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Determination of plasma topotecan and its metabolite *N*-desmethyl topotecan as both lactone and total form by reversed-phase liquid chromatography with fluorescence detection

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Abstract

Topotecan (TPT) undergoes hepatic *N*-demethylation forming *N*-desmethyl topotecan (NDS). To evaluate the effect of drug–drug interactions on NDS disposition in children receiving TPT we developed and validated a sensitive and specific HPLC–fluorescence detection method for lactone and total (lactone plus carboxylate) TPT and NDS. Deproteinized plasma is vortexed, centrifuged, and the methanolic extract diluted with water for the lactone form of NDS and TPT or diluted with 1.5% phosphoric acid for NDS and TPT total. A 100 μL sample is injected onto a Varian ChromGuard RP column attached to an Agilent SB-C_{18} reversed-phase analytical column held at 50 °C. The mobile phase (flow-rate, 0.8 mL/min) consists of methanol–aqueous buffer (27:73, v/v) (75 m*M* potassium phosphate and 0.2% triethylamine, pH 6.5). TPT and NDS were detected with excitation and emission wavelengths set at 376 and 530 nm, respectively. The standard curves for both forms of TPT ranged from 0.25 to 80 ng/mL, and for NDS ranged from 0.10 to 8.0 ng/mL. Within-day and between-day precision (% RSD) was ≤4% for TPT and ≤6.2% for NDS, respectively. Within-day and between-day percentage error ranged from 1.4 to 6.3% and from 1.4 to 2.4% for TPT, and from 1.6 to 3.1% and from 0.0 to 3.7% for NDS, respectively. No significant on-column conversion from TPT or NDS lactone to carboxylate was observed. With one method we can measure lactone and total TPT and NDS with adequate sensitivity to allow for evaluation of the disposition of these compounds in children receiving TPT.

Keywords: Fluorescence detection; Topotecan; *N*-Desmethyl topotecan

1. Introduction

Topotecan, a water-soluble analogue of camptothecin, is approved for second line therapy of patients with ovarian carcinoma and small-cell lung cancer [1–3]. In addition, we have used this agent widely to treat many forms of childhood cancer, including neuroblastoma, medulloblastoma, rhabodomyosarcoma, and leukemia [4–6]. Topotecan has been reported to undergo both renal and hepatic elimination. Early reports suggested that as much as 70% of a topotecan dose was excreted, unchanged in the

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urine; however, more recent reports indicate that approximately 49% of an intravenously administered dose and 20% of an oral dose are collected in the urine as parent drug [7]. The remainder of topotecan elimination is thought to occur through biliary or hepatic mechanisms. The primary hepatic metabolite of topotecan is *N*-desmethyl topotecan (NDS; Fig. 1). Although it is the primary hepatic metabolite, data from adults show that NDS urinary recovery accounts for less than 3% of the total topotecan dose. However, unpublished reports indicate that the antitumor activity of NDS is approximately equivalent to that of TPT.

We have previously reported the topotecan lactone pharmacokinetics in children with solid malignancies and leukemia [8–10]. In these studies we have shown that topotecan disposition is highly variable in this patient population as demonstrated by at least a 10-fold range in topotecan clearance. Part of this variability may be due to inter-patient differences in drug metabolism as well as the presence of drug–drug interactions. Thus, even though the NDS metabolite accounts for a relatively low percentage of the topotecan dosage, evidence indicates that it was clinically relevant to quantitate the effect that frequently used ancillary drugs (e.g., enzyme-inducing anticonvulsants, dexamethasone) might have on NDS disposition, especially given that NDS has pharmacologic activity of its own.

