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Sensitive high-performance liquid chromatographic method for the determination of the lactone form and the lactone plus hydroxy-acid forms of the new camptothecin derivative DX-8951 in human plasma using fluorescence detection

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Abstract

A sensitive quantitation of the lactone form and the lactone plus hydroxy-acid forms of DX-8951, a camptothecin derivative, in human plasma has been investigated by high-performance liquid chromatography (HPLC). This assay method consisted of two analytical procedures. In Procedure I, the lactone form was collected by the stepwise separation on a C_{18} cartridge. In Procedure II, the lactone plus hydroxy-acid forms were collected using another batch of the plasma sample by co-elution of the two forms from a C_{18} cartridge with acidic solution. The hydroxy-acid form of DX-8951 was quantitated from the difference of the lactone plus hydroxy-acid forms and the lactone form. Thereafter, these pre-treated samples were assayed by HPLC under the same HPLC conditions with a spectrofluorometer and a reverse-phase ODS column. The mobile phase was acetonitrile/0.05 *M* potassium dihydrogen phosphate (pH 3) (18:82, v/v) at a flow-rate of 1.0 ml/min. For the assay of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 in plasma, analytical method were validated over the range 0.2–50 ng/ml.

Keywords: Camptothecin

1. Introduction

DX-8951f, a camptothecin derivative, is a promising new antitumor agent currently undergoing Phase I clinical trials. The drug exhibited high topoisomerase I inhibition and also high antitumor activity in vitro. Furthermore, the drug was found to affect the transport of P-glycoprotein-mediated multidrug resistant cell lines [1–3].

The anhydrous free base of DX-8951f is referred to as DX-8951. DX-8951 contains a lactone ring in its molecule. As shown in Fig. 1, the lactone form is in equilibrium with the hydroxy-acid form. In a pharmacological study in vitro, the antitumor activity of the lactone form was found higher than that of the hydroxy-acid form. Thus, quantitation of the separation of these two forms is important to evaluate the pharmacokinetics and pharmacodynamics of DX-8951f in clinical trials.

Other camptothecin derivatives such as irinotecan also contain lactone rings. In the assay of irinotecan, simultaneous quantitation of the lactone and the hydroxy-acid forms was reported by HPLC, but the

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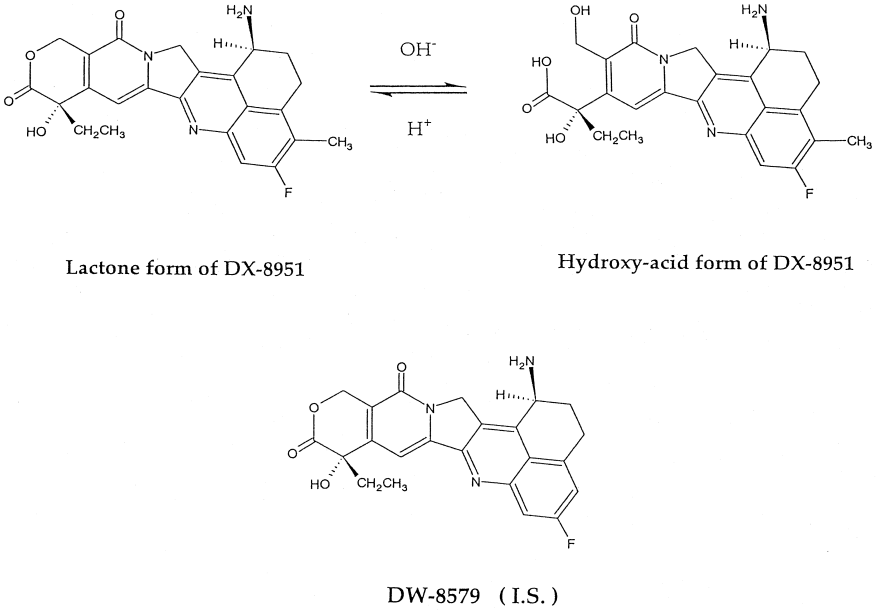

Fig. 1. Chemical structures of the lactone and hydroxy-acid forms of DX-8951 and DW-8579.

method was less sensitive [4–6]. In this paper, we described a new sensitive HPLC method for the lactone form and the lactone plus hydroxy-acid forms in human plasma using a fluorometric detector. The validation of this HPLC method was also described.

