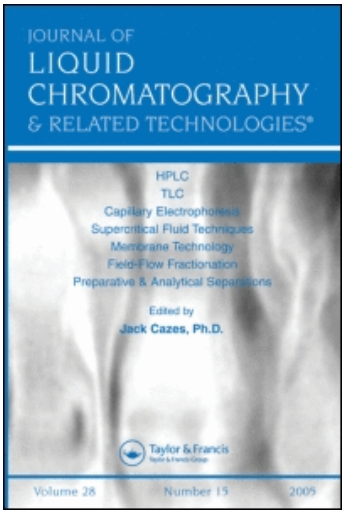

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Hsu, L. C.(1996) 'High Performance Reverse Phase Liquid Chromatographic Method for Topotecan Trace Analysis', Journal of Liquid Chromatography & Related Technologies, 19: 4, 611 — 624
To link to this Article: DOI: 10.1080/10826079608005524
URL: http://dx.doi.org/10.1080/10826079608005524

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 611–624 (1996)

HIGH PERFORMANCE REVERSE PHASE LIQUID CHROMATOGRAPHIC METHOD FOR TOPOTECAN TRACE ANALYSIS

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ABSTRACT

A highly sensitive and selective reversed-phase high performance liquid chromatography (RP-HPLC) with fluorescence detection for topotecan (SK&F 104864A) trace analysis is presented. Topotecan is the prototype of a novel class of antitumor agents, which exert their activities exclusively by inhibition of cellular topoisomerase I. The analytical method is based on an efficient HPLC separation using a Zorbax® StableBond® narrow bore (150 x 2.1 mm i.d.) C_8 column (particle size 5 mm). A secondary-retention phenomenon was noticed during the studies, however, and the problem was solved by adding 0.1% (v/v) triethylamine. Zorbax® StableBond® columns represent the new class of silane-modified silica, and have proved to be stable at least four months with continuously analyzed samples containing bleach and hydrogen peroxide. Column effluents were monitored via fluorescence with the excitation wavelength set at 225 nm and the emission wavelength at 520 nm. The limit of quantification (LOQ) for topotecan was 0.2 ng/mL. Linear response was observed for concentration of topotecan from 0.2 to 20 ng/mL. The method not only provided sensitive, precise and accurate support to photolysis, biodegradation and deactivation treatment system studies but also

reduced the organic solvent waste by two-thirds as compared to conventional HPLC columns (4.6 mm i.d.).

INTRODUCTION

Topotecan ((S)-9-Dimethylaminomethyl-10-hydroxy-camptothecin hydrochloride SK&F 104864A, Fig. 1), is a semisynthetic analogue of camptothecin. Topotecan demonstrated considerable cytotoxic and antitumour activity by exclusive inhibition of topoisomerase I.[1,2] Topoisomerases are intranuclear enzymes that transiently break and rejoin DNA strands to facilitate processes like replication, recombination and transcription that are essential for cell survival. Currently, one SmithKline Beecham facility has been modified to handle secondary production for topotecan clinical trials. Due to its potential health hazards, a topotecan deactivation treatment system was required to treat the waste generated from the packaging process. This system was set up prior to the sewer discharge system and consisted of eight UV lamps with an auxiliary hydrogen peroxide delivery system. Photolysis and biodegradation pathways have also been investigated. To support the large-scale waste treatment system, biodegradation and photolysis studies, an efficient HPLC method capable of analyzing more than three samples per hour was required.

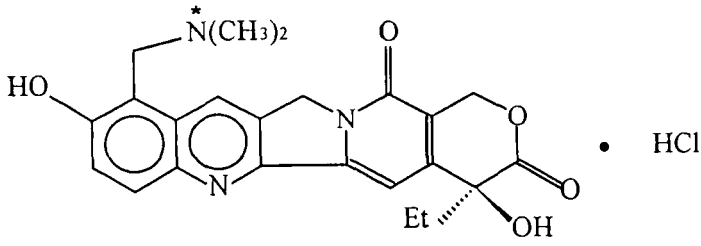

Figure 1. Topotecan (SK&F 104864A) structure. • is the possible adsorption site for silanol groups.