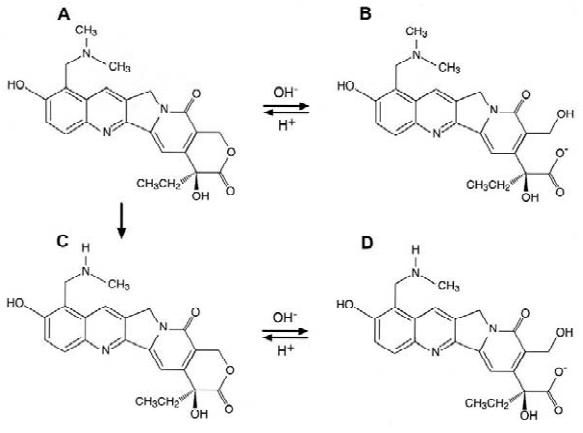

Fig. 1. Chemical structures for (A) topotecan lactone undergoing reversible pH-dependent hydrolysis to (B) the open-ring carboxylate form. Topotecan metabolite (C), *N*-desmethyl topotecan lactone undergoing reversible pH-dependent hydrolysis to (D) the carboxylate form.

Both topotecan and NDS exist with an α-hydroxy lactone moiety in the E-ring, which undergoes reversible pH-dependent hydrolysis to the carboxylate form [11–14]. A previously published HPLC–fluorescence method describes measurement of TPT and NDS as both lactone and total (lactone plus carboxylate) forms [15]; however, this method requires that two separate HPLC systems are used. Moreover, with this published method we were unable to measure NDS in our methanolic samples that we had obtained from patients due to a lack of sensitivity. Therefore, we developed a more sensitive assay to simultaneously measure both lactone and total TPT/NDS in one single system, which would allow us to utilize our previous extraction procedure (methanol extraction: plasma–methanol, 1:4, v/v). Here, we describe the development and validation of an HPLC method for both lactone and total topotecan and NDS in human plasma, which we have used to evaluate the effect of drug–drug interactions on NDS disposition in children receiving TPT.

2. Experimental

2.1. Chemicals

Topotecan (Hycamtin) and *N*-desmethyl topotecan used for preparation of standards and quality control samples were supplied by GlaxoSmithKline (Collegeville, PA, USA). All water was distilled, deionized, and further purified via a Millipore Ultra-Pure Water System (Tokyo, Japan). Potassium dihydrogen phosphate (99.7%) and hydrochloric acid (Reagent A.C.S.) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Triethylamine (minimum 99%) was obtained from Sigma (St Louis, MO, USA). Methanol (HPLC grade) was obtained from Burdick and Jackson (Muskegon, MI, USA).

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Shimadzu LC-10ADVP pump and a Jasco Fluorescence FP-920 detector (Tokyo, Japan). A Shimadzu Class-VP

chromatography data system was used for data acquisition and processing. An Hitachi F-2000 fluorescence spectrophotometer (Japan) was used for the selection of the detection wavelength.

Separations were obtained on a SB-C_{18} 3.5 μm, 3.0×150 mm column (Agilent, Palo Alto, USA) with a ChromGuard RP SS, 10×3 mm column (Varian, San Diego, CA, USA). A 100 μL sample was injected manually onto a 20 μL loop. Analytes were isocratically eluted with methanol–aqueous (27:73, v/v) mobile phase containing 75 m*M* potassium dihydrogen phosphate, and 0.2% triethylamine. The aqueous mobile phase was adjusted to pH 6.5 with potassium hydroxide and filtered with a 0.2 μm PALL membrane (Port Washington, NY, USA). The resultant mobile phase was degassed with helium for 10 min before use. The flow-rate was 0.8 mL/min during analysis. A Keystone Column Hot Pocket (Bellfonte, PA, USA) was used to maintain the column temperature at 50 °C. An Orion 250A pH meter (Beverly, MA, USA) was used for mobile phase preparation and a Labnet microcentrifuge (Germany) was used for sample preparation.

2.3. Sample preparation

2.3.1. Stock solutions

The topotecan and *N*-desmethyl topotecan stock solutions were prepared by dissolving each compound in ice-cold acidified methanol (containing 1.0 m*M* HCl) separately at a concentration of 1.0 mg/mL. Aliquots of the stock solutions were stored in screw cap tubes at −80 °C, and are stable (less than 5% loss) for at least 3 years for TPT and 1 year for NDS.

