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Application of a Highly Specific and Sensitive Fluorescent HPLC Method for Topotecan Lactone in Whole Blood

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Abstract

Individualization of topotecan dosing reduces inter-patient variability in topotecan exposure, presumably reducing toxicity and increasing efficacy. However, logistical limitations (e.g., requirement for plasma, intensive bedside plasma processing) have prevented widespread application of this approach to dosing topotecan. Thus, the objectives of the present study were to develop and validate an HPLC with fluorescence detection method to measure topotecan lactone in whole blood samples and to evaluate its application to individualizing topotecan dosing. Plasma samples (200 μ L) were prepared using methanolic precipitation, a filtration step, and then injection of 100 μ L of the methanolic extract onto a Novapak® C18, 4 μ m, 3.9 \times 150 mm column with an isocratic mobile phase. Analytes were detected with a Shimadzu Fluorescence RF-10AXL detector with an excitation and emission wavelength of 370 nm and 520 nm, respectively. This method has a lower limit of quantification of 1 ng/mL (S/N \geq 5; RSD 4.9%), was validated over a linear range of 1 to 100 ng/mL, and results from a 5-day validation study demonstrated good within-day and between-day precision and accuracy. Data are presented to demonstrate that the present method can be used with whole blood samples to individualize topotecan dosing in children with cancer.

Keywords

topotecan; HPLC; whole blood; pharmacokinetics

INTRODUCTION

Topotecan, a topoisomerase I inhibitor, has been widely evaluated in many pediatric cancers including neuroblastoma, medulloblastoma, rhabdomyosarcoma, and acute leukemias (Furman *et al.*, 2002, Houghton *et al.*, 1995, Houghton *et al.*, 1992, Pratt *et al.*, 1994, Santana *et al.*, 2003, Stewart *et al.*, 2004). Due to the inherent variability in topotecan disposition, investigators have used pharmacokinetically guided dosing to individualize topotecan for patients based upon their systemic clearance and the goals of the clinical trial (Metzger *et al.*, 2007; Montazeri *et al.*, 2002; Santana *et al.*, 2005; Stewart *et al.*, 2004).

As depicted in Figure 1, topotecan undergoes a reversible, pH-dependent hydrolysis to an inactive carboxylate form. This complicates the approach to pharmacokinetically guided topotecan dosing since measurement of the lactone form is preferable as it is the

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pharmacological active moiety (Staker *et al.*, 2002). Thus, in our institution topotecan dosing is individualized for each patient based upon that patient's topotecan lactone systemic clearance with the goal to achieve a pre-defined drug exposure or area under the concentration-time profile (Metzger *et al.*, 2007; Santana *et al.*, 2005; Stewart *et al.*, 2004).

The response rate for this approach has been promising, but individualized dosing of topotecan has limitations. Measurement of topotecan plasma lactone concentrations requires immediate bedside processing into dry-ice methanol in order to minimize interconversion between lactone and carboxylate forms of the drug (Bai *et al.*, 2003; Beijnen *et al.*, 1990). This process is logistically demanding and requires well-trained personnel. Thus, the availability of trained personnel and the presence of equipment needed for immediate sample processing may hinder implementation of individualized topotecan dosing at an institution. To increase the likelihood that this approach can be incorporated into routine clinical practice, it will be important to make all aspects of the current dosing approach accessible to the clinician.

By removing the need for intensive bedside sample processing and using a biological matrix like whole blood, individualized topotecan dosing could be made much more accessible to clinicians and patients. An HPLC technique for the determination of topotecan in whole blood has been previously published (Loos *et al.*, 2000a; Loos *et al.*, 2000b). However, this technique requires extensive sample preparation as well as a run time of 20 minutes. Hence, the objectives of the present study were to develop and validate an HPLC with fluorescence detection method for the measurement of topotecan lactone in whole blood samples and to evaluate its application to individualizing topotecan dosing in pediatric patients with cancer.

