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Simple and sensitive high performance liquid chromatographic method for the simultaneous quantitation of the lactone and carboxylate forms of topotecan in human plasma

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Abstract

A selective and highly sensitive isocratic high performance liquid chromatographic (HPLC) method is described for simultaneous determination of lactone and carboxylate species of topotecan, in plasma. The method utilizes a protein precipitation step with cold methanol (−20 °C) for sample preparation followed by separation on a Novapack C_{18} column using ammonium acetate buffer, acetonitrile and triethylamine (84:16:1.5, v/v) containing tetrabutyl ammonium hydrogen sulfate (TBAHS) (2 mM) with a pH of 5 as the mobile phase. The eluted peaks were detected by a fluorescence detector was set at an excitation wavelength of 380 nm and an emission wavelength of 527 nm. The method was validated in the range of lactone and carboxylate forms of topotecan concentrations from 0.05 to 75 ng/ml. Intra- and inter-day precision expressed by the relative standard deviation was less than 8.50% and inaccuracy did not exceed 10% for lactone and carboxylate forms of topotecan. The limit of quantitation was 0.05 ng/ml using 0.50 ml plasma. Stability studies in plasma and plasma extract indicated that topotecan is stable for at least 2 weeks at −70 °C.

Keywords: Topotecan; HPLC; Plasma; Lactone; Carboxylate

1. Introduction

Topotecan [SK&F 104864, (S)-9-dimethylaminomethyl-10-hydroxy camptothecin, NSC 609669; Fig. 1] is a semi-synthetic water-soluble analogue of the alkaloid camptothecin, which has been shown to be a potent inhibitor of topoisomerase I, an enzyme essential for replication of DNA [1]. Topoisomerase I is present in both proliferating and non-proliferating cells and therefore, inhibition of this enzyme provides a unique therapeutic approach to the treatment of slow growing tumor types [2]. This drug has shown encouraging anti cancer activity against a variety of human tumor types, including colorectal cancer, ovarian cancer, non-small cell lung cancer and non-lymphocytic haematological malignancies [3–9]. Compared with camptothecin, topotecan has reduced protein binding, and shows promising efficacy with a strongly reduced toxicity profile [3,10–12].

Topotecan differs from its parent compound because it incorporates a stable basic side chain at the nine-position of the A-ring of 10-hydroxycamptothecin, which allows the drug to be formulated as a hydrochloric acid derivative. The lactone ring is quite labile thus topotecan undergoes a

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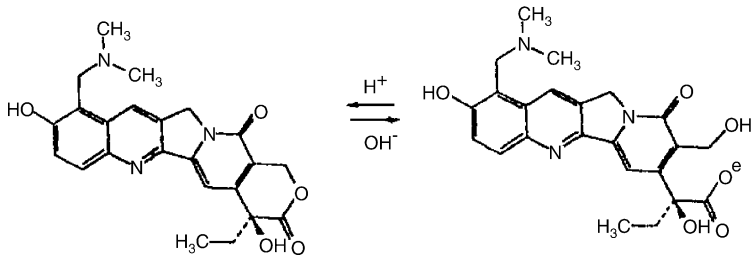

Fig. 1. Chemical structure of the lactone and carboxylate forms of topotecan.

pH-dependent reversible hydrolysis of its lactone moiety, yielding a carboxylate form (SK&F 105992; Fig. 1) [10,13]. In vitro and in vivo pharmacological studies have shown that the antitumor activity of the lactone form is higher than that of the carboxylate form [14]. Therefore, development of analytical methods that could measure the lactone and carboxylate forms separately was important to further analyse the pharmacokinetics and pharmacodynamics of this compound.

Several HPLC methods using fluorescence detection have been developed for the determination of topotecan as lactone form and as the total of the lactone plus carboxylate forms in human plasma [13,15,16], but a few methods exist for the simultaneous separation of the carboxylate and lactone species of this compound [10,17,18]. Beijnen et al. [10] developed an HPLC assay with a limit of quantitation of 1 ng/ml for the analysis of closed-ring lactone form of topotecan and the open-ring form in human plasma. The sensitivity of this method was insufficient for performing precise pharmacokinetic studies [13]. Loos et al. [17] developed a new method because the plasma concentrations in their clinical study, where topotecan was administered orally for prolonged periods of time, were anticipated to be much lower than the lower limit of quantitation of the previously developed method. In spite of improved sensitivity (lower limit of quantitation of 0.10 ng/ml for both compounds) their method fails to distinctly separate the peak of carboxylate form from the plasma endogenous peaks. The HPLC assay reported by Warner and Burke [18] employed a mobile phase included only triethylamineacetate buffer (TEAA) and acetonitrile, which compared to previously reported rather complex mobile phases was a simplified system. However, their assay suffers from low sensitivity (LOQ: 5 ng/ml) and broad appearance of lactone peak.

