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Simple non-ion-paired high-performance liquid chromatographic method for simultaneous quantitation of carboxylate and lactone forms of 14 new camptothecin derivatives

Kazumi Sano[a,*], Megumi Yoshikawa[a], Shinya Hayasaka[a], Kurita Satake[a], Yoji Ikegami[a], Hisahiro Yoshida[a], Toshihisa Ishikawa[b], Seigo Sawada[c], Shinzo Tanabe[d]

[a]*Department of Drug Metabolism and Disposition, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose-si, Tokyo 204-8588, Japan*

[b]*Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan*

[c]*Yakult Central Institute for Microbiological Research, Tokyo, Japan*

[d]*Department of Analytical Biochemistry, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan*

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Abstract

SN-38 (7-ethyl-10-hydroxycamptothecin) is an active metabolite derived from the semi-synthetic compound camptothecin (CPT) named Irinotecan (CPT-11). The antitumor activity of SN-38 is 1000-fold more potent than the parent CPT-11. Fourteen new derivatives of camptothecin have recently been developed by Yakult Honsha (Tokyo, Japan). Here we describe a simple and cost-effective high-performance liquid chromatography (HPLC) method without an ion-pairing agent, which allows the simultaneous determination of both lactone and carboxylate forms of SN-38 and other camptothecin derivatives. A weak linear relationship between the HPLC retention factors ($\ln k'$) and the cellular concentrations of these compounds was observed. These results suggest that low-polarity compounds easily accumulate in cancer cells and may circumvent drug resistance. The HPLC analysis herein described is expected to greatly assist in derivative synthesis and chemical modification of camptothecin-based antitumor drugs.

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Keywords: Derivatization, LC; Camptothecin; SN-38; CPT-11

1. Introduction

Irinotecan hydrochloride (CPT-11) is a water-soluble derivative of camptothecin (CPT) [1]. CPT is a potent antitumor alkaloid extracted from the Chinese tree *Camptotheca acuminata* [2]. The antitumor activity of CPT is due to irreversible inhibition of DNA topoisomerase I (Topo I) [3–6]. However, clinical use of CPT was discontinued due to its high toxicity, and the water-soluble analog CPT-11 was developed. CPT-11 is metabolized in vivo by carboxyesterase to produce 7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite. This metabolite is 1000-fold more potent than the parent compound in vitro [7,8].

SN-38 has an α-hydroxy-δ-lactone ring that under-

*Corresponding author. Tel.: +81-424-958-470; fax: +81-424-958-469.

E-mail address: ksano@my-pharm.ac.jp (K. Sano).

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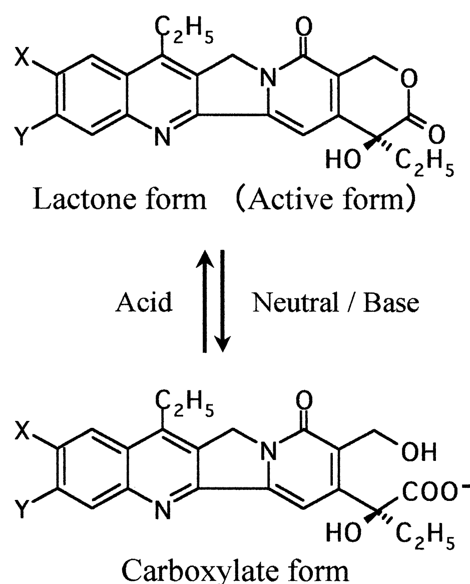

Fig. 1. Molecular structure of the lactone and carboxylate forms of SN-38.

goes reversible hydrolysis (Fig. 1) at a rate dependent on pH, ionic strength and protein concentration [9,10]. Human serum albumin (HSA) preferentially binds the carboxylate form over the lactone form with a 150-fold higher affinity. These interactions result in the more rapid and complete conversion of SN-38 to the carboxylate form [11]. This information demonstrates the importance of simultaneous quantitation of the carboxylate and lactone forms of SN-38. To date, several methods have been described for such determinations [12–18]. Direct measurement of the carboxylate form was impossible with these methods except that in Ref. [15], because it was eluted with the solvent front during HPLC separation. Therefore, carboxylate concentrations were estimated based on total lactone concentrations following acidification of the sample. In the present paper, we report a new and simple HPLC method using a C_{18} column and ordinary buffer systems without an ion-pairing agent.

About 10 cell lines have hitherto been reported to be resistant to CPT-11 or SN-38 [19,20]. Several in vitro mechanisms of resistance to CPT-11 or SN-38 have been reported. The resistant cell lines play an important role in elucidating the in vivo drug resistance mechanism, although results from experiments using resistant cell lines do not always accurately reflect the phenomenon.