This paper describes an efficient, sensitive and specific HPLC method for the determination of topotecan. By using narrow bore columns (2.1 mm i.d.) with fluorescence detection, the limit of detection and the limit of quantification were found to be at 0.06 ng/mL and 0.2 ng/mL, respectively. This method is also mass spectrometry compatible and, as such, would be suitable for further planned LC-MS investigations.

EXPERIMENTAL

Instrumentation

All measurements were made with a Hewlett-Packard 1050 HPLC system with an HP 1046A fluorescence detector (Hewlett Packard, Palo Alto, CA, USA). The excitation wavelength was set at 250 nm (2 x 2 mm slit) and emission wavelength was set at 520 nm (4 x 4 mm slit). The PMT gain was set according to the sample concentration range and the Xenon lamp flash frequency was set at 200 Hz and the response time was set at 1 sec. A Nelson 900 Series interface and PE Access*Chrom VAX chromatographic software (Perkin-Elmer Nelson System, Cupertino, CA, USA) were used for data collection and analysis. The sampling rate was 1 pt/s and the samples were injected at least three times. Chromatographic separations were carried out on a 150 x 2.1 mm i.d. Zorbax C_8 column (MacMod, Analytical Inc., Chadds Ford, PA, USA) at room temperature. The injection volume was 10 µL. Triethylamine (J. T. Baker, Phillipsburg, NJ, USA) 0.1 % (v/v), adjusted to pH 3 with trifluoroacetic acid (J. T. Baker, Phillipsburg, NJ, USA), was used as mobile phase A and acetonitrile (J. T. Baker, Phillipsburg, NJ, USA) as mobile phase B. The gradient profile was 8% B isocratic for 1 minute; to 35% B in 6 minutes; back to 8% B in 1 minute; and re-equilibrate for 10 mins. Total run time was 18 minutes. The optimal excitation and emission wavelengths were determined by trapping the sample in the flow cell, and the best illumination wavelength was found by scanning the wavelength characteristic of the flow cell contents.

Reagents and Solutions

Mobile phase A was prepared by adding 10 mL triethylamine (TEA) to 970 mL HPLC water followed by pH adjustment to 2.9-3.1 through addition of about 15 mL trifluoroacetic acid (TFA). The final volume was brought to 1000 mL with HPLC water. The solution was mixed and filtered through a separate 0.4-0.5 mm polycarbonate, PVDF, or equivalent membrane filter. A 100 µg/mL topotecan stock solution (SmithKline Beecham Pharmaceutical, King of Prussia, USA) was prepared by accurately weighing about 10 mg of topotecan into a 100 mL volumetric flask. Ten mL's mobile phase A was added to totally dissolve the compound, and then solution was diluted to volume with mobile phase A. The standard solution was stored at 4°C and protected from light.

Standard dilutions of the stock solution were prepared, as needed, using volumetric glassware and mobile phase A.

RESULTS AND DISCUSSION

Narrow Bore Columns and Column Characteristics

Reducing organic solvent waste and the associated disposal cost has become a priority for many HPLC users, especially when the organic waste is potentially hazardous as in the present study. The feasibility and desirability of using capillary zone electrolysis (CZE) in the biodegradation studies of a pharmaceutical compound has been previously demonstrated.[3] With no organic solvent consumption, CZE is an environmentally friendly method for the assay analysis. However, CZE lacks the sensitivity needed for the trace analysis, although laser-induced fluorescence detection has been used with CZE.[4-6] Unfortunately, these lasers are available in quantity only for visible and near-IR wavelengths. Thus, an alternative method was needed for topotecan trace analysis.

Columns with internal diameters of less than 4.6 mm, (i.e., 3.0 mm or 2.1 mm) will dramatically reduce solvent consumption and solvent waste, because a smaller flow rate is used (0.2-0.4 mL/min). Simpson and Brown[7] have compared the performance characteristics, such as column efficiency, selectivity, resolution and detection limit obtained in the narrow bore mode with those obtained by conventional HPLC. There are two additional benefits to use columns with smaller internal diameters.