In this study, we document a simple and reliable HPLC method for the simultaneous separation of lactone and carboxylate forms of topotecan in plasma. The distinct high sensitivity (limit of quantitation of 0.05 ng/ml) along with lack of any interfering peaks at the same retention times of both lactone and carboxylate forms of drug and use of an available and inexpensive internal standard, tetracycline, to increase assay reproducibility are the main advantageous of present method over the previously reported ones.

2. Experimental

2.1. Chemicals and reagents

Topotecan was purchased from Ohua Pharmaceutical Technology Company (China). Tetracycline HCl (Internal standard) was kindly provided by Razak pharmaceutical company (Tehran, Iran). HPLC grade acetonitrile, methanol and triethylamine (TEA) and spectrophotometric grade dimethylsulfoxide (DMSO), were purchased from Merck (Darmstadt, Germany). Tetrabutyl ammonium hydrogen sulfate (TBAHS) was a product of Sigma (St. loise, MO, USA). All other chemicals were of analytical grade from BDH (Poole, UK). Phosphate buffered saline (PBS) refers to an aqueous solution of sodium chloride (8.00 g/l), potassium chloride (0.20 g/l), disodium hydrogen phosphate (1.15 g/l) and potassium dihydrogen phosphate (0.20 g/l). Drug-free human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Tehran, Iran)

2.2. Instruments

The liquid chromatographic system consisted of a model 510 solvent delivery pump, an auto injector system (Rheodyne) equipped with a 100 μl loop and a model 474 fluorescence detector connected to a model 746 data integrator (all from Waters Assoc., MA). Chromatographic separation was achieved at ambient temperature using a Novapack C_{18} column (4 μm, 250 mm × 4.60 mm) and a C_{18} precolumn (10 μm, 20 mm × 3.90 mm) (Waters Assoc., MA).

2.3. Chromatographic conditions

The mobile phase consisted of 0.05 M ammonium acetate, acetonitrile and TEA (84:16:1.50, v/v) containing TBAHS (2 mM) adjusted to pH 5 with hydrochloric acid. All separations were carried out at room temperature and a flow-rate of 1 ml/min, detector sensitivity of 0.10 absorbance units and a chart speed of 0.25 cm/min. Eluted peaks were detected fluorimetrically with an excitation wavelength of 380 nm and an emission wavelength of 527 nm.

2.4. Preparation of stock and working solutions

Stock solution of 1 mg/ml of topotecan was prepared in DMSO and stored at −20 °C. The working solutions of 0.50 mg/ml of topotecan in lactone and carboxylate forms

were prepared by diluting 50 μl of stock solution by 50 μl of PBS, pH 3; or PBS, pH 10, respectively. These solutions were serially diluted with PBS of appropriate pH to prepare the solutions with known concentration of each compound.

2.5. Plasma sample preparation

To 500 μl of plasma samples were added, methanolic solution of tetracycline HCl (10 μl; 1 mg/ml) as internal standard (I.S.) and 500 μl of cold methanol (−20 °C) in a micro tube. The mixture was vortex-mixed for 30 s. After centrifugation at 5500 × *g* for 10 min the supernatant was separated and a sample of 100 μl was injected into the liquid chromatograph immediately.

2.6. Standard preparation

Known amounts of both working solutions of lactone and carboxylate form of topotecan in PBS (pH 3.00 or 10) were added to pooled drug free plasma to achieve standard solutions containing 0.05–75 ng/ml of each topotecan carboxylate and lactone forms. These solutions were used to constructing standard curves.

2.7. Validation procedures

Validation of the method was performed according to ICH guidelines [19].