Table 1
Characteristics of 15 derivatives

Compound	Structure		Ratio of fluorescence intensity	
	X	Y	Em_{max} (nm)	Carboxylate/lactone
SN-38	OH	H	**543**	1.2/1.0 (1.2)
SN-22	H	H	439	3.4/2.1 (1.6)
SN-343	Me	H	434	20.7/10.9 (1.9)
SN-348	Br	H	438	3.4/2.4 (1.4)
SN-349	Cl	H	436	17.4/14.1 (1.2)
SN-392	NH_2	H	**527**	1.0/1.6 (0.6)
SN-351	H	Br	438	2.7/2.1 (1.3)
SN-352	H	Cl	434	16.6/15.0 (1.1)
SN-353	H	F	432	19.3/14.0 (1.4)
SN-355	H	OH	440	0.5/0.5 (1.0)
SN-364	Cl	Cl	442	2.9/4.5 (0.6)
SN-398	OH	F	**534**	2.8/1.4 (2.0)
SN-397	OMe	F	416	35.1/32.6 (1.1)
SN-443	Me	F	434	23.5/14.7 (1.6)
SN-444	F	F	436	17.0/11.1 (1.5)

Excitation, 380 nm.

Fourteen derivatives of camptothecin were recently developed by Yakult Honsha (Table 1). These derivatives were synthesized by replacing the hydroxy residue with others (hydrogen, halogen methyl, methoxy residues, etc.) to overcome SN-38 resistance. In the present study, we have developed a new HPLC method to measure cellular concentrations of other derivatives to gain insight into the correlation between cellular concentrations with retention on the C_{18} column. Information of the relationship between the chemical properties and antitumor activities of these 14 compounds would provide a useful path to the development of new antitumor drugs.

2. Experimental

2.1. Materials and reagents

SN-38 and 14 new derivatives of camptothecin (Table 1) were provided by Yakult Honsha. The method of synthesis will be reported elsewhere. Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque (Kyoto, Japan). The water used was

of Milli-Q grade, and all other chemicals were of analytical or HPLC grade.

2.2. Cell lines and growth conditions

The human non-small-cell lung cancer cell line PC-6 was kindly donated by Dr. M. Oka (Nagasaki University). PC-6 is selected for the reason that it is the parent of PC-6/SN2-5H, which has high breast cancer resistant protein (BCRP), although PC-6 has less BCRP. The purpose of our project is an elucidation of the mechanism of ATP-dependent SN-38 transport activities of BCRP and a development of new resistant drugs against BCRP. This HPLC method was developed for the project. The doubling time of PC-6 cells was 23 h. The cell line was maintained as monolayers in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Gibco BRL) as described previously [21].

2.3. Fluorescence intensity

Fluorescence spectra were measured using a Hitachi F-4010 Fluorescence spectrometer (Hitachi, Tokyo, Japan).

2.4. Apparatus

The HPLC system consisted of a Jasco PU1580 pump, a Jasco FP920 fluorescence detector (Jasco, Tokyo, Japan) and a Shimadzu C-R4A integrator (Shimadzu, Kyoto, Japan). Isocratic elutions were performed using a Mightysil RP-18 (L) GP column (5 μm, 150 mm×4.6 mm I.D.; Kanto Chemical, Tokyo, Japan) with a guard column (5 μm, 5 mm× 4.6 mm I.D.). The excitation and emission wavelengths were 380 and 550 nm, respectively, for SN-38, SN-392, and SN-398. Emission wavelength was set at 440 nm for the other 12 compounds. The mobile phase for separation of SN-38 consisted of 50 m*M* phosphate buffer (pH 6.0)–acetonitrile–tetrahydrofuran (THF) (80:20:2, v/v). For the derivative compounds, the ratio of acetonitrile was adjusted to achieve an analyte retention time of 12–18 min. The flow-rate was 1.0 ml/min and all separations were carried out at room temperature (23–25 °C).

2.5. Sample preparation of standard solutions

The method of quantitation used external standards. Stock solutions containing 2.5 m*M* of the derivative in DMSO were prepared and stored at −20 °C. A 20-μl aliquot of stock solution was diluted with 980 μl of 0.01 *M* phosphate buffer (pH 3.0) for the lactone form or 0.05 *M* sodium hydroxide (NaOH) for the carboxylate form. HPLC analysis determined that these secondary stock solutions, 50 μ*M*, were 95% stable for at least 2 months at −20 °C. Sequential dilutions of 5.0, 0.5, 0.05 μ*M* were always freshly prepared by diluting each 50 μ*M* solution with the appropriate buffer. These diluted solutions were allowed to stand at room temperature for at least 30 min to ensure equilibration. All stock and standard solutions were stored in polypropylene tubes, in order to prevent concentration decreases by adsorption of the drugs, particularly the lactone form, to the containers.

