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Liquid chromatographic quantitation of the lactone and the total of lactone and carboxylate forms of 9-nitrocamptothecin in human plasma

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Abstract

Simple and sensitive high-performance liquid chromatography (HPLC) assays were developed and validated for the quantitation of the investigational anticancer drug 9-nitrocamptothecin (9-NC) as the lactone form and as the total of the lactone(I) and carboxylate(II) forms in human plasma. For the assay of lactone form (9NC-lac), the analytical method involved a protein precipitation step with adding a mixture of cold acetonitril–chloroform (5:1 (v/v), −20 °C) to plasma sample that stabilized the pH-dependent conversion of I to II. After evaporation under gentle stream of nitrogen gas (40 °C) the dry extract was dissolved in mobile phase (pH 5.5). For determination of the total of the lactone and carboxylate forms of the drug (9NC-tot), plasma samples were deproteinated with cold acetonitril (−20 °C) acidified with perchloric acid (5%), which resulted in the conversion of the carboxylate into the lactone form. After centrifugation the upper solvent was evaporated (nitrogen, 40 °C) and the dry extract was dissolved in mobile phase (pH 3.5). All separations were performed on a RP-C_8 column, using a mixture of acetonitril–water as eluent (pH 3.5 for total form and pH 5.5 for lactone form) and UV detection. The presented assay was linear over a concentration range of 25–1500 ng/ml with lower limit of quantitation of 25 ng/ml for both 9NC-tot and 9NC-lac. Within-run and between-run precision was always less than 7.5% in the concentration range of interest. The reported assay method showed good characteristics of linearity, sensitivity, selectivity and precision allowing applying in pharmacokinetic studies.

Keywords: 9-Nitrocamptothecin; HPLC; Lactone; Total; Plasma

1. Introduction

9-Nitrocamptothecin (9-NC, RFS2000, Fig. 1) is a novel, lipophilic analogue of the natural plant alkaloid camptothecin, which has demonstrated high antitumor activity against advanced pancreatic carcinoma, ovarian epithelial cancer and leukemia [1–5].

Camptothecins are an important class of anti-cancer drugs that exert their antitumor activity by specifically inhibiting of DNA unwinding enzyme Topoisomerase I through binding the enzyme, stabilising the "cleavable complex" along the DNA replication fork which results in an accumulation of single and double strands DNA breaks and ultimately cell death [6–9].

Chemically, the analogues share the common features of a planner aromatic five-ring system with a lactone moiety that is required for optimal inhibition of Topoisomerase I activity but all of these drugs undergo a rapid, reversible, non enzymatic hydrolysis from closed lactone form to the inactive hydroxy carboxylated form with loss of pharmacologic activity [10–12]. Both the lactone/carboxylate ratio at equilibrium and the rate of conversion between the two forms is affected by the pH. In acidic medium (pH <4) the lactone structure predominants, while at alkaline pHs, including physiological pH the formation of the carboxylate is favoured [13–15]. Fassberg and Stella [13] studied the kinetic and mechanism of the hydrolysis of camptothecin and some analogues in

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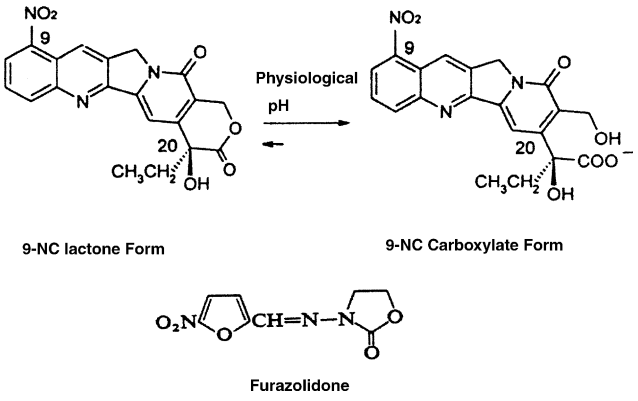

Fig. 1. Chemical structures and equilibrium reaction between the lactone and carboxylate forms 9-NC and chemical structure of furazolidone (I.S.).

aqueous solutions with different pHs. The results showed that at pH values ≤4 camptothecins were exclusively in lactone form and at pH values ≥8, they were exclusively in carboxylate form and the conversion was very fast in both directions [13].

Since an intact lactone form of camptothecins is of vital importance for the biological activity, several high-performance liquid chromatography (HPLC) methods have been developed for the analysis of lactone and total (lactone plus carboxylate) forms of camptothecin derivatives [16–23].

