrifugation (5 min, 2500 g). Plasma proteins were precipitated with cold methanol. The methanol extracts were stored at −30 °C and analyzed for total levels of topotecan (lactone plus the ring-opened carboxylate form) according to a previously validated method [16].

Before treatment a sample of each patient's urine was collected and stored at −30 °C. This sample was used as a blank. During treatment, 24-h urine collections were done for 6 days. The total volume was recorded, and approximately 20 ml of each 24-h sample was stored at −30 °C. Urine was diluted 50 times in methanol. Before HPLC analysis the samples were acidified (1:1, v/v) with 2% (v/v) perchloric acid to convert any ring-opened form to topotecan.

Fluorescence spectra

To 750 µl of 24-h urine from a patient (see above), 750 µl of 2% (v/v) perchloric acid was added. A stock solution with a mixture of topotecan and *N*-desmethyl topotecan references was diluted in 2% (v/v) perchloric acid to obtain a 35-ng/ml concentration for both compounds. From these two solutions, 25 µl was injected on HPLC System II with the emission wavelength set to 527 nm and the excitation wavelength rising from 300 to 420 nm in steps of 10 nm. This procedure was repeated with the excitation wavelength set to 380 nm, and the emission wavelength was increased from 450 to 610 nm in 10-nm increments. The peak areas of topotecan, *N*-desmethyl topotecan reference, and the metabolite were recorded.

Lactone moiety

To investigate the presence of a lactone function in the metabolite, the pH of the samples described above were adjusted to 13 with 1 *N* sodium hydroxide. Volumes of 25 µl were injected and analyzed using HPLC System II. Subsequently the pH was lowered to 1 with 4 *N* hydrochloric acid and the samples were reanalyzed.

Isolation and purification

Urine was concentrated using solid-phase extraction columns (C18, Bakerbond, 1 ml, 100 mg, J.T. Baker, Deventer, The Netherlands). Ten columns were first conditioned with consecutive washings with 1.0 ml of 0.1 *N* hydrochloric acid in methanol and 2.0 ml of distilled water. Next, 1.0-ml aliquots of urine were diluted with 1.0 ml of 0.1 *N* hydrochloric acid and each diluted sample was applied to a column. The columns were then washed with 2.0 ml of distilled water and dried under vacuum (15 mmHg) for 3 min. The analytes were eluted from the columns with 2.0 ml of 0.1 *N* hydrochloric acid in methanol. The elution solvent was pooled from all ten columns and evaporated to dryness under a nitrogen stream at 40 °C. The residue was reconstituted in 200 µl of methanol and 200 µl of 2% (v/v) perchloric acid was subsequently added.

The metabolite was isolated with HPLC System I. The retention time of the metabolite was 46 min (capacity factor 76). Fractions were collected in 12 separate chromatographic runs. After vacuum evaporation of the eluent in a Speed-Vac Plus SC210A system (Savant, Farmingdale, N.Y., USA) at 43 °C, the residues were dissolved in methanol. The relevant methanol fractions from each chromatographic run were pooled and evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted in 50 µl of methanol and 50 µl of 2% (v/v) perchloric acid was subsequently added. Aliquots of the reconstituted sample were injected on HPLC System I and the metabolite fractions were collected in three separate chromatographic runs. The eluent collected from each chromatographic run was evaporated to dryness and the residues were then dissolved in methanol. These three methanol fractions were combined (about 9 ml) and a sample (50 µl) was diluted with 50 µl of 2% (v/v) hydrochloric acid. This sample was injected on HPLC System II to quantify the amount of isolated metabolite and to determine the purity of the compound. The remainder was used for LC-MS analysis.

Liquid chromatography-mass spectrometry

Topotecan, *N*-desmethyl topotecan reference, and the isolated and purified metabolite (about 50 ng absolute) were injected on HPLC System III. MS detection was carried out with a VG Platform II Benchtop LC-MS (Fisons Instruments, Altricham, UK). An electrospray interface was used to ionize the molecules (positive-ion mode). The cone voltage was set to 25 V, and spectra were acquired every 4 s by scanning at m/z 280–480.