2.7.1. Linearity, accuracy, precision and specificity

The linearity of the analytical procedure for each compound was evaluated by processing a seven points calibration curve, analyzed on three occasions. Calibration curves were made by linear regression analysis of detector response (peak area) versus the nominal concentration of topotecan present in the plasma sample. The linearity was tested according to Bressolle et al. [20] and by employing Student's *t*-test. The reproducibility of the analytical procedure was evaluated by determining the intra- and inter-day relative standard deviations (R.S.D.s). The intra-day precision and accuracy of the assay was assessed from the results of five replicate analyses of spiked quality samples (SQCs) prepared at three concentrations using human plasma (one near lower limit of quantitation, one in the middle range and one approaching the high end of the calibration range). The inter-day precision and accuracy of the assay was determined from the results of the same SQCs as for the intra-day variability analyzed on 5 consecutive days.

In order to study the specificity of the present method, six independent blank human plasma were subjected to the same sample processing and analyzed.

2.7.2. Limit of detection and limit of quantitation

Limit of detection (LOD) was determined at the lowest concentration to be detected, taking into consideration of a signal-to-baseline noise ratio of 3.

Limit of quantitation (LOQ) was defined as the lowest concentration at which the precision expressed by relative standard deviation (R.S.D.) is lower than 20% and accuracy expressed by relative difference of the measured and true value (RE) is also lower than 20%. The LOQ was repeated five times for confirmation.

2.7.3. Absolute recovery

The amount of topotecan recovered from the spiked plasma samples were determined by comparing to the response of the external standard dissolved in the PBS for each analyte concentration.

2.7.4. Stability

The stability of lactone and carboxylate forms of topotecan was tested in plasma and plasma extracts at different temperatures. In plasma and plasma extract, the stability of lactone and carboxylate forms of topotecan was tested by incubating plasma and plasma extract containing topotecan for 24 h at room temperature (22 °C), 4 and −20 °C, respectively. Long-term stability at −70 °C was tested during a period of 2 weeks. Three replicates at three levels of topotecan in plasma and plasma extract were analyzed at every time point.

In order to study the freeze and thaw stability one set of SQCs at three levels was prepared and aliquots of each of the SQCs were processed and analyzed. Another aliquots were processed and analyzed after one and two freeze–thaw cycles (at −20 and 22 °C).

The stability of topotecan in DMSO stored at −20 °C was determined over a 6-month period.

3. Results and discussion

3.1. Chromatographic condition and sample preparation procedure

Topotecan is a basic compound with a highly polar character in its protonated form; therefore, method development for the analysis of topotecan using HPLC can be time-consuming and complicated. Normally, the following three or four parameters must be optimized for a successful separation: type and concentration of ion-pairing reagent; type, concentration and pH of buffer; amount of organic modifier and concentration of agents capable of masking silanols [18].

To get acceptable chromatographic results, we found that reversed-phase HPLC with a C_{18} column was most suitable.

Our mobile phase, composed of ammonium acetate buffer, acetonitrile, TEA and TBAHS enabled the simultaneous separation of carboxylate and lactone species of topotecan and I.S. in less than 10 min (Fig. 2). Retention times were 4.77, 6.07 and 7.59 min for carboxylate and lactone form of topotecan and I.S., respectively.

Ammonium acetate buffer was used to control the pH of the mobile phase. The percentage of acetonitrile in the mobile phase was selected to minimize the analytical time while

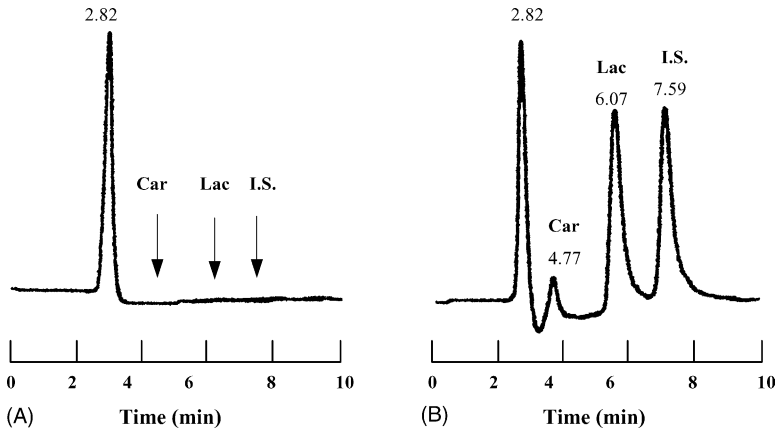

Fig. 2. Chromatograms obtained from blank plasma (A), a plasma spiked with 5 ng/ml of lactone (Lac) form of topotecan, 0.05 ng/ml of carboxylate (Car) form of topotecan and 10 μg/ml tetracycline HCl as I.S. (B).

maintaining baseline resolution of lactone—carboxylate forms of topotecan and lactone form of topotecan—I.S. peak pairs.