2.3.2. Calibration curve and quality controls

The calibrators and quality controls were prepared at the time of assay. To prepare calibrators, TPT and NDS were spiked in single drug-free donor plasma at the following concentrations: TPT/NDS 0.25/0.10, 0.50/0.50, 1.00/1.00, 4.00/2.00, 20.0/4.0, 80.0/8.0 ng/mL. To prepare quality controls, TPT and NDS were likewise spiked as follows: TPT/NDS 2.50/0.30, 75.0/5.0 ng/mL. A 200 μL volume of the resultant spiked plasma was then deproteinized in 800 μL dry-ice cold methanol. This was then vortexed for 10 s and centrifuged at 7000 *g* for 2 min. These methanolic supernatants were stored at −80 °C and are stable for at least 9 months. Prior to injection, they were diluted with either water (supernatant–water, 2:1, v/v) to measure lactone TPT/NDS, or 1.5% H_3PO_4 (supernatant–1.5% H_3PO_4, 2:1, v/v) to measure total TPT/NDS. A 100 μL volume was then injected onto the HPLC. The linear regression of the peak height of TPT and NDS was weighted by $1/x^2$, and the squared correlation coefficient was used to evaluate the linearity of the calibration curve.

2.3.3. Patient sample collection

Patient whole blood samples were collected in 5 mL green-top (Heparin sodium) Vacutainer tubes (Franklin Lakes, NJ, USA), decanted into eppendorf microcentrifuge tubes, and then centrifuged at 7000 *g* for 2 min to separate the plasma. Samples were then processed as previously described [8,16].

2.4. Assay validation

To determine the within-day and between-day precision and percentage error, TPT and NDS as lactone and total were analyzed over 5 days by measuring the quality control samples. Calibration standards were analyzed in duplicate.

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the ratio of signal/noise ≥ 3 and ≥ 5, respectively. These were determined by triplicate analyses of an extensive calibration curve in the low concentration range (e.g. 0.01–0.1 ng/mL).

2.5. In-vitro lactone stability studies

Since the mobile phase is at pH 6.5 we assessed the extent of conversion of TPT or NDS lactone to carboxylate that occurred during analysis on the HPLC column. Methanolic plasma samples containing TPT and NDS were acidified with 1.5% H_3PO_4 at a ratio of 2:1 (v/v) first, then were diluted with the mobile phase, pH 6.5, at a ratio of 1:6 (v/v), and then incubated at either 4 or 50 °C for up to 30 min (exact incubation times were 0, 5, 8, 10, 15, and 30 min) before injection onto the HPLC. The final concentrations of TPT and NDS were 60.0 and 6.0 ng/mL, respectively.

3. Results and discussion

We are able to measure topotecan and NDS as lactone and total using a single system. This method represents an improvement over currently published assays in that (a) it is able to measure the two forms of TPT/NDS in one system, which is more simple than the previously published assay, (b) it is more sensitive than previously published assays, and (c) we have shown that no significant conversion of TPT or NDS lactone to carboxylate occurs during analysis.

To obtain maximum NDS sensitivity and optimum resolution between TPT and NDS peaks, we evaluated the effect of the following parameters: wavelength, mobile phase pH, organic modifier percentage, analytical column, column temperature, and plasma sample treatment.

We have previously determined the optimal wavelength for topotecan (370 nm excitation and 530 nm emission) [8,9,17], thus we needed to determine the wavelengths that would give maximum signal for analysis of both topotecan and NDS. To determine the optimal detection wavelengths for NDS, the fluorescence spectrum of the reference NDS was scanned on an Hitachi fluorescence spectrophotometer. It was determined that at 376 nm (excitation) and 530 nm (emission), the peak height of NDS was at its maximum, compared to other wavelengths at the same concentration of NDS. Topotecan also maintained a similar response at those wavelengths.

