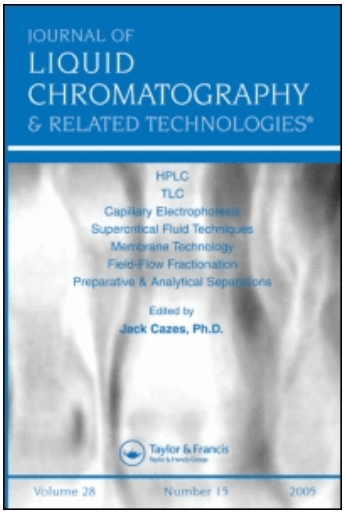

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Comparison of Filter and Tunable Fluorescence Detection for the HPLC Simultaneous Quantitation of Lactone and Carboxylate Forms of Topotecan in Plasma

Deanna L. Warner[a]; Thomas G. Burke[a]

[a] College of Pharmacy Division of Medicinal Chemistry and Pharmaceutics University of Kentucky, Lexington, Kentucky

To cite this Article Warner, Deanna L. and Burke, Thomas G.(1997) 'Comparison of Filter and Tunable Fluorescence Detection for the HPLC Simultaneous Quantitation of Lactone and Carboxylate Forms of Topotecan in Plasma', Journal of Liquid Chromatography & Related Technologies, 20: 10, 1523 — 1537

To link to this Article: DOI: 10.1080/10826079708010992

URL: http://dx.doi.org/10.1080/10826079708010992

J. LIQ. CHROM. & REL. TECHNOL., 20(10), 1523–1537 (1997)

COMPARISON OF FILTER AND TUNABLE FLUORESCENCE DETECTION FOR THE HPLC SIMULTANEOUS QUANTITATION OF LACTONE AND CARBOXYLATE FORMS OF TOPOTECAN IN PLASMA

Deanna L. Warner, Thomas G. Burke*

College of Pharmacy
Division of Medicinal Chemistry and Pharmaceutics
University of Kentucky
Lexington, Kentucky 40506-0286

ABSTRACT

The hydrolysis of the α-hydroxy-δ-lactone ring moiety in topotecan is routinely monitored using high performance liquid chromatography (HPLC) with fluorescence detection. While both tunable and filter fluorescence detectors are commercially available, only the tunable detector has been studied for clinical and in vitro applications of topotecan. In the present study we have developed a simple HPLC method for the simultaneous separation of the lactone and carboxylate forms of topotecan in plasma, which, can be utilized for both clinical and in vitro studies. Limits of detection, percent relative standard deviation, and linear range for both the lactone and carboxylate forms of the drug in plasma are presented and compared using a tunable and filter fluorescence detector. Limits of detection in plasma of 0.10 ng/mL for carboxylate and 0.26 ng/mL for lactone have been obtained using a tunable fluorescence detector. A filter

fluorescence detector produced limits of detection of 0.15 ng/mL for carboxylate and 0.30 ng/mL for lactone. Reproducible quantitation using a tunable fluorescence detector from 0.25 to 250 ng/mL for carboxylate and from 0.50 to 250 ng/mL for lactone was achieved, which is an improvement over existing methods. The filter detector, which has not been previously studied, provided reproducible detection from 0.50 to 250 ng/mL for carboxylate and from 0.75 to 250 ng/mL for lactone.

INTRODUCTION

Topotecan ((S)-9-dimethylaminomethyl-10-hydroxy-camptothecin, SK&F 104864, NSC609669) is the first DNA topoisomerase I inhibitor to gain approval by the Food and Drug Administration. Camptothecin analogues, such as topotecan, halt the growth of various human tumors[1-5] by stabilizing the DNA-topoisomerase I complex and preventing normal enzyme function.[6-9] Due to encouraging results in clinical trials, the general use of topotecan in the United States has recently been approved for patients with ovarian cancer.

As shown in Figure 1, topotecan (I) has an α-hydroxy-δ-lactone ring moiety which undergoes a pH dependent hydrolysis yielding the carboxylate form (II) of the drug.[10] Evidence has shown that an intact lactone ring is necessary for biological activity of camptothecin and camptothecin analogues.[9,11-13] Understanding the pharmacokinetic and pharmacodynamic parameters of lactone ring opening in topotecan requires methodologies which allow for reproducible quantitation of I and II at various concentrations in plasma. High performance liquid chromatography (HPLC) using fluorescence detection has been routinely used to monitor the lactone ring opening of topotecan in plasma for both clinical and *in vitro* studies. HPLC separations of topotecan have been described for both the lactone only,[14] and simultaneous separation of both I and II.[15,16,17] All of the separation schemes to date employ fluorescence detection using a tunable detector. Tunable fluorescence detection has been shown to provide reproducible quantitation from 0.50 to 20 ng/mL (15) and from 5.0 to 500 ng/mL[17] for both I and II. As yet, no quantitative studies have been published using a filter fluorescence detector for clinical applications of topotecan. To date, no one HPLC method has been studied over the wide concentration range capable of covering both clinical and *in vitro* studies.