First, there is an increase in sensitivity. Samples are eluted in smaller peak volumes on 2.1 mm i.d. columns. Therefore, the concentration of the analyte is greater and sensitivity is increased (assuming same injection volumes). Second, narrow bore columns are compatible with most HPLC instruments with little or no system modification; they can also interface directly to other detectors. The lower flow rates (0.2-0.4 mL/min) of small bore columns make it possible to interface directly with many current LC/MS systems. As suggested by Snyder et al.[8] gradient elution was also used to improve peak shape should 10 μL injection cause any noticeable losses in resolution on a 2.1 mm i.d. column. The combination of gradient elution and the narrow bore column provided enough separation power to positively identify topotecan from other possible interference compounds (Fig. 2).

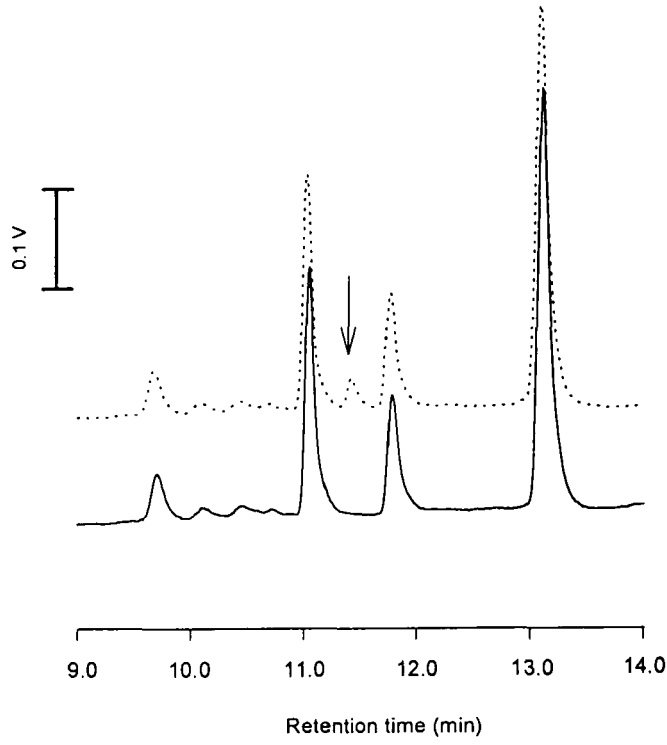

Figure 2. Chromatograms of treated waste samples. Solid line is the treated waste sample chromatogram; dotted line is the same sample spiked with a 2 ng/mL topotecan standard (arrow). HPLC condition: Injection volume: 10 mL; Column temperature: Ambient; Instruments: Hewlett-Packard 1050 with 1046A fluorescence detector; Mobile phase A: 0.1 % TEA and TFA pH 3 solution. Mobile phase B: acetonitrile. Gradient profile: 8% B isocratic for 1 minute; to 35% B in 6 minutes; and back to 8% B in 1 minute; re-equilibrate for 10 mins.; Detection: fluorescence (ex =225 nm, em=520 nm PMT gain =16).

Zorbax StableBond C_8 columns used in this study represent the new type of siliane bond stationary phases which have been prepared with monomeric "sterically-protected" bonded silanes containing diisopropyl- and diisobutyl-silane side groups and a variety of interactive ligands (C_8, C_{18}, etc.)[9,10] It is the steric bulk of the side group (from dimethyl to diisopropyl) which greatly

Table 1

System Suitability Report During Four Months Studies

Parameter	k'[a]	R[b] (x10^6)	N[c]	A_s[d]
Month 0	4.44	1.96	69295	1.41
Month 1	4.44	2.01	68957	1.40
Month 2	4.57	2.04	70120	1.35
Month 3	4.49	2.02	66453	1.45
Month 4	4.43	1.94	65413	1.46
Average	4.47	1.99	68048	1.41
%R.S.D.[e]	1.32	2.11	2.95	3.12

[a] Capacity Factor; [b] Response Ratio;
[c] Theoretical Plate Number, calculated usign Foley-Dorsey approximation.[14] [d] Asymmetry Factor = ratio of the distances from the perpendicular to the rear side and the front side of the peak, along the 10% horizontal line. [e] % RSD = (Standard deviation (σ) divided by the average) x 100.