The column temperature had a significant effect on the detection of NDS and also influences the separation of TPT and NDS, their retention times, and the column pressure. From our experiments, sufficient baseline resolution with a good peak shape, acceptable retention time, and column pressure was achieved at 50 °C. We observed less separation at 60 °C and no separation at 30 °C (Fig. 2), therefore 50 °C was chosen as the ideal temperature for the separation. A flow-rate of 0.80 mL/min was ideal for separation of NDS from TPT. Higher flow-rates accelerated the analysis, but in doing so also increased the column pressure; slower flow-rates resulted in slightly improved resolution but also in a longer run time.

To evaluate the effects of varying aspects of the analytical column, different internal dimensions (4.6, 3.0 and 2.1 mm I.D.) and lengths (50, 75, 100 and 150 mm) of the SB-C_{18} column (3.5 μm) at different column temperatures (30, 40, 50 and 60 °C) were compared for the best resolution and peak heights. Using the 3.0×150 mm column at 50 °C, the two compounds were completely separated with good shape and showed the highest peak height of NDS compared to the results from other combinations (Fig. 2).

When a sample was injected with a high per-

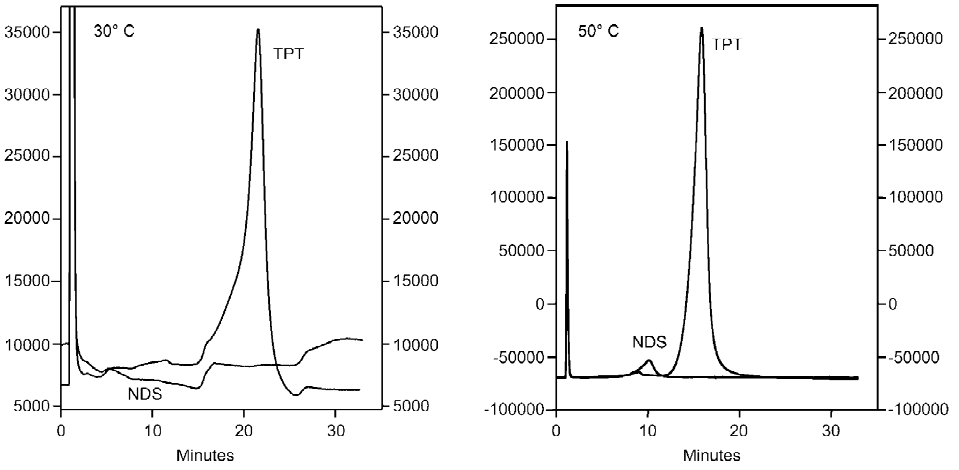

Fig. 2. Chromatograms for (A) TPT (100 ng/mL) and NDS (10 ng/mL) at 30 °C and (B) at 50 °C. HPLC conditions: Zorbax SB-C_{18} (75×4.6, 3.5 μm) with Varian Guard column (10×3 mm); 22% MeOH with 75 m*M* KH_2PO_3+0.2% TEM and flow-rate 1.0 mL/min.

centage (>80%) of methanol we were not able to maintain a flat chromatogram baseline. This effect had been seen in the previously published method by Rosing et al. [14,15] and in our preliminary experiments. Thus we diluted our methanol-extracted plasma sample with water at a ratio of 2:1 (supernatant–water, v/v), prior to HPLC analysis. We then observed a very clean and flat baseline and clear resolution of the two peaks, as can be seen in Fig. 3.