Filter fluorescence detectors differ from tunable detectors in that filters of the appropriate wavelength range are employed as wavelength selectors instead of a monochromator. Because of the larger bandpass, the throughput of filters

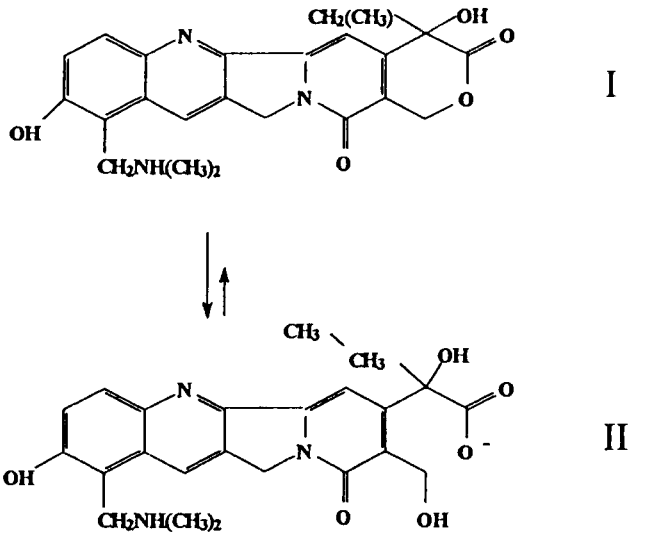

Figure 1. Chemical structure and hydrolysis of the lactone (I) and carboxylate (II) forms of topotecan.

are generally much greater than that of a monochromator. This allows for a very inexpensive lamp such as quartz halogen to be used in filter detectors, rather than, much more expensive lamps such as xenon, which are common in tunable detectors. For adequate results, lamps must be changed after 1000 hours of use in either detector, which correlates to twice per year for routine analysis. This represents a yearly maintenance cost of approximately $3000 for tunable detectors, as compared to $100 per year for filter detectors. Because of low maintenance costs, filter detectors have been utilized in analyses similar to that of topotecan in plasma, which have low background fluorescence matrices over a large wavelength range. In these instances, filter detectors can provide similar reproducibility and limits of detection as found with tunable detectors.

In the present study we have employed a simple HPLC methodology utilizing fluorescence detection for the simultaneous separation I and II in plasma. Limits of detection, percent deviations, and linear range, using both a tunable and filter fluorescence detector, are determined for I and II. Analyte concentrations are then back-calculated from peak using a log-log plot. A comparison of filter versus tunable detection in terms of the above-mentioned parameters is presented.

EXPERIMENTAL

Chemicals and HPLC Reagents

Samples of topotecan were graciously provided by the National Cancer Institute, Division of Cancer Treatment (Bethesda, MD). HPLC grade acetonitrile and triethylamine were purchased from Fisher Scientific (Fair Lawn, NJ). High purity water was provided by a Milli-Q UV PLUS purification system (Millipore, Bedford, MA). Unless noted, all other chemicals were reagent grade and used without further purification. Stock solutions of topotecan were prepared in A.C.S. spectrophotometric grade dimethylsulfoxide (Aldrich Chemical Company, Milwaukee, WI) at a concentration of 100 μM and stored in the dark at 4°C until use. Phosphate buffered saline (PBS) refers to an aqueous solution of 8 mM dibasic sodium phosphate (Na_2HPO_4), 1 mM potassium phosphate monobasic crystals (KH_2PO_4), 137 mM sodium chloride (NaCl) and 3 mM potassium chloride (KCl). Outdated plasma was obtained from Central Kentucky Blood Center (Lexington, KY) and stored at -20°C until use.