EXPERIMENTAL

Chemicals and reagents

Topotecan used for preparation of standards and quality control samples was supplied by GlaxoSmithKline (Collegeville, PA, USA). HPLC grade methanol was purchased from Burdick & Jackson (Muskegon, MI, USA), whereas dioctylsulfosuccinate sodium salt, 96% (DOSS), was obtained from ACROS Organics (New Jersey, NJ, USA). Monobasic sodium phosphate, dibasic sodium phosphate, hydrochloric acid, and 85% *o*-phosphoric acid were acquired from Fisher Scientific (Fairlawn, NJ, USA). Triethylamine (TEA), 99%, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Blank human whole blood was obtained from volunteers with informed consent. All water was distilled, deionized, and further purified via a Millipore Milli-QUV plus and Ultra-Pure Water System (resistance 18.2 M Ω) (Tokyo, Japan).

Equipment and chromatographic conditions

The HPLC system consisted of a Shimadzu LC-20AD pump, a Shimadzu SIL-20AC autoinjector which was cooled at 4°C and a Shimadzu Fluorescence RF-10AXL detector with an excitation and emission wavelength of 370 nm and 520 nm, respectively. All the modules were controlled by a Shimadzu CBM-20A communications bus module (Tokyo, Japan). A Shimadzu Class-VP chromatography data system was used for data acquisition and processing. Separations were obtained on a Novapak® C18, 4 μ m, 3.9 \times 150 mm column (Waters, Milford, MA, USA) with a μ Bondapak C18, 3.9 \times 20 mm, 10 μ m guard column (Waters, Milford, MA, USA) kept at ambient temperature. Analytes were isocratically eluted using a mobile phase delivered at 1 mL/min consisting of methanol-aqueous (58:42, v/v) mobile phase containing 36mM sodium phosphate buffer (pH 6.0), 8.75 mM DOSS, and 0.003% TEA, adjusted to pH 6.5 with phosphoric acid and filtered with a 0.2 μ m PALL membrane (Port Washington, NY, USA). An Orion 250A pH meter (Beverly, MA, USA)

was used for mobile phase preparation and a Labnet microcentrifuge (Edison, NJ, USA) was used for sample preparation.

Preparation of stock and working stock solutions

A 1 mg/mL stock solution was prepared by dissolving topotecan hydrochloride into 0.01 N HCl. Aliquots of the stock solution were stored in amber vials at -80°C . Appropriate dilutions of the stock solution were made in 0.01 N HCl to obtain working stock solutions at concentrations of 10, 1, and 0.1 $\mu\text{g}/\text{mL}$. When not in use, these working stock solutions were stored at -80°C .

Calibration curves

Whole blood calibration standards were prepared by adding topotecan working stock solutions into drug-free, lysed (frozen overnight at -20°C) whole blood to achieve the following concentrations: 1, 5, 20, 70, and 100 ng/mL. Quality control samples were prepared in the same manner to obtain final concentrations of 7, 30, and 80 ng/mL. Two separate calibration curves were obtained by using linear regression of peak height of the calibration standard samples weighted against the inverse square of the concentrations ($1/X^2$). To consider an analytical run acceptable, the correlation coefficients (r^2) of the calibration curves was at least 0.99, and quality control samples must be within 10% of their nominal values.

Sample preparation procedure

Calibrators and controls were deproteinized by adding 200 μL of spiked sample to 800 μL dry-ice cold methanol. After deproteinization, samples were vortexed for 10s, incubated on wet ice for 5 minutes, vortexed again for 10s, and centrifuged at 15000 g for 2 min (4°C). Methanolic supernatants were transferred to Spin-X HPLC microcentrifuge tubes (0.2 μm nylon filter, Costar, Corning, NY, USA) and re-centrifuged at 15000 g for 2 min (4°C) in order to separate out any particulates remaining in the sample. The filtrate was decanted to a screw-top vial and stored at -80°C . The supernatant was transferred into an HPLC sample vial and 100 μL aliquots were injected onto the HPLC system.

Specificity and selectivity

Drug-free whole blood obtained from three different healthy donors was processed and analyzed to determine whether endogenous whole blood constituents interfered with the topotecan lactone signal. In addition, drug-free whole blood was spiked with 50 ng/mL topotecan lactone in order to identify the retention time for topotecan.