To design a mobile phase, we first considered the notion that a lower pH (e.g., pH 2–5) would promote conversion from the carboxylate form of TPT and NDS to lactone form, and a higher pH (e.g. pH 8–10) would reverse the lactone back to the carboxylate form and could damage the stationary phase of the column. Thus, we tested mobile phases at three different pH values: 6.0, 6.5, and 7.0. Based upon the best resolution and peak shapes without peak tailing (Fig. 4), we selected a pH of 6.5 for the mobile phase. The final mobile phase consisted of potassium phosphate, methanol, and triethylamine (pH 6.5). The pH of the eluent was adjusted to 6.5 not only to produce better resolution with good peak shape of both compounds, but also in an attempt to limit the on-column conversion of the lactone analyte to the respective carboxylate forms, or vice versa. Since the mobile phase was pH 6.5 we investigated

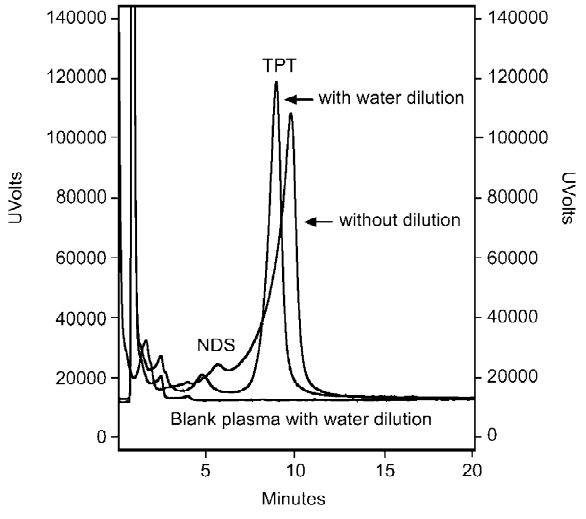

Fig. 3. Chromatograms of plasma TPT/NDS (50/5.0 ng/mL) with and without water dilution compared to blank plasma with water dilution. The same HPLC conditions as described in Fig. 2.

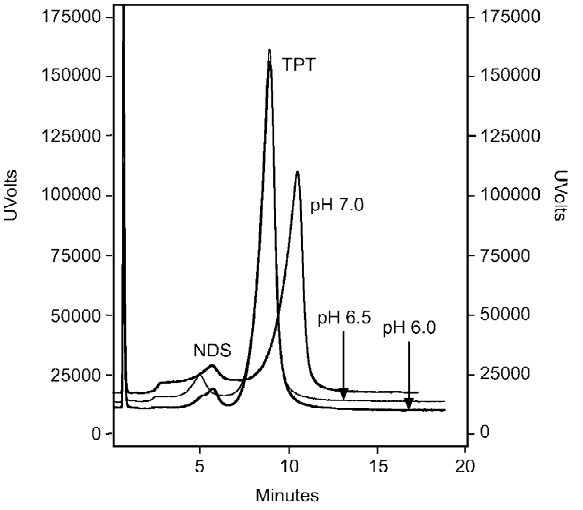

Fig. 4. Chromatogram overlays for plasma TPT/NDS (50/5.0 ng/mL) at pH 6.0, 6.5, and 7.0. Each sample was diluted with water at a ratio of 2:1. The same HPLC conditions at 50 °C in Fig. 2.

the extent of on-column conversion of lactone TPT or NDS to carboxylate at 50 °C. We determined that, under these conditions, total topotecan and NDS remain unchanged over 30 min at 50 °C, retaining 100±8 and 100±5%, respectively.

Next, we considered the amounts of the individual components of the mobile phase. The organic component of the mobile phase, methanol, has a strong effect on the peak resolution. We found that good results were seen when the mobile phase consisted of 20–30% methanol. Testing mobile phases with greater than 30% or less than 20% methanol was not done because the overall duration of the analysis would become too long or too short without adequate resolution. The best peak resolution and run time were obtained when the mobile phase consisted of 27% methanol with 0.2% triethylamine (Fig. 5).