HPLC Apparatus

The isocratic HPLC system utilized consisted of a Waters 501 HPLC pump (Millipore Corp., Milford, MA), a Rheodyne injector (Rainin Instrument Co., Woburn, MA) fitted with a 200 μL sample loop and one of the following detectors. A Gilson Model 121 Fluorescence Detector (Gilson Medical Electronics, Middleton, WI) equipped with filters of 350 to 470 nm for excitation and 510 to 650 nm for emission was used as the filter detector. A Waters Model 470 Scanning Fluorescence Detector (Millipore Corp., Milford, MA) with 16 μL flow cell and excitation wavelength of 390 nm and emission of 520 nm (18 nm bandwidth) was used as the tunable detector. Separations were carried out at ambient temperature using a Waters NovaPak-C_{18} 4 μm particle-size reversed-phase 3.9 x 150 mm column preceded by a Waters Guard-Pak C_{18} Nova-Pak precolumn insert (Millipore Corp., Milford, MA). The mobile phase consisted of 88% triethylamine acetate buffer (3% triethylamine in water (v/v) adjusted to pH 5.5 with glacial acetic acid) and 12% acetonitrile. All mobile phases were filtered and vacuum degassed using sonication prior to use. In all instances, a flow rate of 1.0 mL/min was employed. Fluorescence output signal was monitored and integrated using Millennium 2010 Chromatography Manager (Millipore Corp., Milford, MA) on a 386 IBM computer.

Sample Preparation

To determine limit of detection, linear range and percent relative standard deviation of topotecan solutions in plasma, the following sample preparation procedure was used. A stock solution containing 100 μM of the drug of interest in DMSO was prepared and stored at -20°C. A 50 μL aliquot of this stock solution was added to 407.5 μL PBS pH 2.0 for lactone or PBS pH 11.0 for carboxylate in order to form 5000 ng/mL PBS solutions of I and II. These 5000 ng/mL solutions were allowed to stand at room temperature for 30 min to ensure equilibration. Serial dilutions with PBS of the appropriate pH were performed to make 2.5-2500 ng/mL solutions of I and II. To make 0.25-250 ng/mL solutions of I and II in plasma, 100 μL of the appropriate PBS solution was added to 900 μL plasma. This solution was then vortexed for 20 sec. A 200μL aliquot was added to 800 μL cold methanol (-40 °C), vortexed for 20 seconds and then centrifuged for 2 min. A 500 μL aliquot of supernate was then added to 500 μL PBS of the appropriate pH. A 200 μL aliquot of the resulting solution was then directly injected.

For percent recovery from plasma studies, 0.25 and 0.50 ng/mL solutions of I and II in PBS were diluted by adding 100 μL of the desired concentration to 900 μL PBS of the appropriate pH. A 200 μL aliquot of this solution was directly injected. This provided the same on column concentration as the 2.5 and 5.0 ng/mL plasma samples after extraction and dilution. Percent recovery was then determined by comparing drug concentrations in PBS with drug concentrations after precipitation from plasma.

Validation

Validation was performed for the detection of I and II in plasma. In all instances, three replicate samples of each concentration were used to determine limit of detection, linear range, between run deviation, and percent recovery for I and II in plasma. In addition, three injections of each sample was made to determine within run deviation. Limit of detection (LOD) was calculated according to Equation 1, with the standard deviation of the blank being determined by six injections of the sample blank.

$$\text{limit of detection} = \frac{3\ (\text{standard deviation of blank})}{\text{slope of calibration curve}} \qquad (1)$$

The linear range of each calibration curve was determined by including the lowest concentration which gave a correlation coefficient greater than 0.9950 for the linear regression equation and log-log regression equation. Between run and within run deviation was determined at those concentrations of I and II in plasma as shown in Equation 2.

$$\text{percent deviation} = 100^* \frac{\text{standard deviation of peak area}}{\text{average peak area}} \quad (2)$$

An upper limit of 15% was set as the maximum between and within run percent deviation which was tolerated. Only those concentrations which had between and within run percent deviations below 15% were considered reproducible. Recovery is calculated for I and II in plasma at 2.5 and 5.0 ng/mL as shown in Equation 3.

$$\text{percent recovery} = 100^* \frac{\text{average peak area in plasma}}{\text{average peak area in PBS}} \quad (3)$$

In addition to the above mentioned validation parameters, the peak areas corresponding to each theoretical concentration were used to back-calculate the measured concentration. This was performed by producing a log/log plot of the average peak areas versus concentration for each concentration used in the calibration curve. The regression equation from this plot was then used to back-calculate the measured concentration from each peak area. The average measured concentration and percent deviation from the theoretical concentration are presented for each concentration.