2. Experimental

2.1. Chemicals and reagents

DX-8951f,(1*S*, 9*S*)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-4-methyl-10*H*, 13*H*-benzo[*de*]pyrano-[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13-dione monomethanesulfonate (salt), dihydrate was synthesized at Daiichi Pharmaceutical Co., Ltd. (Tokyo) [7]. DW-8579, (1*S*,9*S*)-1-amino-9-ethyl-5-fluoro-1,2,3,9,12,15-hexahydro-9-hydroxy-10*H*,13*H*-benzo[*de*]pyrano-[3′,4′:6,7]-indolizino-[1,2-*b*]quinoline-10,13-dione hydrochloride hydrate, was synthesized at Daiichi Pharmaceutical Co., Ltd. (Tokyo) and used as an internal standard (I.S.). Methanol (MeOH) and acetonitrile (MeCN) were of HPLC-grade (Kanto Chemical Co. Inc., Tokyo, Japan). All other chemicals were of analytical reagent grade and used without purification. Water purified by a Mill-Q system (Millipore, Milford, MA, USA) was used. Drug-free human plasma samples were obtained from healthy donors.

2.2. Instruments and chromatographic conditions

The chromatographic system consisted of a Waters 600E Multi solvent pump system (Millipore Co., Milford, MA, USA), a reverse-phase TSKgel ODS-80Ts column (250×4.6 mm I.D.) (TOSOH Co., Tokyo, Japan), and a Waters 474 scanning fluorescence detector (Millipore Co., Milford, MA, USA) set at an excitation wavelength of 365 nm and an emission wavelength of 445 nm. The column temperature was kept at 30°C in a model RCM-100 column oven (Millipore Co., Milford, MA, USA). To a HPLC system with a Waters 717plus autosampler (Millipore Co., Milford, MA, USA), 0.1 ml aliquotes of samples were injected automatically. The mobile phase, which had been degassed in an ultrasonic bath before use, was a mixture (18:82, v/v) of MeCN–0.05 *M* potassium dihydrogen phosphate (pH 3 adjusted with phosphoric acid). The flow-rate was

1.0 ml/min. Fluorescence output signal was monitored and integrated using Millennium 2010J (ver. 2.10.) Chromatography Manager software (Millipore Co., Milford, MA, USA).

2.3. Preparation of standard solutions

Standard solutions of the lactone form were prepared in 0.04 *M* Britton–Robinson buffer (pH 3) and diluted with the same solvent. Standard solutions of hydroxy-acid form for Procedure II were prepared in 0.04 *M* Britton–Robinson buffer (pH 11) followed by standing for more than 24 h at 4°C to complete the hydrolysis of the lactone ring, and diluted with the same solvent. Standard solutions of DW-8579, I.S., was prepared in 0.04 *M* Britton–Robinson buffer (pH 3) (20 ng/ml). The standard solutions were stored at 4°C under dark conditions. The drug concentration was expressed as equivalents of DX-8951 (the free from of DX-8951f).

2.4. Sample preparation

For the analytical procedure of the lactone form (Procedure I, Fig. 2), a C_{18} Bond Elute cartridge (1 g) (Varian, Harbor City, CA, USA) was used for the extraction of the lactone form from a 0.5 ml human plasma sample. The cartridge was pre-conditioned with 4 ml of MeOH, followed by 4 ml of purified water prior to sample application. The 0.5 ml human plasma sample was diluted with 2 ml of 0.01 *M* phosphate buffer (pH 7.4) and then applied to the conditioned cartridge. For removing of the hydroxy-acid form, the cartridge was washed with 4 ml of purified water followed by 4 ml of water–MeOH (50:50, v/v). Then the lactone form was eluted with 4 ml aliquots of MeOH–1 *M* HCl (99:1, v/v). To the eluent, I.S. was added [0.05 ml of DW-8579, 20 ng/ml in 0.04 *M* Britton–Robinson buffer (pH 3)]. And the eluent was evaporated to dryness by a centrifugal evaporator (Model EC57C, Sakuma, Tokyo, Japan). The residue was dissolved in MeCN–