increases the stability of these monomeric silane coatings against degradation during use and maintains high column efficiency and reproducibility.[11,12] The columns' characteristics have been reviewed elsewhere.[9-13] The Zorbax StableBond columns appeared to be very stable during the course of our experiments (Fig. 3). Table 1 is the system suitability report during the four months of studies. Although the peak is broader than desired, which may be due to 10 μL injections used to increase the sensitivity, the assymmetry factor is still acceptable (<1.6). It is very important, once the desired separation method has been obtained, that column characteristics should remain unchanged for as long as possible so that any need for further adjustment of separation conditions (or replacement of the column) is minimized.

Secondary-Retention

A secondary-retention phenomenon was observed during the topotecan method development (Fig. 4A). Secondary - retention in the reverse phase

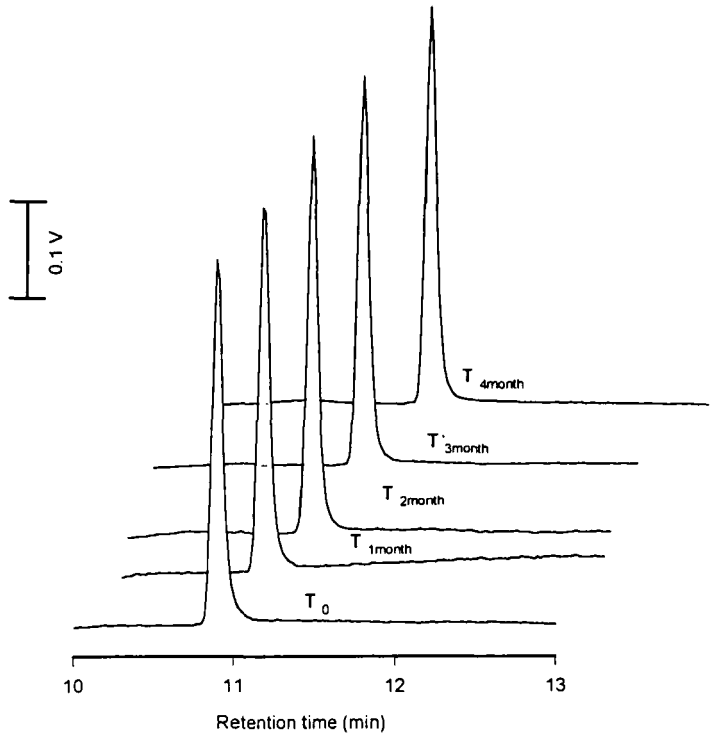

Figure 3. Chromatogram of 20 ng/mL topotecan standard during four months treatibility studies. HPLC conditions are the same as Fig. 2.

separations has been variously attributed to silanol groups of different kinds and to the presence of trace metal impurities in the column packing.[15,16] It is believed that this strong retention in reverse phase systems is due to two kinds of silanol interactions: hydrogen bonding or ion exchange. The benzyldimethylamine functional group (* in Fig. 1) with a pKa of 10.5 [17] is likely the main contributor to the secondary-retention with silanol groups. When a secondary effect such as that of Fig. 4A is present, the most effective solution generally is the addition of some mobile phase modifier that will preferentially interact with (and block) these secondary-retention sites (silanol groups in the present example). Amine-additives such as triethylamine (TEA) are commonly used for this purpose.[18] Fig. 4B shows the effect of adding 0.1 %