A few methods, which have been reported for the determination of 9-NC in plasma, analyze the drug only as the total (lactone plus carboxylate) form [24,25]. Since lactone moiety is the active form of this anticancer agent, it is important to design a sensitive analysis method for measurement of both lactone (9NC-lac) and total (9NC-tot) forms in plasma.

We therefore developed a sensitive and specific reversed-phase HPLC method with UV detection for the analysis of 9-NC as the lactone and total forms in human plasma.

2. Experimental

2.1. Chemicals and reagents

9-NC, 99.8% pure, was purchased from Yuanjian Pharmaceutical Technology Develop Co. (China). Furazolidone as the internal standard was obtained from Fluka.

HPLC grade acetonitril and the analytical grade dimethyl sulfoxide (DMSO), chloroform and perchloric acid (70%, w/v) were purchased from Merck (Darmstadt. Germany).

Drug-free human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Tehran, Iran).

2.2. Instruments

The HPLC system consisted of a model 1001 solvent delivery system and a model 2700 UV detector, equipped with Millennium chromatography manager for integration (all from Knauer, Germany).

Separations were achieved on a Nucleosil-100 C_8 column (250 mm × 4 mm i.d., 5 μm particle size) that protected by a Nucleosil-100 C_8 Encapped guard (4 mm × 4 mm i.d., 5 μm particle size) obtained from Knauer (Germany).

2.3. Chromatographic conditions

The mobile phase was a mixture of acetonitril–water (43:57, v/v) with the pH adjusted to 5.5 and 3.5 with perchloric acid (5%, v/v) for the assay of 9NC-lac and 9NC-tot, respectively.

The mobile phase was degassed by ultrasonication and delivered at a flow rate of 1.3 ml/min for both assays. The ultraviolet detector was set at wavelength of 370 nm.

2.4. Stock solutions and standards

9-Nitrocamptothecin stock solution (100 μg/ml) was prepared by dissolving the appropriate amount of the drug in DMSO and stored at −20 °C prior to further dilutions.

Working solutions were prepared daily at concentrations of 0.5, 1, 2, 5, 10, 20, 30 μg/ml by serial dilutions of stock solution with acetonitril and were stored at −20 °C prior to use.

Spiked plasma samples used as calibration standards were prepared daily by addition of appropriate amounts of the working solutions to drug-free human plasma, resulting in calibration standards of 25, 50, 100, 250, 500, 1000, 1500 ng/ml of 9-NC. In the case of the lactone form (9NC-lac) the prepared calibration standards were used immediately after preparation.

The internal standard solution was prepared by dissolving 20 mg of furazolidone in 100 ml acetonitril to give a final concentration of 200 μg/ml. This solution was diluted further by acetonitril yielding a final concentration of 40 μg/ml and stored at 2–8 °C prior to apply.

2.5. Sample preparation for the lactone form of 9-NC

To 250 μl of human plasma in a 2.0 ml polypropylene eppendorf cup a volume of 10 μl of I.S. solution in acetonitril (40 μg/ml), 500 μl of cold acetonitril (−20 °C) and 100 μl of cold chloroform were added. The mixture was vortex-mixed for 30 s and subsequently was centrifuged for 3 min at 14,000 rpm at ambient temperature. The supernatant was collected in a glass tube and evaporated at 40 °C under a gentle stream of nitrogen gas, until a completely dried residue was left over. One hundred and twenty five microliter of the mobile phase (prepared for the assay of lactone form) was added to the residue and after vortex mixing for 30 s a volume of 100 μl of the solution was injected into the HPLC system.

2.6. Sample preparation for total 9-NC

To 250 μl of human plasma a volume of 20 μl of IS solution in acetonitril (40 μg/ml), 500 μl of cold acetoni-

tril (−20 °C) and 20 μl of perchloric acid solution (5%) were added. The mixture was vortex-mixed for 1 min and allowed to stand for at least 10 min. Then the sample was centrifuged for 1 min at 14,000 rpm at ambient temperature. The clear supernatant was collected in a glass tube and evaporated at 40 °C under a gentle stream of nitrogen gas.

The dried residue was dissolved in 125 μl of mobile phase (prepared for the assay of 9NC-tot) and after 1 min vortex mixing a volume of 100 μl of the supernatant was injected into the HPLC system.