Results

During HPLC analysis of topotecan in human plasma (Fig. 2) and urine (Fig. 3), an additional peak was observed in the chromatograms that was not present in the chromatograms of blank calibration samples, quality controls, or samples originating from stability studies. The capacity factors recorded for topotecan and the unknown metabolite on HPLC System II were 13.5 and 15.7, respectively. The capacity factor noted for

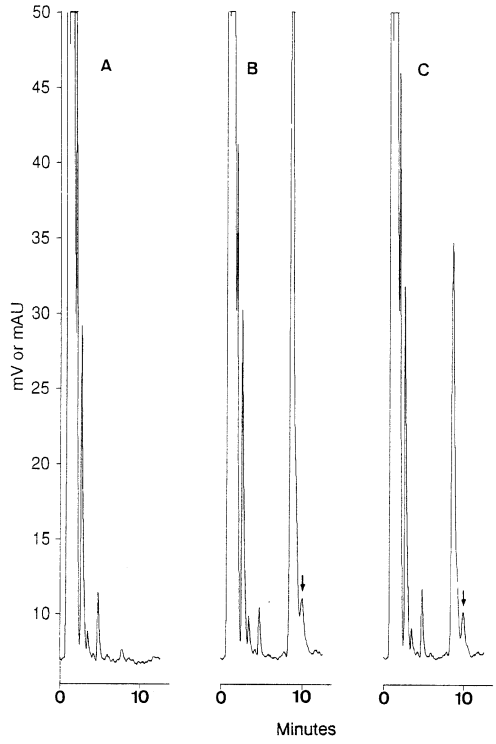

Fig. 2A–C HPLC chromatograms for the analysis of topotecan in plasma: **A** a blank and **B, C** two plasma samples taken at **B** 3 and **C** 6 h after the end of the infusion (topotecan concentrations 11.0 and 5.2 ng/ml, respectively). Topotecan is eluting after 9 min; the metabolite is indicated by an *arrow*

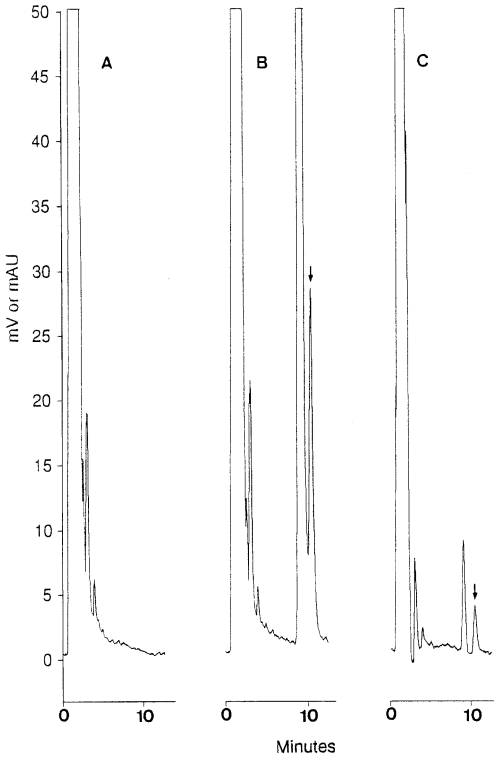

Fig. 3A–C HPLC chromatograms for the analysis of topotecan in urine: **A** a blank, **B** a 24-h urine sample (day 5), and **C** a 48-h urine sample (day 6), with the latter containing topotecan concentrations of 900 and 30 ng/ml, respectively. Topotecan is eluting after 9 min; the metabolite is indicated by an *arrow*