Lower limit of quantification (LLOQ)

To determine the lower limit of quantification (LLOQ), three separate samples within the lower range of the calibration curve (1, 2.5, and 5 ng/mL) were prepared in whole blood, extracted, and analyzed. The LLOQ was defined as the lowest concentration of the analyte that could be reproducibly quantified with suitable precision within (\pm) 10% relative standard deviation (RSD), an accuracy \pm 10% deviation from the nominal value, and a signal to noise ratio ≥ 5 .

Precision and accuracy

Intra-day precision and accuracy was evaluated by analyzing ten low (7 ng/mL), ten medium (30 ng/mL), and ten high (80 ng/mL) replicates of each concentration within a single analytical run in one day. Inter-day precision and accuracy was evaluated by analyzing five low (7 ng/mL), five medium (30 ng/mL), and five high (80 ng/mL) replicates of each concentration for three consecutive days. All samples were prepared in bulk and processed

in the same manner as outlined in the sample preparation procedure section. The criteria for acceptability of the data included an accuracy $\pm 10\%$ of deviation from the nominal value and a precision within $\pm 10\%$ of RSD.

Stability experiments

The short-term stability of topotecan lactone in lysed whole blood was determined by analyzing quality control (QC) whole blood samples at two levels (7 ng/mL and 80 ng/mL) kept at room temperature ($\sim 25^\circ\text{C}$) for 0, 1, and 3 hours, and at 4°C for 0, 1, 3 and 6 hours. Long-term stability of topotecan lactone in whole blood was assessed by analyzing the QC samples stored at -80°C for 0, 1, 7, 15, and 28 days. Topotecan stability in lysed whole blood was also assessed after two freeze-thaw cycles. In addition, the stability of topotecan in processed samples was determined by analyzing the extracted samples kept at 4°C in the autoinjector for up to 8 hours. All samples were prepared in bulk and processed in the same manner as outlined in the sample preparation procedure section. Sample stability limitations were defined as the last time point where values were $\pm 10\%$ of the value obtained from the freshly prepared solutions.

Application of method

The pharmacokinetic analysis of topotecan lactone in plasma versus topotecan lactone in whole blood was carried out in 14 pediatric patients. Serial blood samples (2mL) were obtained from a site contralateral to the site of infusion after an intravenous dose of topotecan between 1.7 and 3.5 mg/m^2 . Each blood sample was collected in a green-top (heparin sodium) vacutainer tube (Franklin Lakes, NJ, USA). Next, 500 μL of whole blood was removed and immediately placed in a polypropylene screw-top tube, then stored on dry ice until transferred to a -80°C freezer. The remaining whole blood was centrifuged at 15000 g for 2 minutes (4°C) to obtain plasma. Plasma was then deproteinized by adding 200 μL to 800 μL of dry-ice cold methanol, vortexed for 10 s, and centrifuged at 15000 g for 2 minutes (4°C). The supernatant was decanted into a polypropylene screw-top tube and stored at -80°C until analysis. On the day of analysis, stored whole blood samples were extracted using the method outlined previously in the sample preparation procedure section. The extracts of both topotecan lactone in whole blood and topotecan lactone in plasma were analyzed against freshly prepared calibration curves in their respective matrices.

To utilize the whole blood topotecan lactone values in our pharmacokinetic analysis, we had to use population priors in a maximum a priori Bayesian (MAP-Bayesian) analysis that were derived from plasma topotecan lactone values (see Discussion). Thus, we determined the relationship between topotecan whole blood and plasma lactone concentrations using linear mixed-effects modeling (Team, 2006). This relationship was used to convert the measured topotecan whole blood concentrations to equivalent plasma topotecan lactone concentrations.