2.7. Validation of the assay

2.7.1. Calibration, accuracy, precision and selectivity

A full validation (three analytical runs) for the analysis of 9NC-lac and 9NC-tot in human plasma was completed.

Seven point calibration curves (ranged from 25 to 1500 ng/ml) were constructed by plotting peak area ratio of each analyte (9NC-lac or 9NC-tot) to internal standard against the analyte concentration. The weighted ($1/x$) linear regression was fitted over the concentration range of 25–1500 ng/ml.

The reproducibility of the analytical procedure was evaluated by determining the intra-day and inter-day relative standard deviations (RSD). 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.

The inter-day precision and accuracy of the assay was determined from the same SQCs as for the intra-day variability analyzed on five consecutive days. The accuracy at each concentration was expressed as the relative differences of measured and nominal concentration. An estimate of the accuracy (%) for each concentration, including LLQ, should be lower than 20%.

In order to study the selectivity of the assays, six independent blank human plasma samples were subjected to the same sample processing and analyzed.

2.7.2. Limit of quantitation (LOQ) and limit of detection (LOD)

The lower limit of quantitation was defined as the lowest concentration at which the precision expressed by relative standard deviation is lower than 20% and accuracy expressed by relative difference of the measured and true value is also lower than 20%. The LOQ was repeated five times for confirmation. Limit of detection was determined at the lowest concentration to be detected, taking into consideration of a signal-to-baseline noise ratio of 3.

2.7.3. Absolute recovery

The absolute recoveries of 9-NC(lac) and 9-NC(tot) were determined by comparing the mean response of processed spiked plasma samples to the mean response of three standard solutions with a concentration equivalent to 100, 500, 1000 ng/ml prepared in corresponding mobile phases in three different runs.

2.8. Stability

The chemical stability of lactone and total form of 9-NC in biomatrix at three concentrations of 100, 500 and 1000 ng/ml was established. The number of replicates was three:

(i) During three consecutive freeze-thaw cycles, in which the samples were kept at room temperature for 30 min before freezing again.
(ii) The stability of the both forms in the biomatrix was investigated at ambient temperature and −20 °C.
(iii) The stability of the drug in the acetonitril–chloroform for lactone form and acetonitril–perchloric acid for total form (precipitation solvents) originating from plasma spiked with 9-NC at concentrations of 100, 500, 1000 ng/ml was evaluated at −20 °C and ambient temperature (stability in plasma extract solutions).
(iv) The stability of dry extracts at −20 °C.
(v) The long term stability of 9-NC at −70 °C at concentrations of 100, 500, 1000 ng/ml in plasma was also tested over a 3-week period.

3. Results and discussion

An intact lactone form of 9-NC is most important factor for the biological activity of this anti-tumor agent. At physiological pH, 9-NC is not stable and hydrolysis of the lactone moiety leads to the formation of the inactive hydroxy carboxylate form [13–15]. Therefore, a bio-analytical method is required for the selective quantitation of the intact lactone form beside total form of 9-NC in human plasma.

In the HPLC methods developed by Schoemaker et al. [24] and Zhong et al. [25] only the total form of 9-NC (lactone plus carboxylate) is analysed. Long time procedure of sample preparation and need of laborious solid-phase or liquid–liquid extraction techniques are other disadvantages of these reported methods [24,25].

The assay described here employed simple and short time procedures for sample preparation using cold acetonitril (−20 °C) or a cold mixture of acetonitril–chloroform (−20 °C) for plasma protein precipitation and fixation of the equilibrium between the lactone and the carboxylate form of 9-NC.

3.1. Chromatography and detection

The presence of the 9-nitro group in the molecule decreased the fluorescence properties dramatically when compared to other camptothecins and use of ultraviolet detector for analysis of 9-NC was a necessity.

Various chromatography systems were tested and finally the use of a RP-C_8 column in combination with a mobile

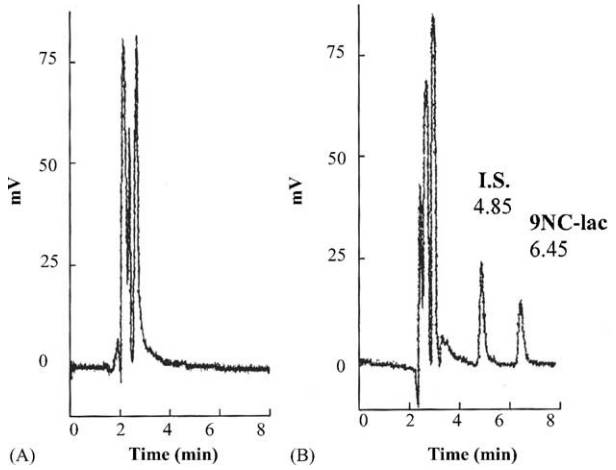

Fig. 2. Chromatograms obtained from blank plasma (A) and a plasma spiked with 250 ng/ml of lactone form of 9-NC (B).