2.6. SN-38 sample preparation for determination of cellular concentrations

Cells (1×10^6) were incubated at 37 °C for 1, 5, 10 or 20 min in the presence of 10 μ*M* carboxylate form of SN-38 in 200 μl of RPMI 1640 medium supplemented with 50 m*M* Hepes/Tris (pH 7.4) and then washed twice in 800 μl of ice-cold PBS(−). After centrifugation at 15 000 *g* for 5 min at 4 °C, cells were disrupted by vigorous sonication in 200 μl of distilled water (pH 6.0), freezing with liquid N_2 and rapid thawing. This was carried out three times and the cell solution was centrifuged at 15 000 *g* for 5 min at 4 °C. The supernatant was then subjected to HPLC. The reason we use distilled water as the disruptive solution of cells is that the distilled water has a pH of 6.0 and low enough molarity to disrupt cells. Moreover, both lactone and carboxylate forms are stable (above 98%) in distilled water for 2 h at room temperature (data not shown).

2.7. Method validation

The within- and between-day reproducibilities were determined for the carboxylate and lactone forms of SN-38. Three different concentrations of both forms of SN-38 were analyzed. Three aliquots

of each sample were analyzed each day for 6 days, and the resulting coefficients of variation (C.V.) and accuracy indicated the within- and between-day reproducibilities.

The limit of detection was defined as the concentration of carboxylate form resulting in a signal-to-noise ratio of 5.

3. Results and discussion

3.1. Detectable emission of derivatives of SN-38

Wavelength optimization revealed that the maximum emission wavelength of the derivatives were different from that of SN-38, when the appropriate excitation wavelength, i.e. 380 nm, was used (Fig. 2). SN-38 derivatives were classified into two groups. One was the SN-38 type that has two emission peaks at around 543 nm (SN-392, SN-398). SN-398 had a similar pattern as SN-38 and the carboxylate form showed a twofold higher fluorescence intensity than the lactone form. On the other hand, the SN-392 carboxylate form had a lower fluorescence intensity than the lactone form. The second group was the SN-22 type that showed a maximum intensity at around 439 nm (SN-355, SN-364, etc.). Although the SN-22 carboxylate form had a higher fluorescence intensity than the lactone form, the lactone form of SN-364 showed a higher intensity than its carboxylate form. Table 2 shows that each derivative has a different maximum emission wavelength, and a different ratio of fluorescence intensity for the carboxylate and lactone forms. The excitation and emission wavelengths were set at 380 and 550 nm for SN-38, SN-392, SN-398, and the emission wavelength was set at 440 nm for the other 12 compounds.

3.2. Method optimization

3.2.1. Effect of acetonitrile and THF

SN-355, a derivative with a hydroxy group in the Y position, was difficult to elute from the ODS column due to strong adsorption. However, addition of THF at 2 ml per 100 ml of acetonitrile–phosphate buffer (pH 6.0) with an increase in the acetonitrile ratio led to more rapid elution of the lactone form

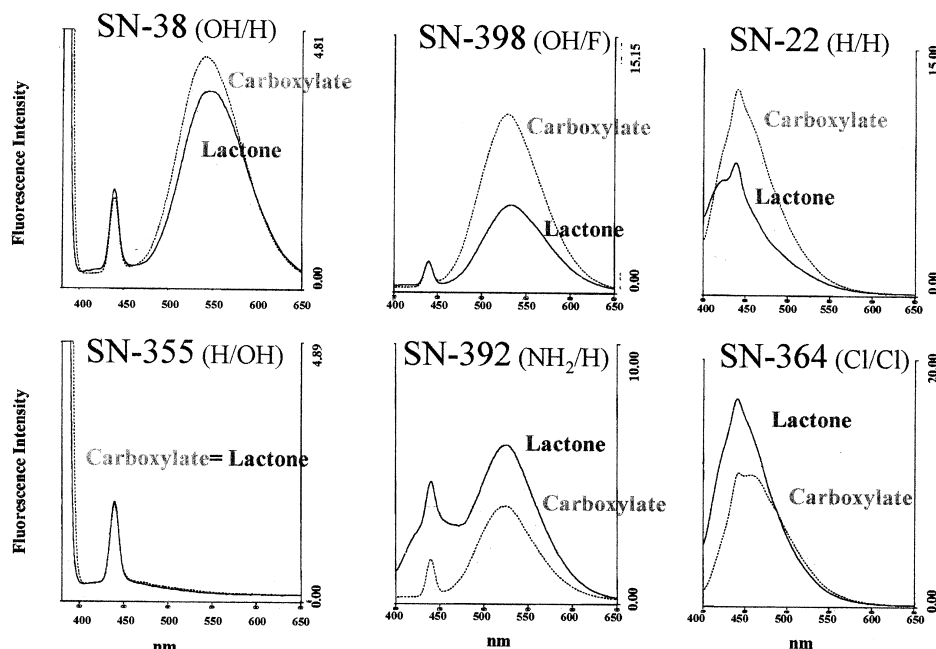

Fig. 2. Representative fluorescent spectrum of SN-38 and five derivatives. Fluorescence spectra were measured using a Hitachi F-4010. The appropriate excitation wavelength, i.e. 380 nm was used.