As topotecan lactone clearance is the primary parameter used to estimate topotecan systemic exposure (i.e., topotecan area under the concentration-time curve from 0 to infinity; $\text{AUC}_{0 \rightarrow \infty}$), a two-compartment model was fit to concentration-time data using a maximum a posteriori (MAP)-Bayesian approach as implemented in ADAPT II (D'Argenio *et al.*, 1997). The sample time points for the present group of patients were obtained from a previously developed limited sampling model described elsewhere (Turner *et al.*, 2006). The topotecan lactone clearance was determined from the plasma concentration data using accepted pharmacokinetic equations. The topotecan whole blood values were adjusted to plasma topotecan lactone concentrations using an equation determined from linear mixed-effects modeling (see Application of Method). The topotecan lactone clearance values determined from the plasma concentration data were compared with the topotecan lactone

clearance values determined from the whole blood adjusted to plasma topotecan lactone concentration data using linear mixed-effects modeling.

RESULTS AND DISCUSSION

Optimization of the experimental conditions

To optimize the detection and selectivity for this method, the fluorescence detector excitation and emission settings were set to $E_x=370$ nm and $E_m=520$ nm (Bai *et al.*, 2003). To stabilize the topotecan lactone, the stock and working solutions were made in 0.01 N HCl instead of methanol. An additional sample preparation step was added to further clean the sample prior to injection onto the HPLC system.

Whole blood samples were not effectively cleaned with a simple methanolic precipitation, so an additional sample preparation step was necessary. A small amount of precipitant from the whole blood remained in the supernatant, which clogged the guard column and increased the pressure on the HPLC system. Thus, different approaches were evaluated including further pretreatment with chemicals such as zinc sulfate. The best results were obtained by centrifuging the methanolic supernatant obtained after spiking whole blood with known amounts of working stock solutions in 0.2 μ m Spin-X HPLC microcentrifuge tubes at 15000 g for 2 min (4°C). Using this approach, a significant portion of unwanted precipitant was retained onto the nylon filter with minimal loss of sample volume to the membrane. This extra step sharpened up the topotecan lactone peak shape and minimized the pressure increase on the HPLC system.

Once the sample was processed and injected on the HPLC system, an endogenous peak that interfered with the topotecan peak was noted when using a mobile phase with a pH of 6.0. Thus, to allow for separation of the endogenous whole blood peak, the mobile phase pH was modified so that the final pH was 6.5 instead of 6.0. In addition, to achieve better topotecan lactone peak shape, a 4 μ m C18 analytical column was used.

Specificity and chromatography

The retention time of topotecan lactone was 3.9 minutes, with a total run time of 7 minutes. Upon analysis of blank human whole blood, no interfering endogenous peaks were found. Chromatograms of representative processed blank whole blood and spiked whole blood with topotecan lactone are depicted in Figure 2. Additional studies were performed to determine whether or not topotecan carboxylate was detectable using this analytical method. Three 0.04 N borax stabilized topotecan carboxylate solutions at low and high concentrations (3 ng/mL and 90 ng/mL) were injected and no carboxylate peak was observed upon analysis of the chromatogram (data not shown). In addition, no conversion from the carboxylate form to the lactone form of topotecan was noted (data not shown).

Calibration curves

Both calibration curves for topotecan lactone in human whole blood were linear over a concentration range of 1 ng/mL to 100 ng/mL with correlation coefficients (r^2) of 0.995 ($y=0.00134x + 0.0635$) and 0.997 ($y=0.00133x + 0.00161$). Three quality control samples were run with each calibration curve, and these values were within 94–110% of their nominal values.

Lower limit of quantification (LLOQ)

The LLOQ of topotecan lactone in human whole blood was established at 1 ng/mL, with a precision within 4.9% RSD and an accuracy within 93% of the nominal value. In addition, the signal to noise ratio was ≥ 5 . The LLOQ of 1 ng/ml is adequate to individualize

topotecan dosages based upon published data from studies (Metzger *et al.*, 2007; Santana *et al.*, 2005; Santana *et al.*, 2003; Stewart *et al.*, 2004).

Precision and accuracy

As shown in Table 1, inter- and intra-day precision and accuracy for topotecan lactone in human whole blood were within acceptable limits. The accuracy of the quality control samples (7 ng/ml, 30 ng/mL, and 80 ng/ml) was close to 100 % (within 93.7 % of the nominal value, Table 1). The inter- and intra-day precision expressed as the relative standard deviation (RSD) of the measurements was less than 10%.