phase composition of acetonitril–water (43:57, v/v) with adjusted pHs of 5.5 (for 9NC-lac) and 3.5 (for 9NC-tot) made it possible to determine the analytes under optimum conditions. Representative HPLC chromatograms of the assays for 9NC-lac and 9NC-tot are shown in Figs. 2 and 3, respectively.

The retention times of I.S and 9-NC in the assay of the lactone form were 4.85 ± 0.2 min and 6.45 ± 0.3 min (Fig. 2), while the corresponding retention times in the assay of 9NC-tot were 4.23 ± 0.2 min and 5.85 ± 0.2 min, respectively (Fig. 3). The overall chromatographic run time was 8 min for both assays.

The selectivity for the analytes is shown by the sharp resolution of the peaks and no significant interfering peaks for both assays in blank plasma samples from six independent donors. After oral administration a small amount (approximately 6%) of 9-NC is converted to 9-aminocamptothecin (9-AC), the major and active metabolite of the drug [26]. Due to a very low plasma concentration of 9-AC in the assay

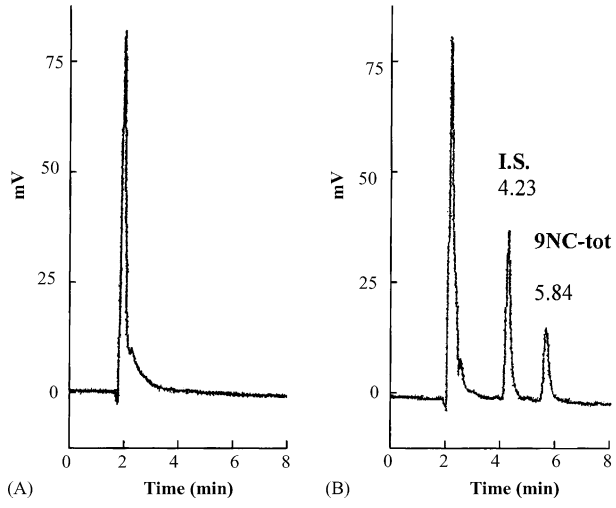

Fig. 3. Chromatograms obtained from blank plasma (A) and a plasma spiked with 250 ng/ml of total form of 9-NC (B).

Table 1
Equations of calibration curves for the analysis of 9-NC (lac) and 9-NC (tot) in plasma ($n = 3$)

Compound	Equation[a]	R^2
9-NC lactone form	$Y = 0.202X - 2.89$	0.994
9-NC total form	$Y = 0.197X + 0.213$	0.997

[a] X is the concentration of 9-NC in ng/ml and Y is the peak area ratio (drug/IS).

method reported by Schoemaker et al. [24] parallel UV and fluorescence detection were used for simultaneous determination of 9-NC and 9-AC in human plasma and the related retention times were 12.5 and 6 min, respectively. Considering the aforementioned data as well as the higher polarity of 9-AC compared to the parent drug, it is unlikely that the metabolite will interfere with analysis of 9-NC.

3.2. Linearity, precision and accuracy

Calibration curves of the lactone and total (lactone plus carboxylate forms) of 9-NC in human plasma were linear in the rang of 25 to 1500 ng/ml. The regression coefficients were 0.994 for 9NC-lac and 0.997 for 9NC-tot (Table 1).

The average of intra-day and inter-day precisions and mean accuracy for 9-NC lactone and total forms are shown in Table 2. Intra-day and inter-day coefficients of variation were not more than 7.34 and 5.08, respectively.

The deviations of the nominal concentrations for all concentrations were less than 5.5%.

3.3. LOD, LOQ and recovery

The LOQ for 9-NC in the assays for the lactone and total forms were 25 ng/ml. The LOD for both lactone and total forms was 10 ng/ml. The mean extraction recoveries of 9-NC and I.S. in the assay for 9NC-lac were 63.51 and 73.87 and the recovery for 9NC-tot were found to be 96.62 and 80.75, respectively (Table 3).