Table 2
HPLC conditions for SN-38 derivatives

0.05 *M* Phosphate buffer–acetonitrile–THF	Em (Ex: 380 nm)	SN-38 derivatives
80:20:2	550 nm	SN-38, SN-392, SN-398
70:30:2	440 nm	SN-22, SN-353, SN-355
60:40:2	440 nm	SN-443, SN-444, SN-349
		SN-348, SN-343, SN-352
		SN-351, SN-364, SN-397

(Fig. 3). Furthermore, the lactone forms of low-polarity derivatives with halogens were eluted in around 20 min by increasing the acetonitrile ratio up to 30 or 40% (Table 2).

3.2.2. Column selection

For column selection, we evaluated four ODS HPLC columns (4.6 mm I.D.×150 mm) under identical conditions (Fig. 4). The mobile phase consisted of 50 m*M* phosphate buffer (pH 6.0)–acetonitrile–THF (80:20:2, v/v). Samples were 10 μl of 100 pmol/ml of the carboxylate and lactone forms of SN-38. In this experiment samples were prepared separately and injected in turn into the same injector loop. As soon as the end of the injection, separations were started. The conversion between carboxylate and lactone does not occur in the mobile phase (pH 6.0) for at least 30 min (data not shown). On column A (TSK-gel ODS 80Ts), carboxylate and lactone forms eluted at 3.44 min and 23.27 min, respectively (resolution value (R_s)=5.73). This was unsuitable for this investigation because the lactone peak was late and broad. Column B (J's sphere ODS-H80, S-4 μm, 8 nm) gave good resolution (R_s=20.8) but was unsuitable due to solvent shock occurring just prior to carboxylate form elution. The retention time on column C (Waters Puresil C_{18}, 5 μ, 120 Å) was not long enough. Carboxylate and lactone forms were eluted at 2.54 min and 7.63 min, respectively (R_s=5.45) and the carboxylate form could not be separated from cellular components. On the Mightysil RP-18 (L) GP column (column D) good separation was observed (R_s=10.39). We finally decided to select the Mightysil RP-18 (L) GP column because of its high separation capacity and reasonable cost (50% less than the others).

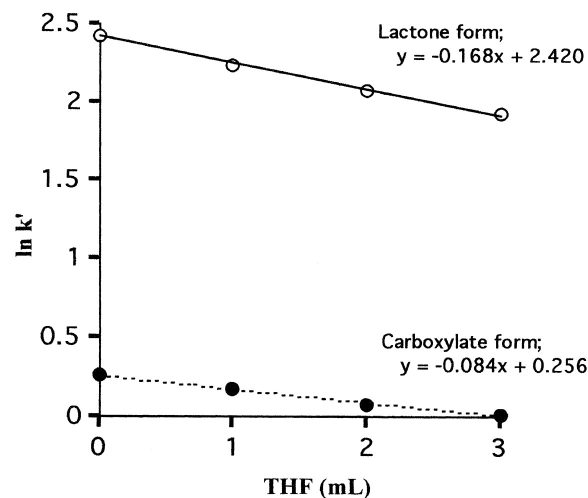

Fig. 3. Effect of tetrahydrofuran (THF) on capacity factor (k') of SN-355. The *X*-axis presents additional volume (ml) per 100 ml of acetonitrile–phosphate buffer (pH 6.0). $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the compound and t_0 is the retention time of unretained molecules. HPLC conditions, the excitation and emission wavelengths were 380 and 440 nm, respectively. The mobile phase consisted of 50 m*M* phosphate buffer (pH 6.0)–acetonitrile–THF (70:30:0–3, v/v). The flow-rate was 1.0 ml/min and all separations were carried out at room temperature (23–25 °C).

3.2.3. Optimum pH

To estimate the stability of SN-38 in elution buffer, we investigated the conversion ratio of SN-38 from the lactone to the carboxylate form in 10 m*M* phosphate buffer after 30 min at pH 3.0 to 9.0 (Fig. 5). The lactone form was stable at pH below 6.0, whereas it started to decrease and rapidly convert to carboxylate in the pH range of 7.0 to 9.0. However, the carboxylate form slowly converted to lactone from pH 9.0 and completely disappeared at pH 3.0. These results indicated that the conversion rate depends on the hydrogen ion concentration. The conversion rate of lactone to carboxylate was substantially delayed in 0.1 *M* phosphate buffer with 97% of the original form existing at pH 7.4 after 30