The final chromatographic conditions selected for both TPT and NDS were as follows: analytical column—SB-C_{18} (3.5 μm) 3.0×150 mm with a Varian guard column C_{18}, 3.0×10 mm; mobile phase—methanol, 75 m*M* potassium dihydrogen phosphate containing 0.2% triethylamine (pH 6.5) in a 27:73 ratio, with a flow-rate of 0.8 mL/min; detection wavelength—emission 376 nm and excitation 530 nm; column temperature was kept at 50 °C. The quantitation limits for this assay (LOQ) ranged

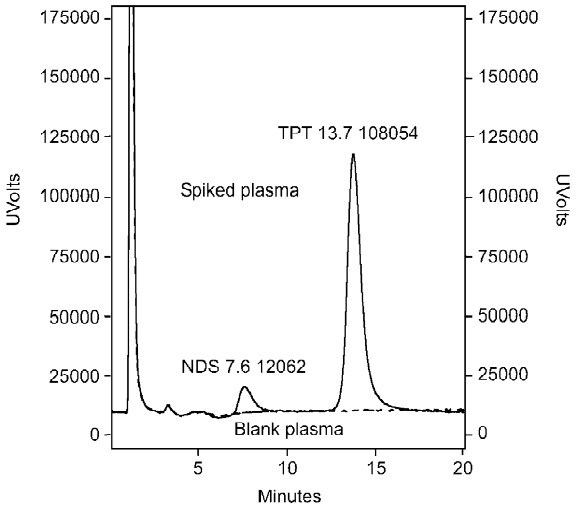

Fig. 5. Chromatogram overlays for plasma TPT/NDS (50/5.0 ng/mL) and blank plasma at optimized HPLC conditions: Zorbax SB-C_{18} (150×3.0, 3.5 μm) with Varian Guard column (10×3 mm); 27% MeOH with 75 m*M* KH_2PO_3 + 0.2% TEM at pH 6.5 and flow-rate 0.8 mL/min.

between 0.10 ng/mL for NDS and 0.25 ng/mL for TPT, while the detection limit (LOD) for NDS was 0.05 ng/mL ($S/N = 3.2$).

These conditions differ from those previously reported [12–15] and provide greater than two times more sensitivity for NDS (LOD 0.05 ng/mL) compared with the published assays (e.g., 0.125 ng/mL). By diluting the extracted plasma samples with water or 1.5% H_3PO_4 before injection, we could use a volume of methanol consistent with our previous sample preparation approach and yet still maintain adequate sensitivity for use in our pharmacokinetic studies (see results below).

3.1. Assay validation

To assess within-day and between-day variability in assay performance, precision, and accuracy, we evaluated validation parameters for lactone and total TPT and NDS (Table 1). Ten injections of low and high quality control samples were made on day 1 to assess within-day variability, and again on days 2 through 5 for between-day variability. The calibration curves for plasma sample were linear ($R^2 = 0.996$) up to 8.0 ng/mL for both forms of NDS and 80 ng/mL for both forms of TPT.

All results of percent recovery were in the 94.2–106.3% range, with the respective RSD (%) (within-day and between-day) between 1.02 and 6.23% for NDS lactone and total, as well as between 1.28 and 3.38% for TPT lactone and total (Table 1).

To demonstrate the applicability of the method, we analyzed plasma samples from a child with high-risk medulloblastoma who received topotecan as a 4-h infusion. Serial plasma samples were collected and processed as described above, and samples were analyzed by the method described in this report. Representative plasma concentration–time profiles

Table 1
Validation parameters of human plasma topotecan and NDS for lactone and total

Quality control sample	Within-day ($n = 10$)		Between-day ($n = 13$, 4 days)	
	RSD (%)	Error (%)	RSD (%)	Error (%)
Topotecan lactone (ng/mL)				
2.5	2.19	1.4	3.08	1.4
75	3.38	6.3	1.86	2.4
NDS lactone (ng/mL)				
0.3	4.35	3.1	5.14	3.7
5.0	2.12	1.6	1.02	0.0
Topotecan total (ng/mL)				
2.5	1.28	2.1	2.78	5.8
75	2.18	0.6	3.01	3.3
NDS total (ng/mL)				
0.3	5.04	4.1	6.23	5.8
5.0	1.87	0.9	3.29	1.6

for lactone and total forms of TPT and NDS are depicted in Fig. 6. With one method we can measure lactone and total TPT and NDS with adequate sensitivity to allow for evaluation of the disposition of these compounds in children receiving TPT.