The recoveries of 9-NC and I.S. in the assay for 9NC-lac were relatively lower than expected for a protein precipitation procedure most probable due to the high affinity of both hydrophobic compounds to the lipophilic sites on plasma proteins. The extraction recoveries of 9-NC and I.S. in the assay

Table 2
Assay performance data for the determination of 9NC-tot and 9NC-lac in plasma ($n = 5$)

Analyte	Inter-day (CV%)	Intra-day (CV%)	RE%	Measured concentration (ng/ml)	Nominal concentration (ng/ml)
9NC-lac	3.82	4.29	5.44	108.56	100.00
	5.08	3.19	0.98	519.48	500.00
	2.91	4.12	0.53	1005.34	1000.00
9NC-tot	3.67	7.34	2.89	102.89	100.00
	1.91	2.34	1.87	514.12	500.00
	3.51	1.89	4.11	1041.01	1000.00

Table 3
Percent recovery of 9NC-tot and 9NC-lac in plasma ($n = 5$)

Analyte	Absolute recovery (mean ± SD)	Concentration (ng/ml)
9NC-lac	63.5 ± 5.5	100
	64.8 ± 6.5	500
	62.25 ± 4.6	1000
9NC-tot	95.83 ± 6.3	100
	97.42 ± 5.7	500
	96.61 ± 3.9	1000

for 9NC-tot were more than respective values in assay for 9NC-lac. This finding can be explained by the fact that the unionized fractions of these weak basic compounds and as a result their affinity to the lipophilic sites on plasma proteins are obviously higher in a solution with a pH 5.5 than pH 3.5. To further evaluate this suggestion we studied the recovery of 9-NC and I.S. from standard solutions prepared with PBS buffer (pH 7.4) in concentrations and assay conditions completely similar to the studies done for plasma samples. Results (not mentioned here) showed the recoveries of 9NC-lac, 9NC-tot and I.S. were obviously higher (>91%) than plasma, which support aforementioned suggestion.

3.4. Stability

The chemical stability of 9-NC at three concentrations of 100, 500, 1000 ng/ml ($n = 3$ for each concentration) were studied in plasma and plasma extracts for both total and lactone forms at different temperatures of 20 and −20 °C. (Figs. 4–7).

The stability of 9-NC in both forms was critical during the time that plasma samples were kept at ambient temperature. From the data of stability experiments it can be concluded that the sample preparation for both forms should be performed within 0.5 h and plasma extracts should be stored in −20 °C until analysis (maximum 24 h).

Since 9-NC is unstable in the biomatrix at ambient temperature, it is recommended to store the plasma samples at −70 °C for long time or at −20 °C for short time storage (24 h) until analysis.

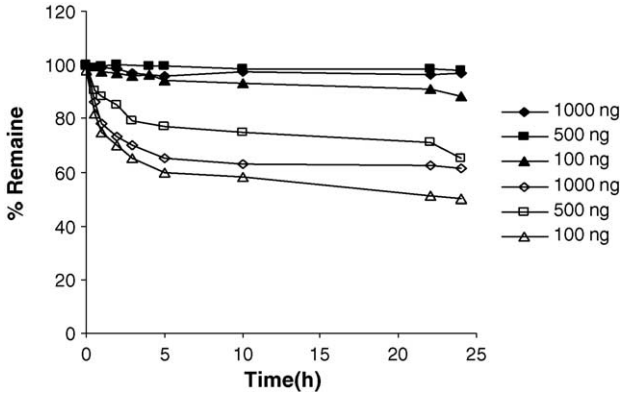

Fig. 4. Stability of 9-NC as the total form in human plasma at three different concentrations for 24 h (number of replicates was three). Stability at −20 °C (solid symbols) and stability at ambient temperature (open symbols).

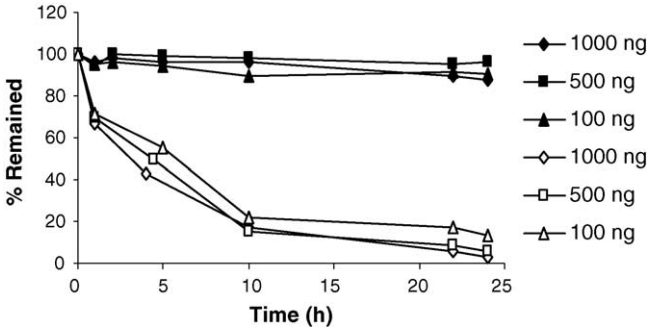

Fig. 5. Stability of 9-NC as the lacton form in human plasma at three different concentrations for 24 h (number of replicates was three). Stability at −20 °C (solid symbols) and stability at ambient temperature (open symbols).