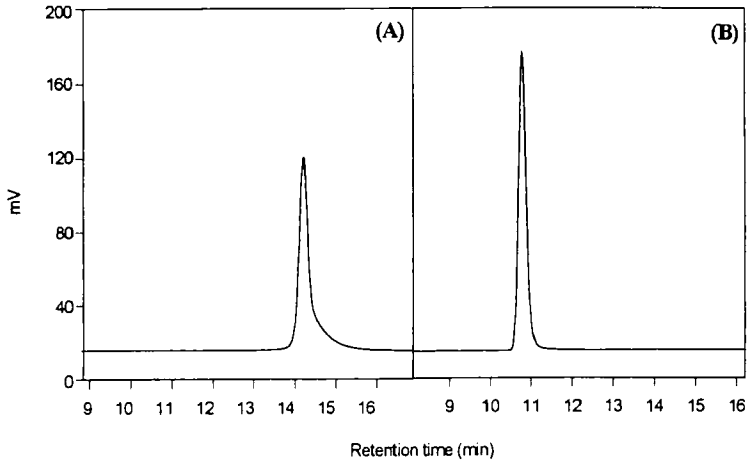

Figure 4. Secondary retention. Topotecan 10 mg/mL in (A) phosphate pH 3 buffer (B) 0.1 %(v/v) TEA/TFA. HPLC conditions are the same as Fig. 2, except fluorescence PMT gain=11. See text for details.

TEA (v/v) to the mobile phase. The result is a pronounced sharpening of the bands for topotecan, with the elimination of any band-tailing. Presumably, the amine-modifier (TEA) effectively eliminates the silanol groups as sorption sites for sample molecules.

Even with the special efforts using the bulky sterically protecting groups in the bonded phase, which have been shown to increase column efficiency and better peak symmetry for basic samples,[13] Zorbax StableBond columns still suffered from the secondary-retention effect. Secondary-retention not only affects the peak shape, but the retention time as well (Fig. 4).

Two interactions are involved in the retention process: from the stationary phase and from the silanol groups. The strong retention caused by secondary-retention will also affect the accuracy of the computer-assisted HPLC method development package such as DryLab® (LC Resources Inc., Walnut Creek, CA) where a single retention is assumed. Currently, the secondary-retention effects on the reverse phase LC retention predictions are under study in our laboratory.

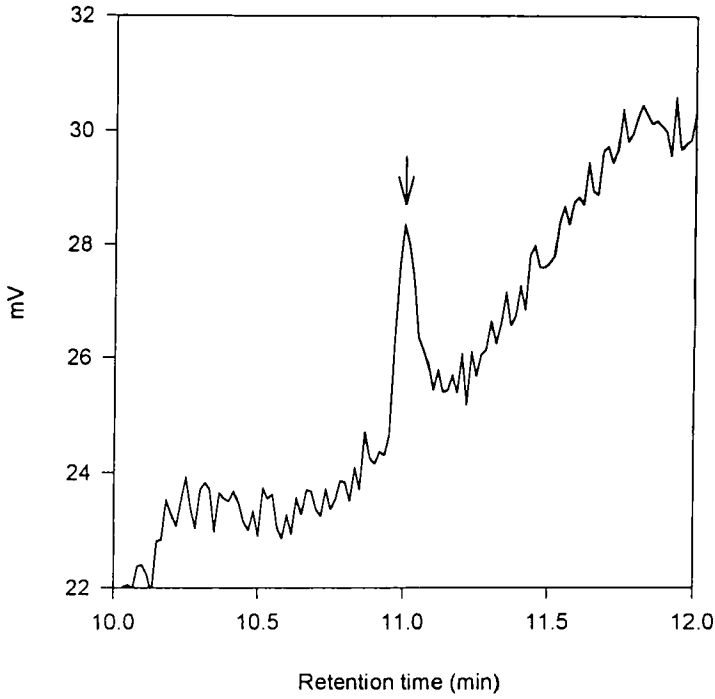

Figure 5. Chromatogram of 0.2 ng/mL topotecan standard. HPLC conditions are the same as Fig. 2.

Sensitivity, Accuracy, Precision and Linearity

The limit of detection (LOD) was determined using Equation 1, derived by Foley and Dorsey:[19]

$$LOD = 3s_B/S \quad (1)$$

where s_B and S are the standard deviation of the noise and the analytical sensitivity (or calibration factor), respectively. The analytical sensitivity is defined as the slope of the calibration curve (signal output per unit concentration). Based on these calculations, the LOD for topotecan is 0.06 ng/mL. The limit of quantification (LOQ) is defined as the amount of analyte