RESULTS AND DISCUSSION

Chromatography

The chromatographic parameters which were employed for this assay were chosen due to the dynamic nature of topotecan. The mobile phase consists of only two components, namely triethylamine acetate (TEA) buffer (3%) and acetonitrile. TEA buffer was used as the aqueous component of the mobile phase for multiple reasons. TEA is known to reduce peak tailing of amino containing compounds such as topotecan on C_{18} columns. Reducing peak tailing improves peak shape which generally lowers the limit of detection.

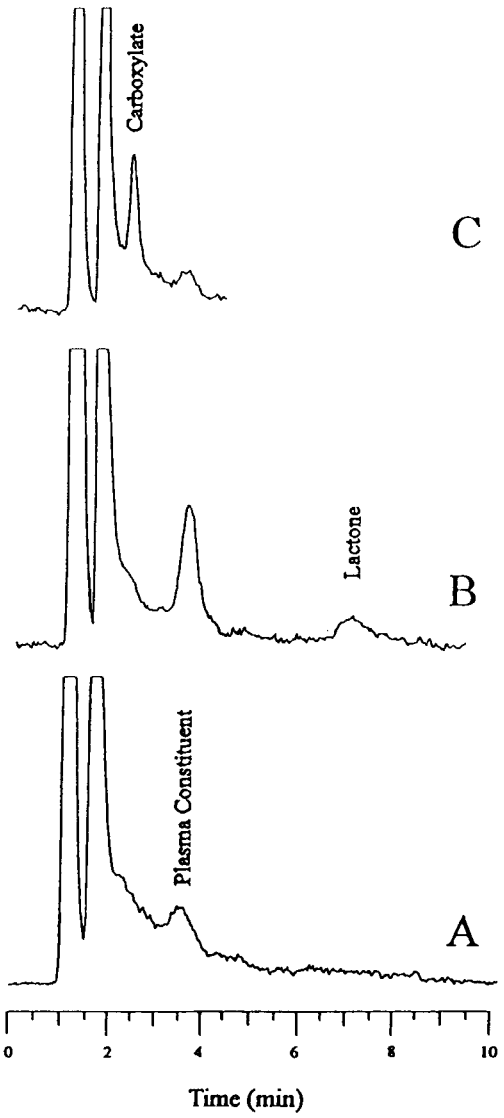

Figure 2. Chromatograms of plasma blank sample (A), plasma sample spiked with 2.5 ng/mL topotecan lactone (B) and plasma sample spiked with 2.5 ng/mL topotecan carboxylate (C) using a tunable fluorescence detector.

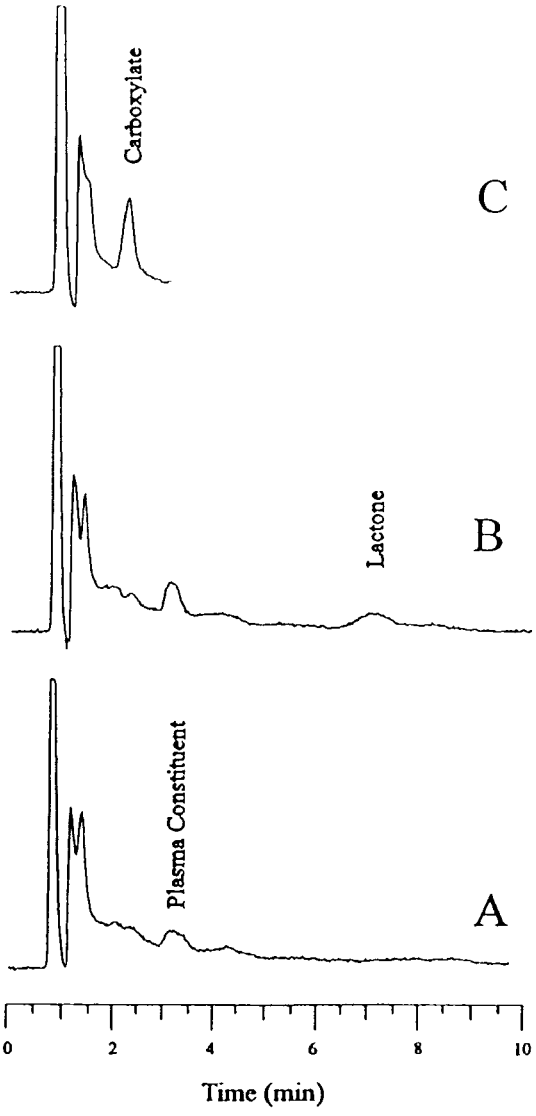

Figure 3. Chromatograms of plasma blank sample (A), plasma sample spiked with 2.5 ng/mL topotecan lactone (B) and plasma sample spiked with 2.5 ng/mL topotecan carboxylate (C) using a filter fluorescence detector.