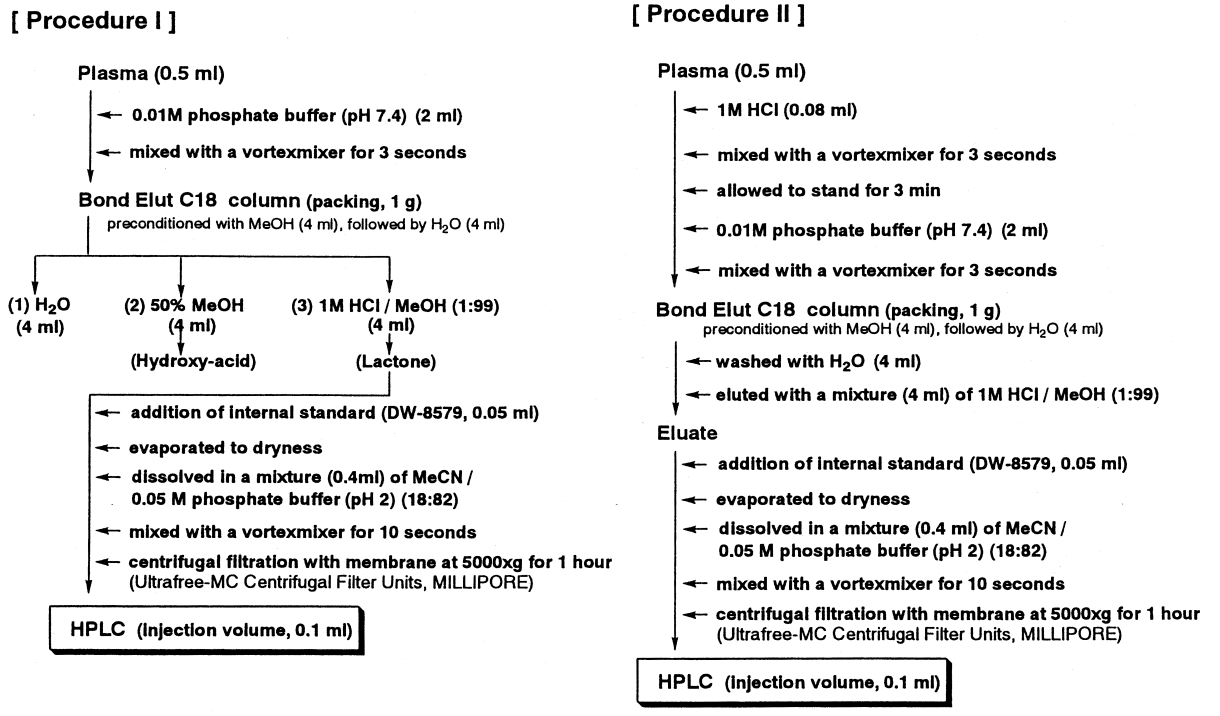

Fig. 2. Determination procedures for the lactone form (Procedure I) and the lactone plus hydroxy-acid forms (Procedure II) of DX-8951 in human plasma by solid-phase extraction.

0.05 *M* phosphate buffer (pH 2) (18:82, v/v) and filtrated with Ultrafree-MC centrifugal filter (Millipore Co., Milford, MA, USA) (5000 *g*, 1 h). A 0.1 ml aliquot of the supernatant was injected onto the chromatographic system.