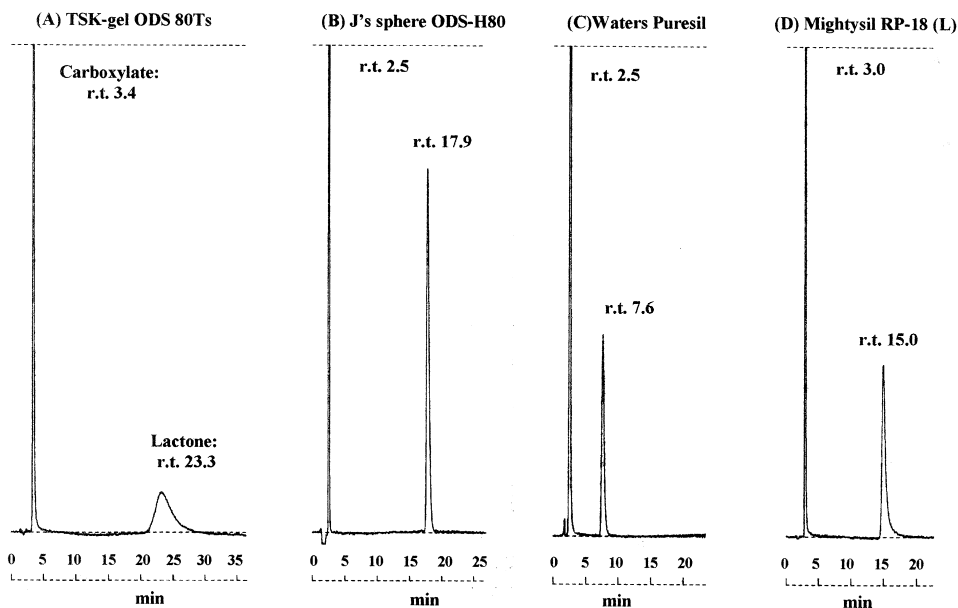

Fig. 4. Comparison of columns for separation of SN-38 carboxylate and lactone forms. Column A, TSK-gel ODS 80Ts (5 μm); B, J's sphere ODS-H80 (S-4 μm, 8 nm); C, Waters Puresil C_{18} (5 μ, 120 Å); D, Mightysil RP-18 (L) GP column (5 μm). HPLC conditions, the excitation and emission wavelengths were 380 and 550 nm, respectively. The mobile phase consisted of 50 m*M* phosphate buffer (pH 6.0)–acetonitrile–THF (80:20:2, v/v). The flow-rate was 1.0 ml/min and all separations were carried out at room temperature (23–25 °C).

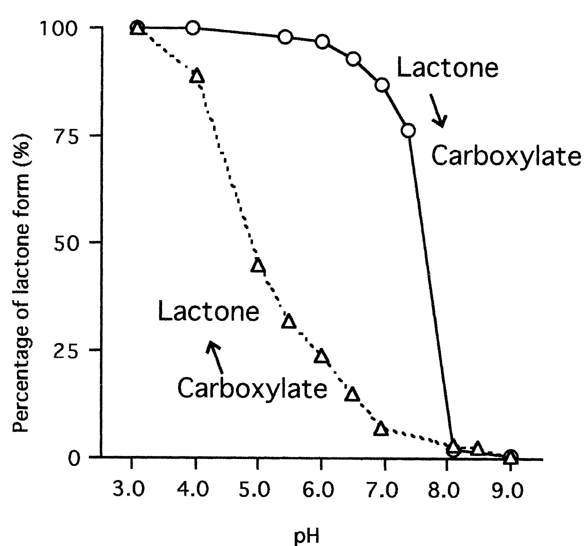

Fig. 5. Conversion ratio between lactone and carboxylate form. Conversion ratio is shown as percentage (%) of lactone form in total SN-38 concentration at each pH in 10 m*M* phosphate buffer after 30 min mixture at room temperature.

min. From these results, the pH of the elution buffer was adjusted to 6.0 and the buffer concentration was set to 50 m*M* to maintain the stability of the SN-38 lactone form. The stability of the lactone form in eluent containing acetonitrile and THF at 15 min, 60 min and 24 h was 99.3%, 97.9% and 90.0%, respectively.

3.2.4. Chromatography

Fig. 6 shows typical chromatograms of carboxylate and lactone forms of SN-38. The retention time of carboxylate and lactone were 3.0 and 15.0 min, respectively. Fig. 6A is 100 μl of supernatant of blank cell lysates. Fig. 6B or C are blank cell lysates with 10 pmol/ml carboxylate or lactone form of SN-38 in the same volume as Fig. 6A. Fig. 6D is a sample of a supernatant of cellular lysates at 1 min into the experiment (see detail in Section 2.6). A mass injection volume (100 μl) gave a dull peak of lactone form.