TEA buffer also has the desired buffering capability at pH 5.5, which minimizes the hydrolysis of topotecan on column. Most importantly, TEA is an effective ion-pairing agent, which allows the carboxylate form of topotecan to be adequately retained on the C_{18} column.

Past HPLC methodologies, utilizing fluorescence detection for the simultaneous separation of I and II in plasma, have not been able to obtain adequate resolution of the carboxylate peak from the plasma or blood matrix peaks.[15,17] Figure 2 and 3 shows chromatograms of plasma and plasma samples spiked with 2.5 ng/mL of I and II using both the tunable and filter detectors, respectively. The plasma blank has only one major constituent with a retention time of 3.2 min and does not interfere with the quantitation of the topotecan samples. Topotecan carboxylate has a retention time of 2.3 min, whereas the lactone species has a retention time of 7.3 min. As is evident from Figures 2 and 3, the use of a filter detector rather than a tunable detector does not degrade the detection of the chromatographic separation.

Figures of Merit

This analytical method has been validated in terms of limit of detection, linear range, percent deviation, percent recovery and back-calculation of concentration, with all results being reported for both the tunable and filter detector.

The limit of detection was determined to be 0.26 ng/mL for I and 0.10 ng/mL for II in plasma using the tunable detector (Table 1). These LOD values are comparable to reported lower limit of quantitation (LLQ) values of 0.1 ng/mL and 0.2 ng/mL for topotecan using a tunable detector (15,17). Although the terms LOD and LLQ differ in that LOD is defined as three times the standard deviation of the baseline and LLQ is an empirical value, they tend to be somewhat comparable. The filter detector yielded limits of detection of 0.30 ng/mL for I and 0.15 ng/mL for II in plasma (Table 1).

The calibration curves of topotecan in plasma are linear over 2.5 decades for I and 3.0 decades for II using the tunable detector and over 2.25 decades for I and 2.5 decades for II using the filter detector (Table 1). In all cases, the correlation coefficients (r^2) are above 0.9990. Using this analytical method, both detectors surpass other published linear ranges, which are generally less than two decades.[15,17] Therefore, this method is recommended for both clinical and *in vitro* studies of topotecan in plasma.

Table 1

Figures of Merit for Topotecan in Plasma Using Tunable and Filter Fluorescence Detectors

Detector	Species	LOD[1] (ng/mL)	Linear Range (decades)	r^2
Tunable	I	0.26	2.5	0.9990
	II	0.10	3.0	1.0000
Filter	I	0.30	2.25	0.9997
	II	0.15	2.5	1.000

[1] Limit of detection (LOD) is calculated as three times the standard deviation of the blank sample divided by the slope of the calibration curve.

Percent relative standard deviations for I and II using the tunable and filter detectors are shown in Tables 2 and 3. Using a tunable detector, within run deviations were less than 7.9%, while between run deviations were under 8.8%. The lowest concentration which produced reproducible quantitation was 0.25 ng/mL for I and 0.50 ng/mL for II. This chromatographic method provides a two-fold improvement for lower limit of reproducible quantitation for II and identical results for I when compared to existing methods.[17] The within run deviations below 13.3% and between run deviations of less than 14.4% were found for the filter detector. The lowest concentration which produced reproducible quantitation using the filter detector was 0.75 ng/mL for I and 0.50 ng/mL for II.

Back-calculation of concentration from peak area for both detectors with percent deviation is shown in Tables 4 and 5. Due to the large linear range of the assay, a log-log plot was utilized for back-calculation. All back-calculated concentrations were determining by using the linear regression equation of the log-log plot of all concentrations studied.

This method is an improvement over existing methods of determining regression equations over each individual decade for concentrations within that decade.[14,15] Deviations from the theoretical concentration ranged from 0.4-11.2% for the tunable detector and from 2.4-17.8% for the filter detector.

Table 2

Within Run and Between Run Deviation of Topotecan Samples in Plasma Using a Tunable Fluorescence Detector

Concentration (ng/ML)	Percent Deviation Within Run[1]		Between Run[2]	
	I	II	I	II
0.25	----	6.7	----	8.8
0.50	4.5	----	0.4	----
2.5	4.1	3.8	2.1	3.7
5.0	7.9	2.8	1.2	4.2
25	2.4	5.3	3.7	2.2
250	2.6	2.6	5.0	2.8

[1] Within run deviation was determined by three injections of each sample.
[2] Betweeen run deviation was determined by injections of three samples.