In the previously published methods for simultaneous determination of lactone and carboxylate forms of topotecan [10,17,18], ion-pairing agents were used to change the chromatographic behavior of the carboxylate form of topotecan, so that it exhibits sufficient retention in the C_{18} column to allow for separation of the carboxylate species from endogenous substances. In this study, we evaluated the effect of TBAHS on chromatograms. Mobile phases composed of ammonium acetate buffer, acetonitrile and different concentrations of TBAHS (0–10 mM) at pH ranges of 4–6.5 were evaluated. In converse to previous studies, TBAHS had no impact on the retention of the carboxylate form of topotecan, but had a large impact on peak sharpness and decreasing the retention of the lactone form of topotecan, leading to achieve good resolution of lactone—IS peaks and short chromatography time.

Topotecan contains a dimethylaminomethyl group that can potentially interact with residual silanols [18]. Therefore, to further minimize peak tailing, TEA was incorporated in the mobile phase and the effect of a combination of TBAHS and the amine modifier TEA in the mobile phase at pH levels between 4 and 6.50 was also investigated. For peak tail decreasing the most suitable percentage of TEA was 1.50% (v/v) and the further increase in TEA content did not have a marked influence on peak shape of the lactone analyte (data not shown).

In the presence of TEA and at pH levels from 5 to 6.5, it was found that the carboxylate form of topotecan exhibited sufficient retention in the C_{18} column. As at pH levels higher than 5 the lactone form of topotecan showed the broadened peak, therefore a mixture of ammonium acetate (0.05 M), acetonitrile and TEA (84:16:1.50, v/v) containing TBAHS (2 mM) with a pH of 5 was chosen as the proper composition for the mobile phase.

In order to improve precision of the method we implemented an internal standard, tetracycline HCl. Plasma samples were injected directly after protein precipitation by methanol. Blank human plasma samples of six individuals showed no substances at the retention times of the carboxylate and lactone form of topotecan and I.S.

3.2. Linearity

The calibration curves were constructed using a weighted linear regression (weighting factor 1/concentration^2). The peak area ratios of lactone and carboxylate forms of topotecan to the internal standard were plotted versus the nominal concentrations of the calibration standards. Results of linearity are presented in Tables 1 and 2. Correlation coefficients of calibration curves for the lactone and carboxylate forms were 0.9957 and 0.9965, respectively and the relevant slopes were statistically different from 0 ($p < 0.001$). Although intercepts of the related calibration curves were significantly different from 0 but did not have any effect on the accuracy of the method [20]. For calibration standards the back-calculated concentrations from the responses were calculated. The R.S.D. values were less than 9% and the deviations of the nominal concentrations for all

Table 1
The assayed concentrations of calibration standards of lactone and carboxylate forms of topotecan in human plasma ($n = 3$)

Nominal concentrations (ng/ml)	Assayed concentration of lactone and carboxylate forms of topotecan (ng/ml)	
	Lactone mean ± R.S.D.%	Carboxylate mean ± R.S.D.%
0.05	0.05 ± 8.56	0.05 ± 7.24
0.20	0.20 ± 4.17	0.20 ± 3.89
1.00	0.92 ± 2.36	0.98 ± 8.90
5.00	5.33 ± 1.51	4.76 ± 3.26
10.00	10.06 ± 1.95	9.95 ± 4.48
25.00	26.27 ± 3.56	26.61 ± 1.40
50.00	48.47 ± 5.65	50.48 ± 5.36
75.00	76.26 ± 4.07	74.52 ± 4.37

Table 2
Characteristics of calibration curves for the analysis of lactone and carboxylate forms of topotecan in plasma ($n = 3$)

Compound	Slope	Intercept	r^2
Topotecan lactone form	0.0798 (0.0009)[a]	0.3705 (0.3210)[a]	0.9957
Topotecan carboxylate form	0.1244 (0.0014)	0.1913 (0.04657)	0.9965

[a] S.E. between parenthesis.