Stability in human whole blood

The results of the stability studies obtained for topotecan lactone in lysed human whole blood are presented in Table 2. The topotecan lactone concentration in lysed whole blood after 1 hour was approximately 75% of the baseline value (data not shown), and after 3 hours it was approximately 49% of the baseline value, suggesting topotecan lactone is not stable at room temperature for any length of time. Thus, whole blood samples used for this assay method should be kept for no more than 3 hours at 4°C or less until processed for analysis. This is because those samples kept at 4°C for 3 hours retained their concentrations within 92% of the nominal value, but did not retain their concentrations after 6 hours (approximately 82% of nominal value, data not shown). Furthermore, the long-term stability of topotecan lactone under storage conditions of -80°C for 28 days was acceptable (87.9% of the value obtained from a freshly prepared solution), showing a gradual degradation over time (data not shown). After two freeze-thaw cycles, the results were within the defined acceptance criteria as shown in Table 2. Processed topotecan lactone samples were stable at 4°C in the autoinjector for 8 hours and at -80°C for 28 days (Table 3).

Application of the method

The proposed HPLC method was used to measure topotecan lactone in whole blood samples obtained from pediatric patients with neuroblastoma and retinoblastoma to compare with topotecan lactone in plasma samples measured by our previously published method (Stewart *et al.*, 1994; Zamboni *et al.*, 1998; Zamboni *et al.*, 1999). As depicted in Figure 3, we found a linear relationship between the topotecan lactone concentrations in both biological matrices. The regression equation that described the linear relation is shown in Figure 3, and on average the topotecan lactone whole blood concentrations were 17% greater compared to the plasma results. This result was expected as it has been previously reported that topotecan binds to red blood cells (Loos *et al.*, 2000). We assessed the relationship between topotecan lactone concentrations in plasma and in whole blood using a linear mixed-effects model.

Depicted in Figure 4 are the plasma and the converted whole blood concentration-vs-time profiles (using the relationship obtained for plasma and whole blood matched samples) for a representative patient. The actual observations are represented as solid circles while the line shows the simulated concentrations according to the pharmacokinetic parameter estimates obtained for that particular patient.

The median topotecan lactone clearance determined from the plasma concentration data was 133 ng*h/ml (range X to Y) compared with the topotecan lactone clearance value determined from the whole blood adjusted to plasma topotecan lactone concentration data which was 134 ng*h/ml. The mean %bias was 2.3%. Data from two different pharmacokinetic studies performed on the same patient were available for six patients. A good concordance was observed between the topotecan AUC values calculated from plasma and the corrected whole blood concentrations using the relationship found with linear mixed effects model. The %bias was 15% and 19% for two studies in one patient, but was less than

14% for the remainder of the studies. No statistically significant difference was found in the values between the two groups ($p > 0.05$, linear mixed effects model).

Although a direct comparison of the topotecan lactone clearance values calculated from whole blood and plasma would be desirable, that was not possible for this particular example. The initial topotecan pharmacokinetic studies conducted over 10 years ago utilized extensive sampling (e.g., 6 to 8 samples per study per patient), but that was burdensome on the patient, families, and the laboratory. Thus, we developed a pharmacokinetic limited sampling model (LSM) to conduct future dosage individualization studies. This LSM utilizes only 2 or 3 plasma samples per patient per study and MAP-Bayesian pharmacokinetic modeling methods to determine the topotecan lactone clearance for the individual patient. The MAP-Bayesian method depends upon the use of population priors (mean and variance) determined from the extensive plasma sampling studies. Now that LSM are exclusively used in topotecan pharmacokinetic studies, it was not possible to obtain extensive sampling specifically for the purposes of evaluating the whole blood method. Thus, the approach utilized was to convert the whole blood topotecan lactone value to an equivalent topotecan plasma value to use the plasma population priors in the MAP-Bayesian analysis.