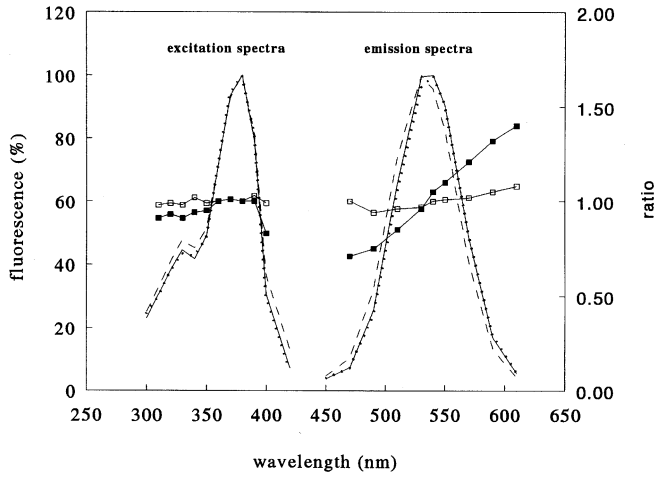

Fig. 4 Normalized excitation and emission spectra of *N*-desmethyl topotecan reference (——), topotecan (– – –), and the metabolite (● ● ●) at a concentration of 35 ng/ml (conditions are described in Materials and methods, fluorescence spectra). Spectra ratios calculated between the metabolite and topotecan (■) and between the metabolite and *N*-desmethyl topotecan (□) are also presented

N-desmethyl topotecan reference was 15.7, identical to that seen for the metabolite. When the pH of a urine sample was adjusted to 13, both the topotecan peak and the metabolite peaks disappeared and two additional front peaks were distinguished. Subsequently, when the sample was acidified, the topotecan and metabolite peaks emerged again. This observation is indicative of the presence of the lactone function in the structure of the metabolite.

The fluorescence spectra of *N*-desmethyl topotecan as a reference compound, topotecan, and the metabolite are depicted in Fig. 4. The optimal excitation wavelength for the three compounds in the eluent was identical: 380 nm. However, there was a slight difference in the emission maxima: the fluorescence intensity for topotecan was maximal at 530 nm, whereas the emission spectra of *N*-desmethyl topotecan reference and the metabolite were shifted to a higher wavelength (540 nm). The fluorescence intensity ratios of the metabolite against the reference compounds are also shown in Fig. 4.

The yield of the metabolite after isolation and purification was only 17%, and the purity was 95.1%. However, the amount was sufficient for the mass-spectrometry analysis. HPLC-MS spectra recorded at *m/z* 380–460 are shown in Fig. 5. The protonated molecular ion ($M + H^+$) of the metabolite at *m/z* 408 was also found for reference compound *N*-desmethyl topotecan.

Total topotecan and metabolite levels (lactone plus ring-opened hydroxycarboxylate forms) detected in plasma and urine from four treated patients are shown in Figs. 6 and 7. As no reference standard for *N*-desmethyl topotecan was available when samples were assayed, the metabolite concentrations were quantified on the calibration line prepared for the determination of topotecan plus its ring-opened hydroxycarboxylate form. Although *N*-desmethyl topotecan has a slight difference in emission maximum (540 nm) as compared with topotecan (530 nm) in the eluent, the fluorescence intensities were almost equal at 380/530 nm (Fig. 4). Moreover, the recovery of the metabolite after the sample pretreatment procedure was not significantly different from that of topotecan. The mean maximal concentration (C_{max}) of topotecan in plasma was reached at the end of the infusion (0.5 h) and was about 40 ng/ml ($n = 4$). The mean C_{max} of the metabolite was 0.29 ng/ml ($n = 4$), thus being only about 0.7% of the peak concentration of topotecan. Immediately following the end of the infusion, plasma concentrations of topotecan started to decline, whereas those of the metabolite increased initially and then remained approximately constant during the first 6 h (Fig. 6).

The average amount of topotecan and metabolite excreted in the 24-h urine aliquots were 41 ± 10% and 2.5 ± 1.0% ($n = 20$; 4 patients treated on 5 days) of the delivered dose, respectively. The average concentration ratio of topotecan to metabolite in the 24-h urine portions as measured on 5 consecutive days was about 17:1 (Fig. 7). At day 6 (24- to 48-h urine) this ratio had