Table 3
The intra-day precision and accuracy of the assay of lactone and carboxylate forms of topotecan in human plasma

	Assayed concentration of lactone and carboxylate forms of topotecan (ng/ml)					
	High SQC		Medium SQC		Low SQC	
	Lactone	Carboxylate	Lactone	Carboxylate	Lactone	Carboxylate
Day1	77.63	76.48	10.42	9.28	0.23	0.22
	73.69	75.93	10.25	9.66	0.21	0.24
	75.62	72.68	10.89	8.98	0.20	0.19
	78.23	71.14	9.56	10.32	0.22	0.23
	72.35	73.58	9.78	10.11	0.22	0.22
Mean	75.51	73.96	10.18	9.67	0.22	0.22
R.S.D. (%)	3.32	3.03	5.18	5.77	5.28	8.50
Nominal concentration (ng/ml)	75.00	75.00	10.00	10.00	0.20	0.20
RE (%)	0.67	−1.38	1.82	−3.30	8.00	10.00

Table 4
The inter-day precision and accuracy of the assay of lactone and carboxylate forms of topotecan in human plasma

	Assayed concentration of lactone and carboxylate forms of topotecan (ng/ml)					
	High SQC		Medium SQC		Low SQC	
	Lactone	Carboxylate	Lactone	Carboxylate	Lactone	Carboxylate
Day 1	78.58	77.34	9.66	9.34	0.20	0.20
Day 2	68.41	75.21	9.66	9.94	0.21	0.22
Day 3	79.04	74.56	10.11	10.19	0.23	0.22
Day 4	72.68	69.32	10.86	10.59	0.20	0.19
Day 5	75.46	70.20	9.60	9.64	0.23	0.23
Mean	74.84	73.33	9.98	9.94	0.22	0.21
R.S.D. (%)	5.90	4.67	5.34	4.86	6.39	7.75
Nominal concentration (ng/ml)	75.00	75.00	10.00	10.00	0.20	0.20
RE (%)	−0.22	−2.23	−0.18	−0.60	8.00	6.00

concentrations were less than 10%. Linear regression of the calculated concentrations versus the nominal ones provided a unite slope for both lactone ($p = 0.70$) and carboxylate ($p = 0.33$] forms of topotecan and also intercepts equal to 0 ($p = 0.87$) for carboxylate and ($p = 0.65$) for lactone.

3.3. Recovery

The recovery of lactone and carboxylate forms of topotecan from human plasma was determined using quality control samples and a PBS buffer, both spiked with three concentra-

Table 5
Stability of lactone and carboxylate forms of topotecan in human plasma after two freeze and thaw cycling

Nominal concentration (ng/ml)	Assayed concentration (ng/ml)					
	Number of freeze thaw cycles					
	0		1		2	
	Lactone	Carboxylate	Lactone	Carboxylate	Lactone	Carboxylate
0.20	0.20	0.21	0.19 (97.09)[a]	0.20 (97.25)	0.18 (92.35)	0.20 (97.45)
10.00	10.11	9.98	9.62 (97.86)	9.82 (98.15)	9.24 (91.65)	9.78 (98.05)
75.00	75.68	74.67	73.15 (98.13)	74.12 (99.08)	69.64 (92.75)	73.59 (97.48)

[a] Percentage of initial concentration; $n = 3$.

tions of lactone and carboxylate forms of topotecan covering the calibration range. The mean recovery obtained as a peak areas ratio (extracts of plasma samples/corresponding samples in the buffer) was 101.16 for lactone form of topotecan and 99.35 for carboxylate form of topotecan.

3.4. Limit of quantitation and limit of detection

Detection limits were 0.015 ng/ml for both lactone and carboxylate forms of topotecan. Limits of quantitation in plasma were determined to be 0.05 ng/ml for both lactone and carboxylate forms of topotecan.

The criteria for precision and accuracy at the limit of quantitation were R.S.D. < 20% and RE < 20%. The criteria were met by the results of the analysis of the lowest calibration standards for lactone and carboxylate forms of topotecan (0.05 ng/ml).

3.5. Intra- and Inter-day precision and accuracy

At three different spiked levels, the intra-day R.S.D.s of analysis method ranged between 3.32 and 5.28% for the lactone form of topotecan and from 3.03 to 8.50% for the carboxylate form of topotecan. The range of the Intra-day RE was 0.67–8.00% for lactone form of topotecan and −3.30–10.00% for carboxylate form of topotecan (Table 3).