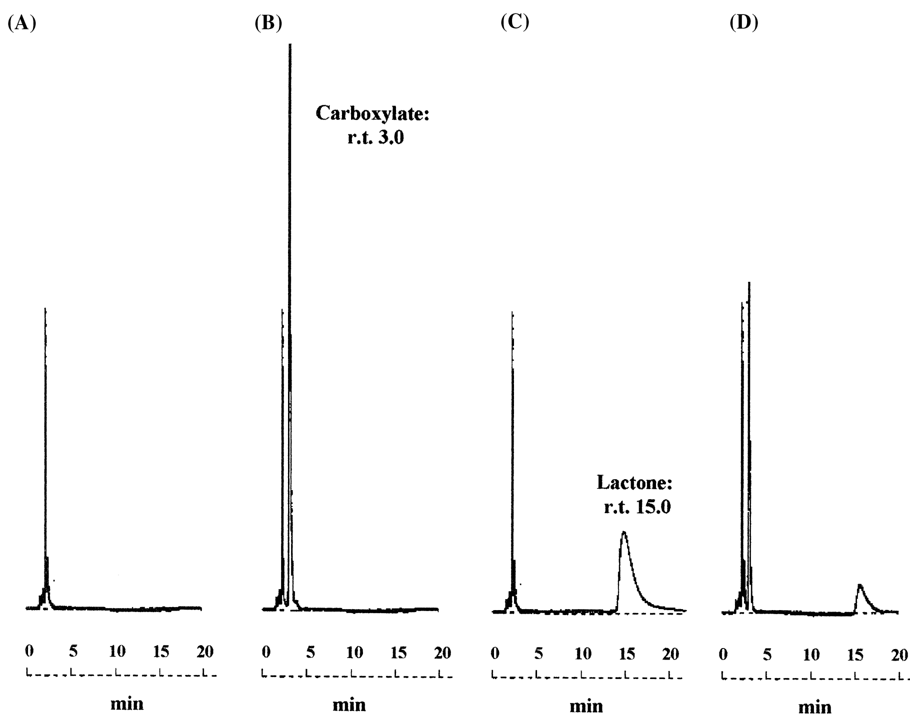

Fig. 6. HPLC chromatograms of carboxylate and lactone form of SN-38. (A) Chromatogram of supernatant of blank cell lysates; (B, C) blank cell lysates with 10 pmol/ml carboxylate or lactone form of SN-38 in the same volume as (A) for a calibration curve; (D) sample of a supernatant of cellular lysates at 1 min of experiment (see detail in Section 2.6).

3.3. Method validation

Within- and between-day reproducibilities for the carboxylate and lactone forms of SN-38 solutions are shown in Table 3. The coefficient of variation (C.V.) of peak areas for each was less than 3%. The accuracy of the method (within-day), expressed by the bias, varied between −1.0 and 1.1% for carboxylate form, and between −0.2 and 1.7% for lactone form, respectively. The between-day accuracy varied between −5.5 and −1.8% for carboxylate form, and between 3.7 and 4.1% for lactone form, respectively. This result suggests that the carboxylate form degraded within a few days.

The linearity study was carried out with concentrations ranging from 4 to 5000 pmol/ml of either the carboxylate form in 0.05 M NaOH or the lactone form in DMSO. The linearity of the calibration lines obtained by plotting peak areas against concentration was examined. The equations for the carboxylate and lactone forms of SN-38 were $y = 2362.5x + 330.6$

Table 3
Precision (C.V.) and accuracy (bias) in the HPLC assay

Concentration (pmol/ml)	Carboxylate ($n = 3$)		Lactone ($n = 3$)	
	C.V. (%)	Bias	C.V. (%)	Bias
Within-day				
50	2.1	−1.0	2.7	1.7
500	2.3	1.1	1.7	−0.2
5000	1.0	0.0	1.5	0.0
Between-day				
50	0.9	−5.5	2.5	4.0
500	2.6	−1.8	2.7	4.1
5000	2.5	−2.8	3.0	3.7

($r^2 = 1.000$) and $y = 3074.5x - 2292$ ($r^2 = 1.000$), respectively. The limit of quantitation (LOQ) was ~4.0 pmol/ml (1.6 ng/ml) for both forms.

3.4. Stability of SN-38 solution

To quantitate the cellular concentrations of the SN-38 lactone form, an aliquot of lactone form solution in 10 m*M* acetate buffer (pH 3.0) was added to several solvents. The stability of the SN-38 lactone form at 37 °C in water (pH 6.0), PBS (pH 7.4), human plasma (pH 7.4) and RPMI 1640 (pH 7.4) using this HPLC method is shown in Fig. 7. In water the proportion of lactone form remained unchanged for 3 h, and thus water is the most suitable solution for sample preparation prior to HPLC quantitation. The half-life ($t_{1/2}$) values of the SN-38 lactone form in PBS, human plasma and RPMI 1640 medium were 0.51, 0.63 and 0.26 h, respectively.