For the analytical procedure of the lactone plus hydroxy-acid forms (Procedure II, Fig. 2), a C_{18} Bond Elute cartridge (1 g) (Varian, Harbor City, CA, USA) was used. The cartridge was pre-conditioned with 4 ml of MeOH, followed by 4 ml of purified water prior to sample application. The 0.5 ml human plasma sample was mixed with 0.08 ml of 1 *M* HCl and the mixture was allowed to stand for 3 min at room temperature. After dilution with 2 ml of 0.01 *M* phosphate buffer (pH 7.4), the sample was applied to the conditioned cartridge. The cartridge was then washed with 4 ml of purified water. The lactone plus hydroxy-acid forms were eluted with 4 ml aliquots of MeOH–1 *M* HCl (99:1, v/v). To the eluent, I.S. was added [0.05 ml of DW-8579, 20 ng/ml in 0.04 *M* Britton–Robinson buffer (pH 3)]. And the eluent was evaporated to dryness by a centrifugal evaporator (Model EC57C, Sakuma, Tokyo, Japan). The residue was dissolved in MeCN–0.04 *M* phosphate buffer (pH 2) (18:82, v/v) and the sample was filtrated with Ultrafree-MC centrifugal filter (Millipore Co., Milford, MA, USA) (5000 *g*, 1 h). A 0.1 ml aliquot of the supernatant was injected onto the chromatographic system.

2.5. Calibration curves

Calibration curves were construction by plotting the peak area ratio of the lactone form of DX-8951 to I.S. against the concentration of the lactone form of DX-8951. The data were least squares linear regression fitted with a weighing factor of 1/concentration.

2.6. Absolute recovery

The absolute recovery of the lactone and hydroxy-acid forms from human plasma were estimated by comparing the peak area obtained from injections of standard solutions with those obtained from the injection of extracts of plasma samples spiked with known concentrations of the lactone and hydroxy-acid form of DX-8951.

2.7. Selectivity

Six blank plasma samples obtained from six healthy volunteers were assayed by the procedure as described above to evaluate the selectivity of the method.

2.8. Precision and accuracy

Intra-day precision and accuracy of the method were evaluated by replicate analyses ($n=6$) of the plasma samples. Inter-day precision and accuracy were determined by assaying the plasma samples on six separate days. The low limit of quantitation was chosen as the concentration of the lowest calibration standard with an acceptable limit of variance (within 20% for both precision and accuracy).

2.9. Stability

The stability of the lactone form was tested by standing plasma samples at 24°C over 24 h and during three quick freeze–thaw cycles. The samples were thawed quickly in cold water and freezed in dry ice–MeOH quickly. The amount of the lactone form was determined by Procedure I.

The long-term stability of the lactone form and the lactone plus hydroxy-acid forms in human plasma stored at −80°C and −20°C over a six-month period was determined in separate experiments by repeating analysis of the same set of plasma samples.

2.10. Analysis of patient samples

To demonstrate the utility of the analytical methods, blood samples from a patient were obtained at 0.25, 0.45, 1, 1.5, 2.5, 3.5, 4.5, 6.5, 8.5, 10.5 and 24 h after the start of the 30 min continuous infusion (0.1 mg/m^2) in a Phase I clinical trial. The plasma was collected immediately and stored at −80°C.

3. Results and discussion

3.1. Analytical procedure using a C_{18} cartridge

3.1.1. Stepwise elution of the lactone form (Procedure I)

Using a C_{18} cartridge, we tried to separate the lactone form from the hydroxy-acid form by a stepwise elution. In this procedure (Procedure I), a separation of the lactone and hydroxy-acid forms was examined using the following plasma samples. Using the plasma samples spiked with the hydroxy-acid form (1.74, 6.95 ng/ml), the hydroxy-acid form was eluted in the first fraction (50% MeOH) with the recoveries of 92%. Using the plasma samples spiked with the lactone form (0.50–9.06 ng/ml), the lactone form was eluted in the second fraction (1 N HCl/MeOH) with sufficient recoveries of 96.6–97.0% (Table 1). From these recovery data, it was confirmed that the lactone form was quantitatively separated from the hydroxy-acid form, and that recovery of the lactone form were acceptable.

3.1.2. Co-elution of the lactone plus hydroxy-acid forms (Procedure II)

As shown in Fig. 1, the equilibrium between the lactone and hydroxy-acid forms was shifted to the lactone form under acidic conditions. Taking into account this chemical property, we tried to co-elute the two forms from a C_{18} cartridge with an acidic eluting solution. In this procedure (Procedure II), recovery was examined as follows: The plasma samples spiked with the hydroxy-acid form (0.50–9.06 ng/ml) were applied to the C_{18} cartridge. Then the elution was conducted under acidic conditions. The recoveries were found to be from 105.0 to 108.4% (Table 1). Judging from these recovery data, we decided to analyze the lactone plus hydroxy-acid forms by co-elution with an acidic eluting solution.