Application of this method has numerous advantages over the previously published methods, which will enhance the accessibility of individualized topotecan to the clinician. First, this method uses whole blood and does not require any processing of the blood in the clinic, which simplifies the sample collection step for the clinician. The one caveat determined from this study is that the whole blood sample must be kept at 4°C or less until processed for analysis to maintain the stability of the topotecan lactone. Second, the sample does not require any complex bedside processing to isolate the topotecan lactone form. The processing of the sample that is required for bioanalysis occurs in the laboratory, and only involves one additional step compared with the previous method used. Lastly, results of the pharmacokinetic analysis showed that the whole blood topotecan lactone data could be used to estimate the topotecan lactone AUC values, which is the ultimate test of the clinical utility of this method.

CONCLUSIONS

An HPLC with fluorescence detection method that can be used routinely to measure topotecan lactone whole blood concentration in patient samples has been successfully developed and validated. In addition, the applicability of this method was demonstrated by showing that the topotecan whole blood concentrations correlated linearly with plasma concentrations and could be used for pharmacokinetically guided drug dosing. Thus, application of this method will enhance the ability to individualize topotecan dosing in children with cancer. Furthermore, the simple collection of whole blood will simplify sample collection and provide for uniformity of sample processing, enhancing the likelihood that individualized topotecan dosing can be more routinely performed.

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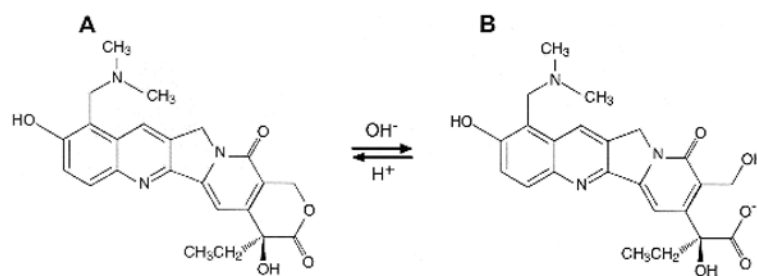


Figure 1.
Structures of topotecan lactone (a) and carboxylate (b).

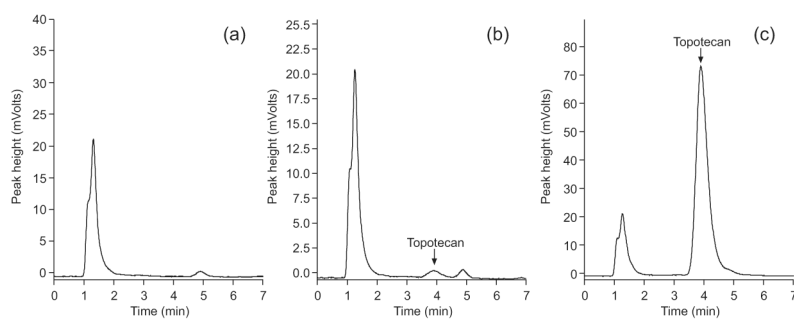


Figure 2. Chromatograms showing no interfering peaks in the blank whole blood sample (a), and topotecan elutes at a point separate from other peaks at low (b) and high (c) concentrations. (Note: the scales are different for each panel)

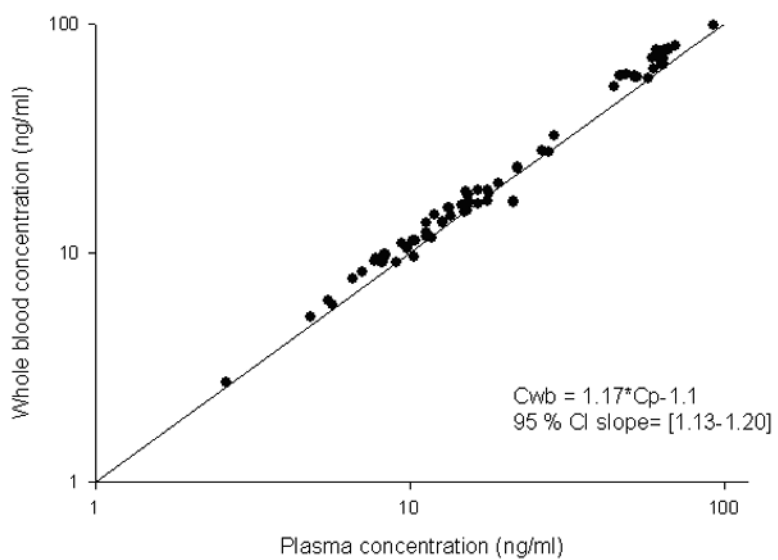


Figure 3. Relation between toptecan lactone measured in plasma and whole blood. Solid circles represent individual measurements and solid line represents the identity line.