3.4.1. Quantitation of cellular SN-38 concentration

To estimate the ratio of carboxylate/lactone forms of SN-38 in PC-6 cells, we measured the cellular concentrations of the carboxylate and lactone forms after incubation with 10 m*M* of SN-38 (Fig. 8). The lactone form accounted for about 50% of the total cellular SN-38 concentration. However, in the RPMI medium, the concentration ratio of the SN-38 carboxylate form to the lactone form was 97:3. These results suggested that a large portion of the carboxylate form was converted to the lactone form

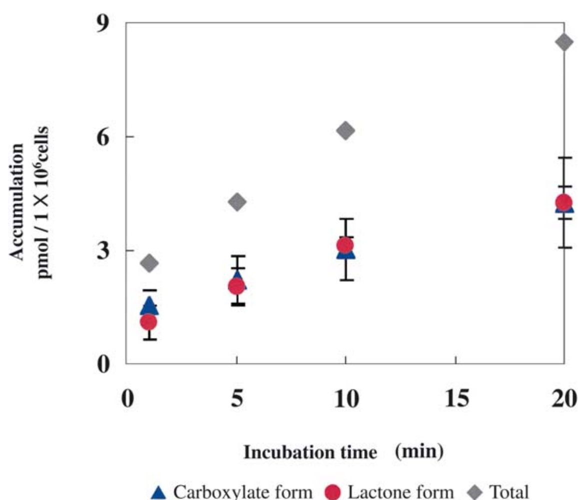

Fig. 8. Cellular concentration of SN-38 in PC-6 cells. Accumulation is shown as cellular concentrations of each form of SN-38 in PC-6 cells (1×10^6) after incubation for 1, 5, 10, 20 min at 37 °C.

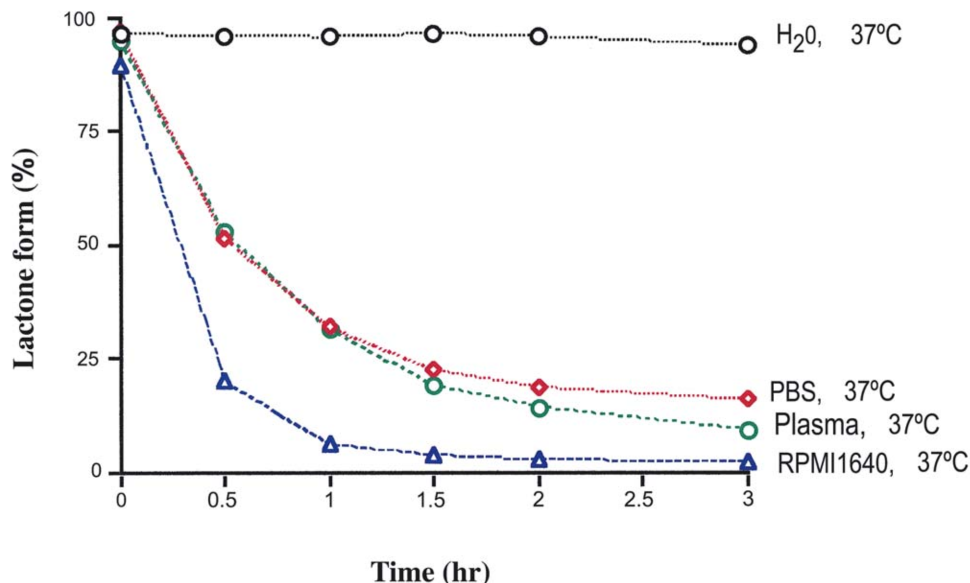

Fig. 7. Stability of SN-38 lactone form in several solutions. Alteration of the SN-38 lactone form concentrations after addition to water (pH 6.0), PBS (pH 7.4), human plasma (pH 7.4) and RPMI 1640 (pH 7.4) for 0.5, 1, 1.5, 2 and 3 h at 37 °C.

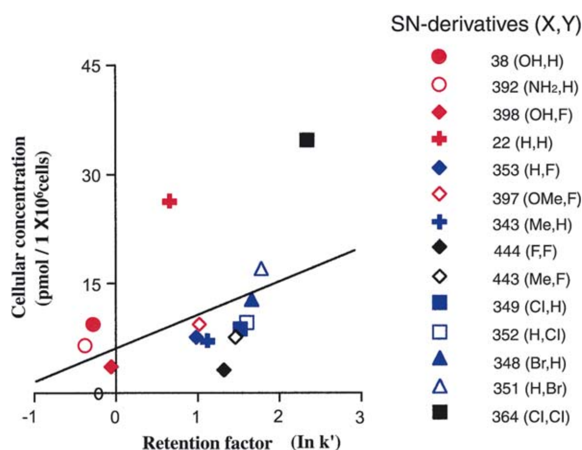

Fig. 9. Relationship between HPLC retention factors (ln k') and the cellular concentration of SN-38 and the other 14 derivatives in PC-6 cells.

under acidic cellular conditions, or that only the lactone form was taken up into cells. A follow-up study is currently underway to investigate this further.