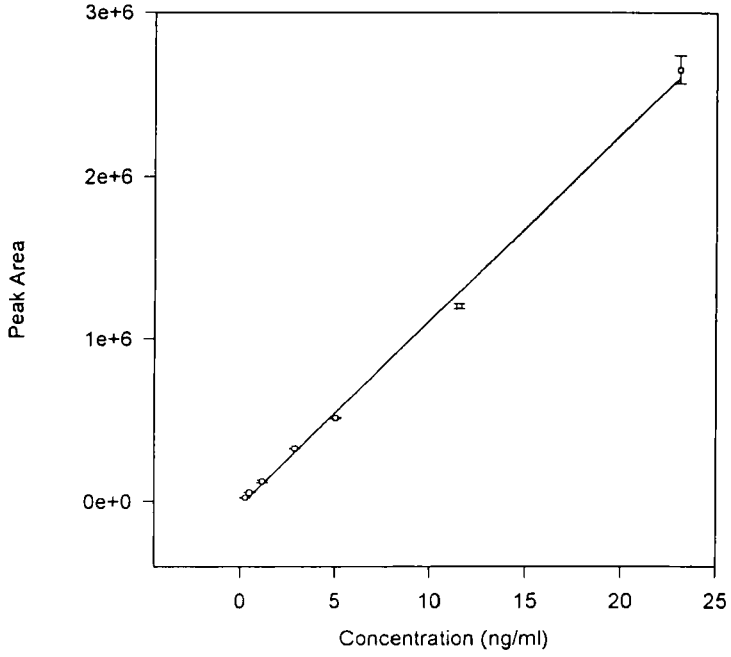

Figure 6. Representative topotecan calibration curve from 0.2 to 20 ng/mL; The error bar is the 95% confidence interval.

detected at ten times the standard deviation of the noise[20] and was calculated using Equation 2:

$$LOQ = 10s_B/S \qquad (2)$$

Using this calculation, the LOQ for topotecan is 0.2 ng/mL. Figure 5 is the chromatogram of 0.2 ng/mL topotecan standard; the signal to noise ratio (S/N) was estimated at 6. An extensive method validation was performed. The within-day precision of the assay was less than 11% for all concentrations within the standard curve range (Table 2). By using 2.1 mm i.d. HPLC columns, the lowest concentration of topotecan that could be determined quantitatively in 1 mL of waste samples was 0.2 ng (475 fmol). The calibration curve obtained was linear over the range of 0.2-20 ng/mL of topotecan. Linear regression analysis of calibration curves provided the equation $y = 1.019 \times 10^{5} x - 5 \times 10^{-3}$ and a correlation coefficient greater than 0.999 (Fig. 6). The

Table 2

Accuracy and Precision Data for Topotecan in Four-Day

Parameter	Concentration (ng/mL)							
	0.2	**0.5**	**1**	**2**	**5**	**8**	**10**	**20**
R.S.D. (%)								
Day1	9.30	4.72	1.89	1.99	0.72	0.70	0.61	1.17
Day2	13.93	2.34	3.77	0.92	0.65	0.57	1.13	1.42
Day3	12.83	4.03	4.58	2.71	2.06	0.64	1.20	1.88
Day4	5.46	5.97	6.04	2.82	1.51	0.84	1.17	0.63
Error (%)[a]								
Day1	10.60	4.52	-5.16	-2.65	-2.20	-5.19	-2.21	-0.29
Day2	10.40	11.48	-2.17	-1.60	-4.07	-4.24	-3.76	0.37
Day3	3.70	0.76	-3.92	-4.22	-3.47	-3.82	-2.13	0.65
Day4	-3.80	3.72	-3.92	-6.72	5.03	-4.36	-2.92	1.97
Day-to-day R.S.D.[b]	13.24	5.78	4.48	2.99	1.73	0.87	1.39	1.58
Within-day R.S.D.[c]	10.38	4.26	4.07	2.11	1.23	0.69	1.03	1.28
Mean Accuracy (%)	92.90	94.9	96.2	96.2	96.3	95.6	97.2	99.0

[a] (Calculated concentration-actual concentration)/actual concentration x 100
[b] Coefficients of variation of daily means
[c] Mean of the daily R.S.D.'s

calibration curves were highly reproducible and the accuracy, estimated by the average concentration back calculated from the composite standard calibration curve, was within 10% of the original value at each concentration. The precision, as measured by the R.S.D.s at each concentration, was within 7% across the calibration range (except for 0.2 ng/mL).