Table 3

Within Run and Between Run Deviation of Topotecan Samples in Plasma Using a Filter Fluorescence Detector

Concentration (ng/ML)	Percent Deviation Within Run[1]		Between Run[2]	
	I	II	I	II
0.50	----	11.1	----	14.4
0.75	13.3	----	9.8	----
2.5	4.5	7.1	2.0	7.2
5.0	6.5	3.8	5.3	2.9
25	3.9	2.7	2.6	6.3
250	1.8	1.9	5.1	6.9

[1] Within run deviation was determined by three injections of each sample.
[2] Between run deviation was determined by injections of three samples.

Table 4

Back-Calculation of Concentration from Peak Areas for Topotecan Lactone and Carboxylate Using a Tunable Fluorescence Detector

Species	Theoretical Conc. (ng/mL)	Measured Conc. (ng/mL)	Percent Deviation[1]
I	0.500	0.507	1.4
	2.50	2.28	8.8
	5.00	5.13	2.6
	25.0	27.8	11.2
	250	238	4.8
II	0.250	0.267	6.8
	2.50	2.26	9.6
	5.00	5.02	0.4
	25.0	25.4	1.6
	250	256	2.4

[1] Deviation calculated as theoretical concentration minus measured concentration divided by theoretical concentration.

Recovery of I and II using protein precipitation with methanol from plasma has been determined from three independent determinations at two concentrations (2.5 and 5.0 ng/mL) of I and II. Percent recoveries range from 80.3-82.2% for I and 86.6-104.9 % for II and are in accordance with other published values.[14]

Comparison of Filter versus Tunable Fluorescence Detector

The limit of detection of topotecan in plasma is similar using the tunable and filter detectors. Although the LOD for both I and II are slightly better using the tunable detector, the values for both detectors are below concentration levels found in clinical studies following drug infusion (1-5 ng/mL). The linear range for the tunable detector is 0.75 decades larger for I and 0.5 decades larger for II, but both exceed previously published values.[15,17]

Reproducible detection was achieved over the range of 0.50-250 ng/mL for I and 0.25-250 ng/mL for II using the tunable detector. For the filter detector the range is slightly smaller, 0.75-250 ng/mL and 0.50-250 ng/mL, but

Table 5

Back-Calculation of Concentration from Peak Areas for Topotecan Lactone and Carboxylate Using a Filter Fluorescence Detector

Species	Theoretical Conc. (ng/mL)	Measured Conc. (ng/mL)	Percent Deviation[1]
I	0.750	0.669	10.8
	2.50	2.86	14.4
	5.00	4.74	5.2
	25.0	27.7	10.8
	250	232	7.2
II	0.500	0.434	13.2
	2.50	2.63	5.2
	5.00	5.89	17.8
	25.0	24.4	2.4
	250	235	6.0

[1] Deviation calculated as theoretical concentration minus measured concentration divided by theoretical concentration.

appropriate for the concentration levels found in clinical trials and *in vitro* studies. At the lowest concentration studied, within run and between run deviations are somewhat better for the tunable detector as compared to the filter detector. At concentrations above 2.5 ng/mL, deviations are similar for both detectors.

Back-calculation of concentrations from peak areas yielded deviations below 11.2 % for the tunable detector. The filter detector produced deviations ranging from 2.4-17.8%, which is still adequate for most applications. This back-calculation is performed using the log-log linear regression over all concentrations studied.

If a smaller concentration range is appropriate, as with clinical samples, a more accurate linear regression equation can be produced for concentrations within that range. Therefore, it is expected that the percent deviations using back-calculation will be smaller in that case.

CONCLUSIONS

An HPLC method has been established for the simultaneous separation of topotecan lactone and carboxylate species in plasma using both a filter and tunable fluorescence detector. Both detection schemes have produced reproducible quantitation of topotecan lactone and carboxylate at concentrations appropriate for clinical and *in vitro* studies, which is an improvement over existing methods. Limits of detection for topotecan lactone and carboxylate in plasma are similar for both the tunable and filter detector. From these studies, it seems that the more cost efficient filter detector is adequate for quantitating topotecan lactone and carboxylate in plasma.

ACKNOWLEDGMENTS

This work was supported in part by NIH CA-63653 and American Cancer Society DHP-138.

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Received October 24, 1996
Accepted November 15, 1996
Manuscript 4315