3.2. Stability studies

The acidified extracted methanolic plasma samples of total TPT and NDS in mobile phase (pH 6.5) were incubated at 4 and 50 °C and immediately analyzed after each incubating time. The comparison results at each incubating time to the initial result did not show a significant difference between incubation at 4 °C (NDS, 98.8–104%; TPT, 102–107%) and at 50 °C (NDS, 99.3–102%; TPT, 93.1–108%). Both TPT and NDS were stable at 50 °C for at least 30 min. Our previous data and others also showed that TPT and NDS stock solutions (1.0 mg/mL in acidified methanol) were stable at −80 °C for at least 3 years and 1 year, respectively. Both compounds in methanolic extracted plasma sample were stable at −80 °C for at least 9 months.

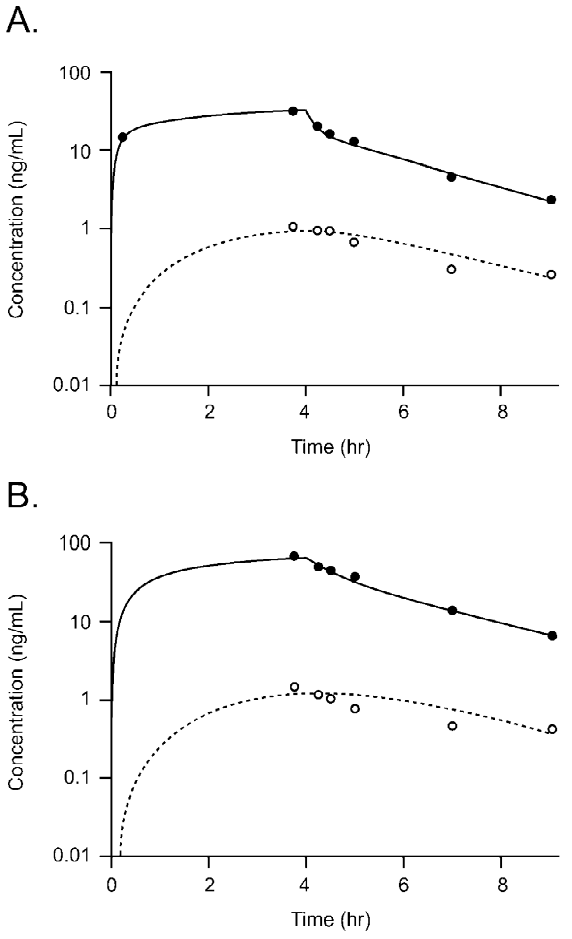

Fig. 6. Concentration–time plots from a patient after treatment with a 4-h intravenous infusion of 3.6 mg/m^2 topotecan depicting (A) lactone concentrations of topotecan (●) and NDS (○) and (B) total (lactone plus carboxylate) concentrations of topotecan (●) and NDS (○).

4. Conclusions

The goal of this study was to develop a simple and reliable method using the same analytical procedure for the separation and quantitation of TPT and NDS in both lactone and carboxylate forms. The proposed method, based on reversed-phase HPLC with fluorescence detection, is suitable for the sensitive and specific determination of the two forms of both TPT and NDS in human plasma. The plasma pretreatment, which is based on a simple deproteinization procedure, requires a very small amount of patient plasma (200 μL) and still has the advantage of satisfactory sensitivity and precision. As we showed with the example of the child with high-risk medulloblastoma this method will be very useful in determining the extent of hepatic metabolism of topotecan in children that are receiving ancillary drugs thought to alter topotecan disposition. Moreover, we have recently utilized this assay to measure topotecan and NDS plasma concentrations in very young children (<2 years old) to determine the disposition of topotecan and NDS in this age group. Thus, this assay will be an extremely useful tool as we gain a better understanding of the disposition of topotecan in children with cancer receiving topotecan.

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