3.5. Interrelation between cellular concentrations of derivatives and HPLC retention

The 15 SN-38 derivatives were separated under the same conditions; 50 m*M* phosphate buffer (pH 6.0)–acetonitrile–tetrahydrofuran (THF) in a ratio of 60:40:2 (v/v). The HPLC retention factors (ln k') were plotted against cellular concentrations in the PC-6 cell line (Fig. 9). A weak linear relationship between the HPLC retention factors (ln k') and the cellular concentrations of these compounds was observed ($Y = 4.875X + 6.568$, $r^2 = 0.202$). This result suggests that low-polarity compounds tend to accumulate in cells.

4. Concluding remarks

We have described a simple and cost-effective HPLC method without an ion-pairing agent that allows the simultaneous determination of the lactone and carboxylate forms of SN-38 and 14 SN-38 derivatives. Using this HPLC method, we determined the cellular concentrations of the lactone and carboxylate forms of SN-38. A linear relationship between ln k' and cellular concentrations of these compounds was observed. This suggests that low-polarity compounds easily accumulate in cells and can overcome drug resistance. Our new HPLC method will be useful for investigations related to the breast cancer resistance protein (BCRP) ABCG2, a transporter that exports SN-38 [21,22]. Furthermore, this observation provides valuable insight into derivative synthesis and chemical modification of antitumor drugs.

References

[1] S. Sawada, S. Okajima, R. Aiyama, K.-I. Nokata, T. Furuta, T. Yokokura, E. Sugino, K. Yamaguchi, T. Miyasaka, Chem. Pharm. Bull. 39 (1991) 1446.
[2] M. Wall, M. Wani, C. Cook, K. Palmer, A. McPhail, G. Slim, J. Am. Chem. Soc. 88 (1966) 3888.
[3] Y.-H. Hsiang, R. Hertzberg, S. Hecht, L.F. Liu, J. Biol. Chem. 260 (1985) 14873.
[4] R.P. Hertzberg, R.W. Busby, M.-J. Caranfa, K.G. Holden, R.K. Johnson, S.M. Hecht, W.D. Kingsbury, J. Biol. Chem. 265 (1990) 287.
[5] M. Redinbo, L. Stewart, P. Kuhn, J. Champoux, W. Holl, Science 279 (1998) 1504.
[6] L. Stewart, M. Redinbo, X. Qui, W. Hol, J. Champoux, Science 279 (1998) 1534.
[7] Y. Kawato, M. Aonuma, Y. Hirota, H. Kuga, K. Sato, Cancer Res. 51 (1991) 4187.
[8] W.J. Slichennmyer, E.K. Rowinsky, R.C. Donehower, S.H. Kaufmann, J. Natl. Cancer Inst. 85 (1993) 271.
[9] J. Fassberg, V.J. Stella, J. Pharm. Sci. 81 (1992) 676.
[10] T.G. Burke, Z. Mi, Anal. Biochem. 212 (1993) 285.
[11] Z. Mi, T.G. Burke, Biochemistry 33 (1994) 10325.
[12] L.P. Rivory, J. Robert, J. Chromatogr. B 661 (1994) 133.
[13] Y. Sasaki, Y. Yoshida, K. Sudoh, H. Hakusui, H. Fujii, T. Ohtsu, H. Wakita, T. Igarashi, K. Itoh, Jpn. J. Cancer Res. 86 (1995) 111.
[14] X.-Y. Chu, Y. Kato, K. Niinuma, K. Sudo, H. Hakusui, Y. Sugiyama, J. Pharmacol. Exp. Ther. 281 (1997) 304.
[15] N. Kaneda, Y. Hosokawa, T. Yokokura, Biol. Pharm. Bull. 20 (1997) 815.
[16] D.L. Warner, T.G. Burke, J. Chromatogr. B 691 (1997) 161.
[17] A. Kurita, N. Kaneda, J. Chromatogr. B 724 (1999) 335.
[18] G. Boyd, J.F. Smyth, D.I. Jodrell, J. Cummings, Anal. Biochem. 297 (2001) 15.
[19] T. Andoh, K. Ishii, Y. Suzuki, Y. Ikegami, Y. Kurunoki, Y. Takemoto, K. Okada, Proc. Natl. Acad. Sci. USA 84 (1987) 5565.
[20] A. Fujimori, M. Gupta, Y. Hori, Y. Pommir, Mol. Pharmacol. 50 (1996) 1472.

[21] K. Nakatomi, M. Yoshikawa, M. Oka, Y. Ikegami, S. Hayasaka, K. Sano, K. Shiozawa, S. Kawabata, H. Soda, T. Ishikawa, S. Tanabe, S. Kohno, Biochem. Biophys. Res. Commun. 288 (2001) 827.

[22] S. Kawabata, M. Oka, K. Shiozawa, K. Tsukamoto, K. Nakatomi, H. Soda, M. Fukuda, Y. Ikegami, K. Sugahara, Y. Yamada, S. Kamihira, L.A. Doyle, D.D. Ross, S. Kohno, Biochem. Biophys. Res. Commun. 280 (2001) 1216.