Table 2 summarizes the results obtained from a four-day validation study in which five replicate standards at eight concentrations, 0.2, 0.5, 1, 2, 5, 8, 10 and 20 ng/mL, were analyzed each day. The mean accuracy of the assay at these concentrations ranged from 92.9 to 99%, whereas the within-day precision, indicated by the mean of the daily R.S.D.'s, varied from 0.69 to 10.38%. The reproducibility of the assay was high with day-to-day precision, indicated by the R.S.D.'s of the daily means, ranging from 0.87 to 13.24%.

CONCLUSIONS

A simple and sensitive HPLC method is presented for the trace analysis of topotecan. The assay can detect 0.2 ng/mL and is compatible with many LC/MS interfaces. The increase in the use of narrow bore columns is largely due to several inherent advantages relative to conventional HPLC, such as smaller sample requirements, reduced mobile-phase consumption, and lower costs. With little or no hardware modification, narrow bore columns are the preferable columns for HPLC trace analysis. Although, larger injection volumes (>20 μL) can be used to increase the sensitivity on 4.6 mm id. columns, this usually results in peak broadening, thus, poor reproducibility and resolution. During the four months topotecan treatment analysis, most of the waste samples contained hydrogen peroxide and bleach; Zorbax® StableBond® columns were still very stable as indicated in Table 1. Further work with the LC/MS, for possible topotecan degradants characterization, is currently underway in our laboratory.

ACKNOWLEDGMENTS

The author will like to thank Drs. D. Orvos, J. Brum and Mr. R. Hannah for their help in preparing this manuscript and the support from the management of the Corporate Environmental Research Laboratory.

REFERENCES

1. M. E. Wall, M. C. Wani, C. E. Cook, K. H. Palmer, A. T. McPhail, G. A. Sim., J. Am. Chem. Soc., **88**, 3888 (1966).

2. M. H. Beijnen, Pharm Weekbl. Sci., **14**, 258 (1992).

3. L. C. Hsu, D. R. Orvos, D. J. C. Constable, R. E. Hannah, J. Chromatogr., B, **699**, 85 (1995).

4. H. Drossman, J. A. Luckey, A. J. Kostichka, J. D'Cunha, L. M. Smith, Anal. Chem., **62**, 900 (1990).

5. V. Dombek, Z. Stransky, Anal. Chim. Acta, **256**, 69 (1992).

6. T. Higashijima, T. Fuchigami, T. Imasaka, N. Ishibashi, Anal. Chem., **64**, 711 (1992).

7. R. C. Simpson, P. R. Brown, J. Chromatogr., **385**, 41 (1987).

8. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, 2nd edition, Wiley-Interscience, New York, 1979, pp. 51-56.

9. J. J. Kirkland, J. L. Glajch, R. D. Farlee, Anal. Chem., **61**, 2 (1988).

10. J. L. Glajch, J. J. Kirkland, U.S. Patent 4,705,725 (1987).

11. J. J. Kirkland, C. H. Dilks, Jr., J. E. Henderson, LC-GC, **11**, 249 (1993).

12. B. E. Boyes, J. J. Kirkland, Peptide Res., **6**, 249 (1993).

13. J. J. Kirkland, J. W. Henderson, J. Chrom. Sci., **32**, 473 (1994).

14. J. P. Foley, J. G. Dorsey, Anal. Chem., **55**, 730 (1983).

15. M. Verzele, LC, Liq. Chromatogr. HPLC Mag., **1**, 217.

16. Vespalec, J. J. Neca, J. Chromatogr., **281**, 35 (1983).

17. J. Fassberg, V. J. Stella, J. Pharm. Sci., **81**, 676 (1992).

18. J. S Kiel, S. L Morgan, R. K. Abramson, J. Chromatogr., **320**, 313 (1985).

19. J. P. Foley, J. G. Dorsey, Chromatographia, **18**, 503 (1984).

20. "Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry," Anal.Chem., **52**, 2242 (1980).

Received August 9, 1995
Accepted September 10, 1995
Manuscript 3948