The inter-day R.S.D.s for analysis of lactone and carboxylate forms of topotecan were less than 6.39 and 7.75%, respectively and the corresponding REs were less than 8.00 and 6.00% (Table 4).

3.6. Stability studies

One set of SQCs at three levels in plasma and plasma extract was prepared and kept at 22, 4 and −20 °C. To determine the effect of temperature on stability of lactone and carboxylate forms of topotecan, their conversion rates were also evaluated at 22, 4 and −20 °C in plasma and plasma extract (Figs. 3 and 4). Lactone form of topotecan hydrolyzed in plasma and plasma extract when incubated at 22, 4 and −20 °C, corresponding half lives were 1.68, 10.82 and 170.01 h in plasma and 3.60, 41.22 and 200.61 h in plasma extract, respectively. The carboxylate form of topotecan was converted to lactone form with $t_{1/2}$s of 25.50, 501.52 and 601.82 h at 22, 4 and −20 °C in plasma and 17.49, 83.59 and 167.17 h in plasma extract, respectively. These results show that the stability of carboxylate in plasma is more than

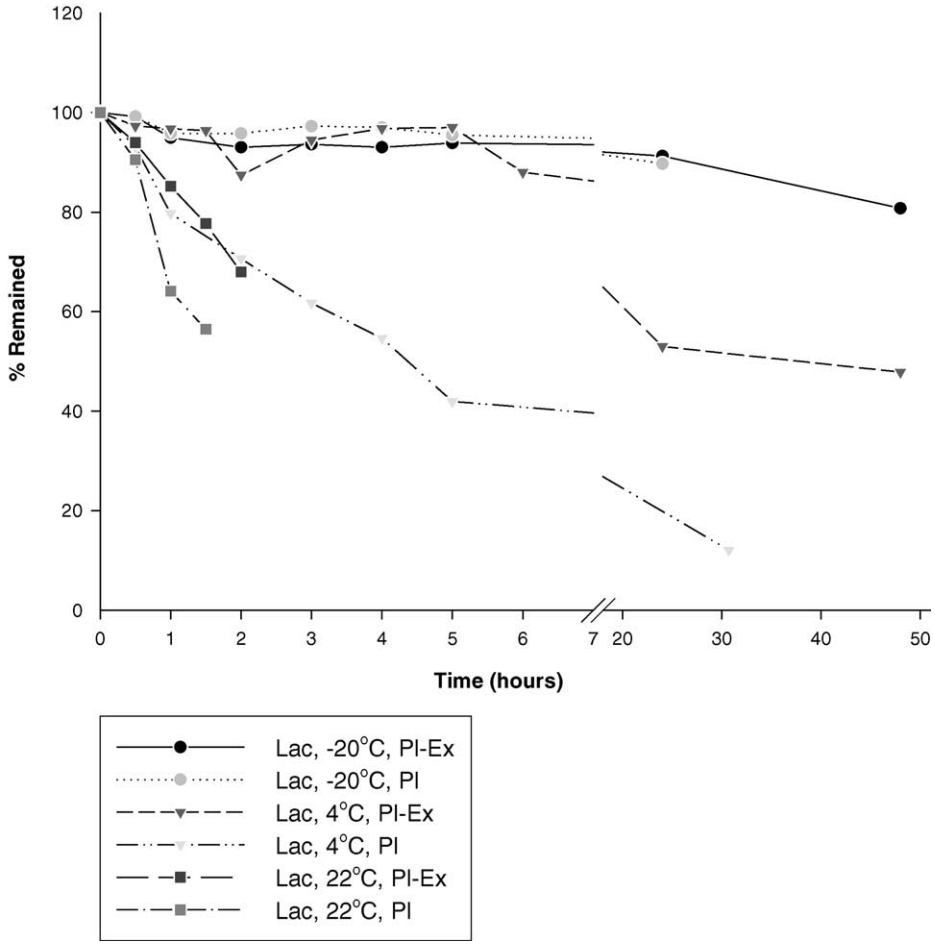

Fig. 3. Stability of lactone form of topotecan (Lac) in plasma (Pl) and plasma extract (Pl-Ex), at different temperatures.