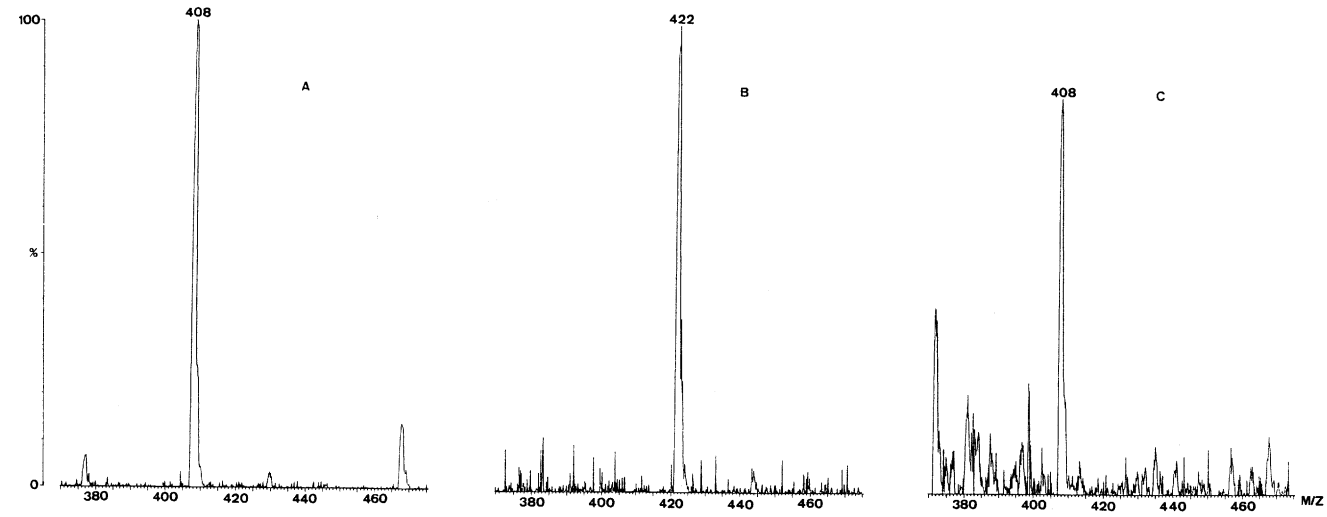

Fig. 5A–C HPLC-MS spectra recorded at m/z 370–480 for **A** *N*-desmethyl topotecan reference, **B** topotecan, and **C** the metabolite. Conditions are described in Materials and methods

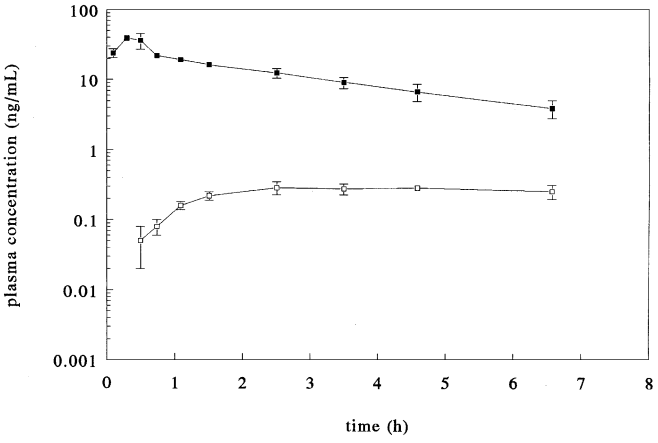

Fig. 6 Total topotecan (■) and metabolite (□) levels (±SD) measured in plasma samples taken on day 1 from four patients treated with topotecan daily for 5 days. Topotecan was given as a 30-min infusion at 1.0 mg/m^2 per day