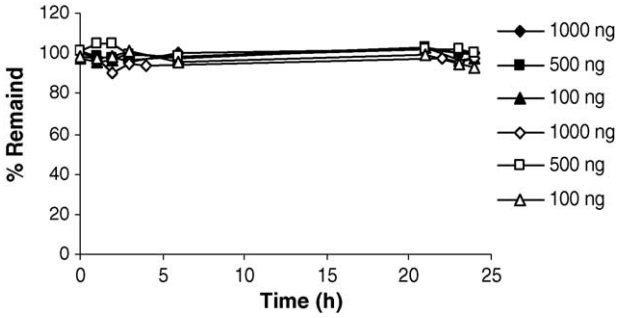

Fig. 6. Stability of 9-NC as the total form in acetonitril–perchloric acid plasma extract solution at three different concentrations for 24 h (number of replicates was three). Stability at −20 °C (solid symbols) and stability at ambient temperature (open symbols).

After 3 weeks storage at −70 °C, the concentrations of lactone and total forms in plasma were >95% of corresponding initial concentrations. Thus, the samples are stable at −70 °C at least for 3 weeks. 9-NC in lactone and total forms, as dry extract, were stable at least 3 weeks at −20 °C.

Stock solution of 9-NC in DMSO was found to be stable up to 6 month when stored at −20 °C. From freeze-thaw results, it is concluded that total form was still stable after

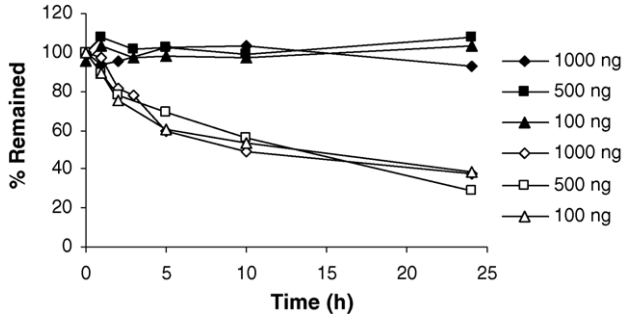

Fig. 7. Stability of 9-NC as the lactone form in acetonitril–chloroform plasma extract solution at three different concentrations for 24 h (number of replicates was three). Stability at −20 °C (solid symbols) and stability at ambient temperature (open symbols).

Table 4
Stability of 9NC-tot after three freeze–thaw cycles ($n = 3$)

Nominal concentration (ng/ml)	Assayed concentration (ng/ml)			
	Zero time	First cycle	Second cycle	Third cycle
100.00	102.50 (102.51)[a]	100.11 (100.11)	101.03 (101.03)	99.98 (99.98)
500.00	499.98 (99.96)	488.26 (97.65)	479.36 (95.87)	486.23 (97.25)
1000.00	1052.73 (105.27)	1032.60 (103.26)	1028.89 (102.90)	1036.99 (103.70)

[a] Percent of initial concentration (at zero time).

Table 5
Stability of 9NC-lac after three freeze–thaw cycles ($n = 3$)

Nominal concentration (ng/ml)	Assayed concentration (ng/ml)			
	Zero time[a]	First cycle	Second cycle	Third cycle
100.00	99.95 (99.95)	95.35 (95.35)	89.65 (89.65)	85.09 (85.09)
500.00	491.73 (98.38)	479.28 (95.85)	459.63 (91.93)	430.57 (86.12)
1000.00	998.45 (99.85)	975.31 (97.53)	938.22 (93.82)	830.41 (83.04)

[a] Percent of initial concentration (at zero time).

three freeze-thaw cycles but the lactone form was stable after the first cycle (Tables 4 and 5).

4. Conclusion

In this paper novel, simple, selective and validated isocratic HPLC methods for the analysis of lactone and total forms of 9-nitrocamptothecin are described. The sample pretreatment procedures are based on deproteination with a mixture of cold acetonitril–perchloric acid solution for 9NC-tot and a mixture of cold acetonitrile–chloroform for assay of 9NC-lac. The methods were linear in the concentration range of 25–1500 ng/ml for both forms of 9-NC. The chromatographic assays reported show good characteristics of selectivity, simplicity, linearity, sensitivity and precision, allowing for numerous samples to be processed in a short period of time in pharmacokinetic studies.

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