3.2. Chromatography and selectivity

Chromatograms of plasma extracts obtained with (A) blank human plasma and (B) blank human plasma spiked with the lactone form of DX-8951 (0.186 ng/ml) and the internal standard (I.S.) after treatment of Procedure I was shown in Fig. 3. These chromatograms indicated that no endogenous compounds interfered with the detection of the lactone form and the I.S. at their retention times. The retention times of the lactone form and the I.S. were approximately 26 and 30 min, respectively.

3.3. Calibration curves

Calibration curves ($n=6$) for the lactone form prepared by Procedure I on every sixth day were linear over the concentrations examined (0.186–11.9 ng/ml) and reproducible with mean±standard deviation values for the constants in the regression equation of $y=(0.618\pm0.019)x+(-0.029\pm0.005)$. Coefficients of determination (r^2) were greater than 0.998. The inter-day coefficient of variation (CV) of the slope of the calibration curves was 0.03%.

Calibration curves ($n=6$) for the lactone plus hydroxy-acid forms prepared by Procedure II on every sixth day were linear over the concentrations examined (0.186–11.9 ng/ml) and reproducible with mean±standard deviation values for the constants in the regression equation of $y=(0.632\pm0.018)x+$

Table 1
Recoveries of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 and I.S. from human plasma by Procedure I and II

	Concentration (ng/ml)	Lactone (Procedure I) Recovery (%) Mean±SD[b] ($n=5$)	Total[a] (Procedure II) Recovery (%) Mean±SD[b] ($n=5$)
DX-8951	0.50	96.9±3.4	108.4±0.8
	4.53	97.0±3.2	105.0±1.2
	9.06	96.6±4.8	105.7±1.2
I.S.	20.33	106.3±3.2	110.7±2.2

[a] Total = the lactone plus hydroxy-acid forms.
[b] SD = standard deviation.

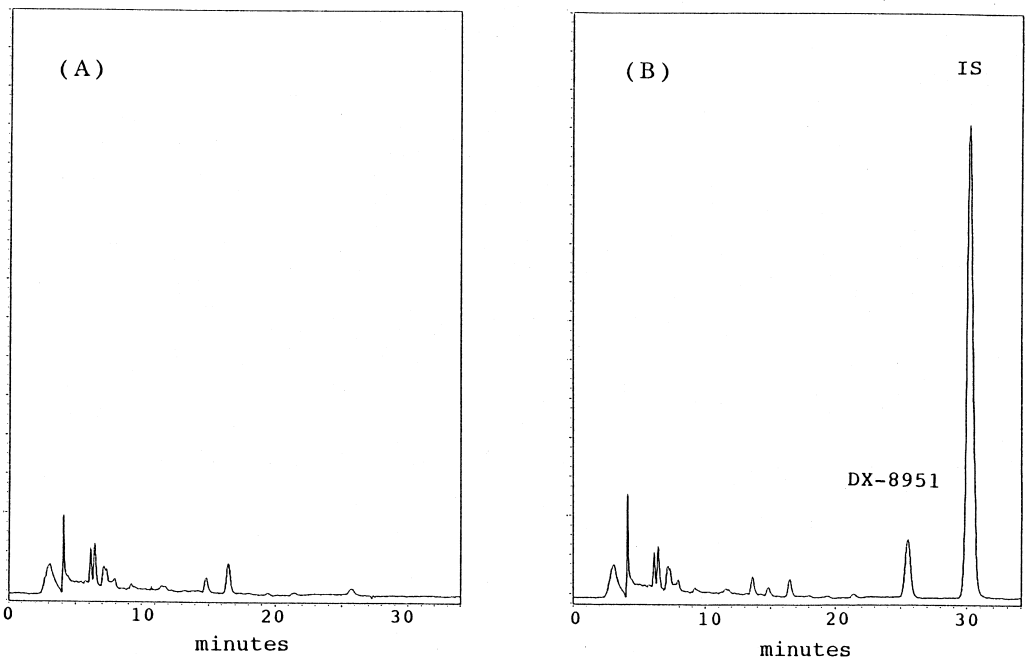

Fig. 3. Representative chromatograms of plasma extracts obtained with (A) blank human plasma and (B) blank human plasma spiked with the lactone form of DX-8951 (0.186 ng/ml) and the internal standard (I.S.) after treatment of Procedure I.