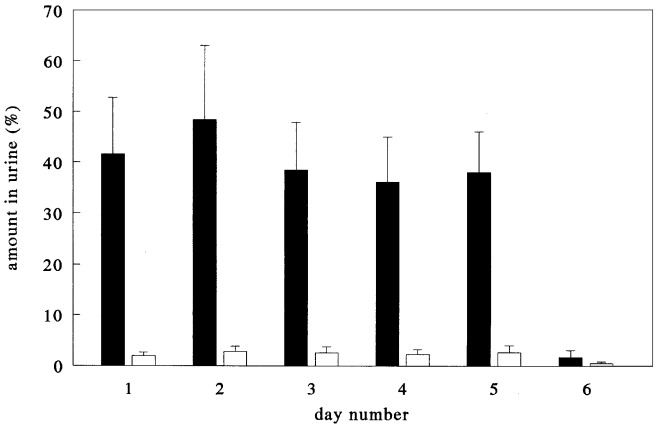

Fig. 7 Total percentages (±SD) of topotecan (■) and metabolite (□) excreted in the urine of four patients treated with topotecan daily for 5 days. Topotecan was given as a 30-min infusion at 1.0 mg/m^2 per day

changed dramatically in favour of the metabolite to 3:1 ($n = 4$).

The four patients presented herein were not solitary cases; metabolism of topotecan was observed in every patient entered in the study ($n = 24$). The metabolite was detected when total levels and lactone levels of topotecan were determined with the HPLC method [16]. An identical metabolic conversion has also been observed in dogs treated with topotecan using a similar HPLC method [17].

Discussion

Thus far, no literature has been published on topotecan metabolism, although it has been suggested that demethylation could occur [15] and that topotecan might undergo cytochrome P450 3A-mediated metabolism [20].

LC-MS data (Fig. 5) showed the protonated molecular ion ($M + H^+$) of the metabolite at m/z 408 that was also found for reference compound *N*-desmethyl topotecan. The 14 mass units of difference from topotecan indicate that a CH_2 moiety has been lost from the parent, consistent with the proposed *N*-desmethylation of topotecan. From plots of the fluorescence-intensity ratios that yielded horizontal straight lines in Fig. 4 (white squares) it can be concluded that *N*-desmethyl topotecan reference and the metabolite have identical fluorescence properties. As compared with topotecan, the emission maximum of the metabolite was shifted from 530 to 540 nm, resulting in a line with a positive slope when the fluorescence-intensity ratio of the compounds is plotted (Fig. 4, black squares). The capacity factors recorded for *N*-desmethyl topotecan reference and the metabolite were identical on three different HPLC systems. The pH experiments were in agreement with the presence of a reversible lactone-hydroxy acid equilibrium in the

molecular structure of the metabolite. As judged from the present data, the structure of the metabolite was consistent with *N*-desmethyl topotecan {(S)-4-ethyl-4,9-dihydroxy-10-[(methylamino)methyl]-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14(4H,12H)-dione}.

Drug metabolites are usually more polar and less lipid-soluble than the parent molecule to enhance their excretion and reduce their volume of distribution. *N*-Desmethyl topotecan would be expected to be more polar than topotecan, although the metabolite showed greater retention on the reversed-phase HPLC systems used. This might be explained by the complex ion-pair mechanism with 1-hexane-sulfonic acid [16]. Oxidative biotransformation reactions, including *N*-dealkylation, are catalyzed by the mixed-function oxygenase system, which resides on the smooth endoplasmic reticulum [12, 14]. The reaction starts with a hydroxylation step catalyzed by the P-450 system. From this reactive intermediate the demethylated product is generated [14]. This mechanism probably also holds for the formation of *N*-desmethyl topotecan from the parent topotecan.

N-Desmethyl topotecan has slightly less antitumour activity as compared with topotecan (Johnson and Wood, Personal communication). The maximal concentrations of *N*-desmethyl topotecan detected in human plasma and urine were very low, and the clinical relevance of the metabolite may therefore be limited. When topotecan was given as a 30-min infusion at 1.0 mg/m^2, total metabolite levels (lactone and carboxylate forms) of 0.29 ng/ml were found in plasma, which were about 0.7% of the maximal concentration of topotecan. The average amount of metabolite excreted in the 24-h urine samples was $2.5 \pm 1.0\%$ ($n = 20$) of the delivered dose.

In conclusion, we isolated and purified a thus far unknown metabolite of topotecan from human urine. Mass spectrometry data along with HPLC retention data and fluorescence data (in comparison with synthetic reference standard) were consistent with the metabolite's being *N*-desmethyl topotecan.

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