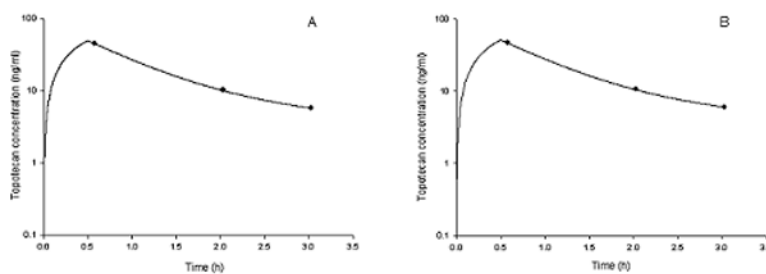


Figure 4.

Topotecan lactone concentrations vs. time profile for a representative patient (A). Topotecan lactone in whole blood concentration (ng/ml) vs. time profile obtained from plasma samples (B). Concentration (ng/ml) vs. time profile was obtained from whole blood samples after transforming the concentrations by the relationship between plasma and whole blood concentrations in the same patient. The line represents the best-fit line from the model fit to the patient concentration data.

Table 1

Inter- and Intra-day precision and accuracy of topotecan lactone in whole blood.

| Nominal concentration (ng/ml) | Mean (SD) Measured concentration (ng/ml) | Accuracy (%) | Within-day precision (%RSD) | Between-day precision (%RSD) |
|-------------------------------|--|--------------|-----------------------------|------------------------------|
| 7.0 | 7.2 (0.3) | 102.3 | 3.2 | 4.1 |
| 30.0 | 28.1 (2.1) | 93.7 | 2.9 | 7.6 |
| 80.0 | 75.8 (5.1) | 94.8 | 2.6 | 6.7 |

RSD. Relative standard deviation (SD*100/mean)

Table 2

Stability of topotecan lactone in lysed human whole blood.

| Nominal Concentration (ng/mL) | Storage condition | Measured Concentration. (ng/mL) ^a | Accuracy (%) ^b |
|-------------------------------|-------------------|--|---------------------------|
| 7.00 | 0 h (Baseline) | 6.5 | |
| | RT (3 h) | 3.18 | 49.3 |
| | 4°C (3 h) | 6.04 | 93.6 |
| | day 0 (Baseline) | 6.80 | |
| | -80°C (28 days) | 5.98 | 87.9 |
| | 2 F-T | 6.77 | 99.6 |
| 80.00 | 0 h (Baseline) | 76.2 | |
| | RT (3 h) | 40.0 | 52.5 |
| | 4°C (3 h) | 70.4 | 92.4 |
| | day 0 (Baseline) | 78.6 | |
| | -80°C (28 days) | 73.9 | 94.0 |
| | 2 F-T | 76.9 | 97.8 |

F-T= freeze thaw-cycle

RT=Room (ambient) temperature

^a Average (n=2)^b (Average measured concentration/average measured concentration at t=0) × 100

Table 3

Stability of processed topotecan lactone in human whole blood samples.

| Nominal Concentration (ng/ml) | Storage condition | Measured Concentration (ng/ml) ^a | Accuracy (%) ^b |
|-------------------------------|-------------------|---|---------------------------|
| 7.00 | 0 h (Baseline) | 6.94 | |
| | 4°C (8 h) | 7.06 | 101.7 |
| | day 0 (Baseline) | 6.82 | |
| | -80°C (28 days) | 6.12 | 89.7 |
| 80.00 | 0 h (Baseline) | 75.6 | |
| | 4°C (8h) | 83.7 | 110.7 |
| | day 0 (Baseline) | 78.6 | |
| | -80°C (28 days) | 74.7 | 95.0 |

^a Average (n=2)^b (Average measured concentration/average measured concentration at t=0) × 100