(-0.029 ± 0.006). Coefficients of determination (r^2) were greater than 0.999. The inter-day coefficient of variation (CV) of the slope of the calibration curves was 0.03%.

3.4. Precision and accuracy

The intra-day precision and accuracy were evaluated by analyzing the plasma spiked with the lactone form (Procedure I) or the lactone plus hydroxy-acid forms (Procedure II) at concentrations of 0.186 to 11.9 ng/ml in six replicates (Table 2). Precision was based on the calculation of the relative error (RE) of the found concentration compared to the theoretical value. The intra-day precision (CV) of this method for the lactone form and the lactone plus hydroxy-acid forms were $<8.4\%$ and $<2.8\%$, respectively. The range of accuracy (RE) for the lactone form was -2.4 to 12.2%. For the lactone plus hydroxy-acid forms, the accuracy ranged from -7.7 to 10.1%. The low limit of quantitation of this method using a 0.5 ml plasma sample was determined as 0.186 ng/ml with acceptable precision and accuracy (within $\pm 20\%$). The inter-day precision and accuracy were assessed by the analyses of plasma samples at concentrations of 0.375 to 6.01 ng/ml on six separate days (Table 3). The inter-day precision (CV) of this method for the lactone form and the lactone plus hydroxy-acid forms were $<2.2\%$ and $<1.5\%$, respectively. The range of accuracy (RE) for the lactone form was -2.2 to 1.0%. For the lactone plus hydroxy-acid forms, the accuracy ranged from -1.9 to -0.7%. These results indicated that the present method has a satisfactory precision, accuracy and reproducibility.

3.5. Stability

Assessment of the stability of the lactone form was very important in this assay method. The stability of the lactone form was measured in vitro in fresh human plasma at 24°C over 24 h. When the samples (lactone: 7.23 ng/ml) were allowed to stand for 0.5 h, the lactone concentration showed 94.0% of the nominal concentration as compared to the freshly spiked samples. However, the concentration de-

Table 2
Intra-day validation of calibration standards for the determination of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 in human plasma by Procedure I and II

Theoretical concentration (ng/ml)	Lactone (Procedure I)			Total[a] (Procedure II)		
	Mean found concentration (ng/ml, $n=6$)	CV[b] (%)	RE[c] (%)	Mean found concentration (ng/ml, $n=6$)	CV[b] (%)	RE[c] (%)
0.186	0.209	8.4	12.2	0.205	2.5	10.1
0.372	0.365	1.6	−1.9	0.369	1.0	−0.9
0.744	0.730	0.7	−1.9	0.686	2.2	−7.7
1.490	1.470	2.5	−1.3	1.540	0.4	3.1
2.980	2.910	3.1	−2.4	2.920	0.6	−1.9
5.950	6.070	1.4	1.9	5.800	2.8	−2.5
11.900	12.000	1.6	1.2	12.800	2.4	7.9

[a] Total = the lactone plus hydroxy-acid forms.
[b] Coefficient of variation.
[c] RE = relative error.

Table 3
Inter-day validation of calibration standards for the determination of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 in human plasma by Procedure I and II

Theoretical concentration (ng/ml)	Lactone (Procedure I)			Total[a] (Procedure II)		
	Mean found concentration (ng/ml, $n=6$)	CV[b] (%)	RE[c] (%)	Mean found concentration (ng/ml, $n=6$)	CV[b] (%)	RE[c] (%)
0.375	0.379	2.2	1.0	0.372	1.5	−0.7
3.000	2.935	1.7	−2.2	2.944	1.0	−1.9
6.010	5.964	0.9	−0.7	5.942	0.4	−1.1

[a] Total = the lactone plus hydroxy-acid forms.
[b] Coefficient of variation.
[c] RE = relative error.

creased rapidly after 1 h (Table 4). This instability necessitates quick freezing of the samples after blood sampling in order to stabilize the relatively unstable lactone form.