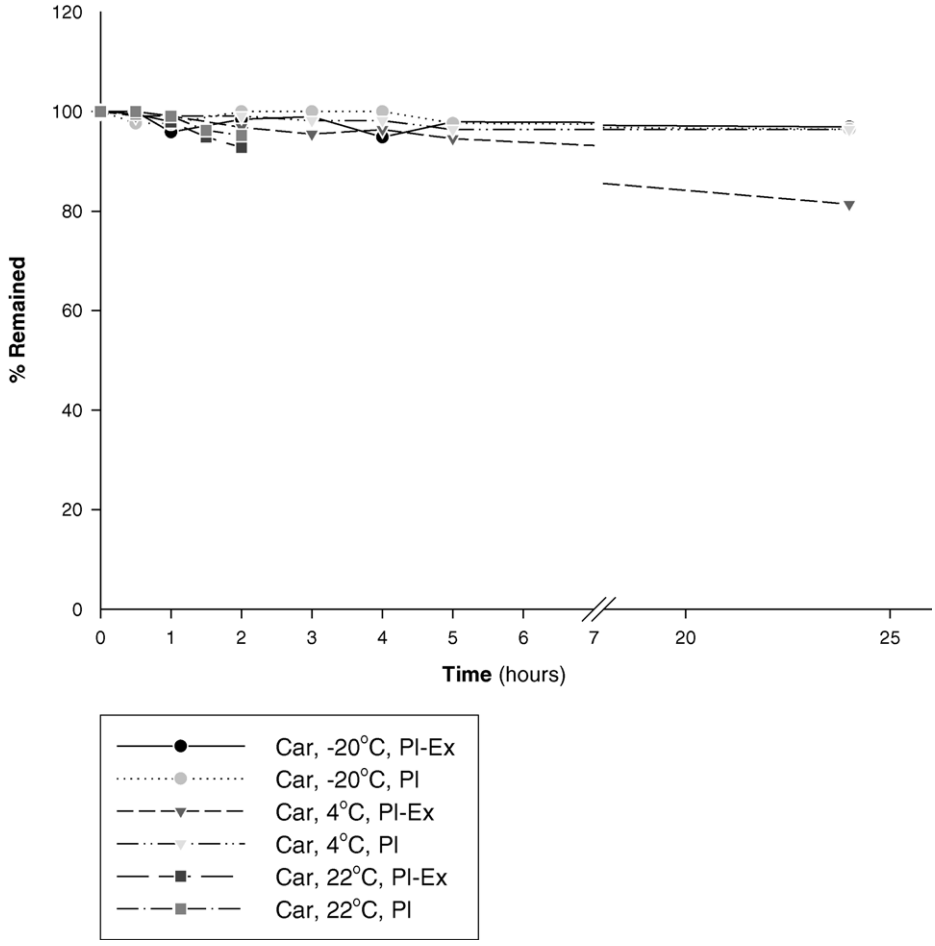

Fig. 4. Stability of carboxylate form of topotecan (Car) in plasma (Pl) and plasma extract (Pl-Ex), at different temperatures.

lactone form. It has been reported that plasma protein binding is an important factor affecting the camptothecin lactone-carboxylate equilibrium and also the affinity of human serum albumin to carboxylate form is 200-fold more than lactone form [21]. Therefore, the higher stability of carboxylate form could be attributed to its higher plasma protein binding. After 2 weeks storage at −70 °C, the concentrations of lactone and carboxylate forms in plasma were >95% of corresponding initial concentrations. Thus, the samples are stable at −70 °C at least for 2 weeks.

The stability of samples after one and two freeze–thaw cycles was also studied. As shown in Table 5, the concentrations found indicated no significant loss after the first freeze–thaw cycle. After the second cycling the carboxylate form was stable but a relatively significant loss was shown in the lactone concentration.

Stock solution of topotecan in DMSO was found to be stable up to 6 months when stored at −20 °C.

4. Conclusion

In this paper an isocratic HPLC method for the determination of lactone and carboxylate forms of topotecan in human plasma is described. The method was validated in the range of lactone and carboxylate forms of topotecan concentrations from 0.05 to 75 ng/ml. The assay is rapid. The analysis time is only 10 min for simultaneous determination of lactone and carboxylate forms of topotecan and I.S. The chromatographic assay reported shows good characteristics of selectivity, simplicity, linearity, sensitivity and precision, allowing for numerous samples to be processed in a short period of time.

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