The samples treated through freeze/thaw cycle three times before analysis showed 100.9, 97.3 and 97.1% of the nominal concentration as compared to the freshly spiked samples. The results are presented in Table 5.

The stability under long-term storage conditions at −80°C and −20°C were evaluated over a period of six months. Plasma samples were analyzed using freshly spiked calibration curves. When the samples (lactone: 0.85, 8.5 ng/ml) were allowed to stand at

Table 4
Stability of the lactone form of DX-8951 in human plasma at 24°C

Time (h)	Theoretical concentration (ng/ml) Lactone (Procedure I)	
	7.23 ng/ml Stability (%) Mean±SD[a] ($n=3$)	28.9 ng/ml Stability (%) Mean±SD[a] ($n=3$)
0	100.0	100.0
0.25	101.8±0.9	105.2±1.6
0.5	94.0±0.2	100.3±2.0
1	86.6±1.7	89.5±2.7
2	69.2±1.4	65.0±2.9
6	60.8±0.1	18.9±0.9
24	33.6±0.9	9.6±4.7

[a] SD = standard deviation.

Table 5
Freeze/thaw stability of the lactone form of DX-8951 in human plasma

Freeze/thaw cycle	Theoretical concentration of the lactone form of DX-8951 (Procedure I)		
	0.484 ng/ml Stability (%) Mean±SD[a] ($n=3$)	2.42 ng/ml Stability (%) Mean±SD[a] ($n=3$)	9.67 ng/ml Stability (%) Mean±SD[a] ($n=3$)
0	100.0	100.0	100.0
3	100.9±4.2	97.3±1.2	97.1±0.9

[a] SD=standard deviation.

−80°C for six months, the concentration of the lactone form showed 117.8 and 103.0% of the nominal concentration as compared to the freshly spiked samples, and the concentration of the lactone plus hydroxy-acid forms also showed 114.4 and 97.8% (Table 6). When the samples (lactone: 0.85, 8.5 ng/ml) were allowed to stand at −20°C for six months, the lactone concentrations were 12.7 and 13.1% of the nominal concentration as compared to the freshly spiked samples, the lactone plus hydroxy-acid forms showed 101.8 and 98.7% (Table 6). Acceptable stability for the lactone form has been demonstrated for storage at −80°C for six months. Instability of the lactone form at −20°C was also demonstrated, suggesting a rapid change of the lactone form to the hydroxy-acid form. The lactone plus hydroxy-acid forms was found to be stable for at least six months when stored at −80°C and −20°C.

3.6. Analysis of patient samples

The plasma concentration versus time profile of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 after the 30 min continuous infusion of DX-8951f (0.1 mg/m^2) was shown in Fig. 4. As the result of the assayed data, the low limit of quantitation of 0.186 ng/ml is sufficient to measure the concentrations of the drug in plasma at the lowest dose level of DX-8951f used in Phase I clinical trials.

Table 6
Stability of the lactone form and the lactone plus hydroxy-acid forms of DX-951 in human plasma at −20°C and −80°C

Temperature (°C)	Time	Theoretical concentration (ng/ml)			
		Lactone (Procedure I)		Total[a] (Procedure II)	
		0.85 ng/ml Stability (%) Mean±SD[b] ($n=3$)	8.5 ng/ml Stability (%) Mean±SD[b] ($n=3$)	0.85 ng/ml Stability (%) Mean±SD[b] ($n=3$)	8.5 ng/ml Stability (%) Mean±SD[b] ($n=3$)
−20	1 day	103.1±3.6	88.0±2.2	109.7±1.8	101.7±2.5
	1 week	80.5±3.1	80.8±2.2	94.7±2.8	96.2±2.2
	3 weeks	72.9±1.6	63.8±5.1	107.3±4.1	95.8±8.1
	3 months	29.7±0.8	28.2±1.1	116.7±4.9	118.4±1.5
	6 months	12.7±1.6	13.1±1.2	101.8±1.6	98.7±6.6
−80	1 day	106.0±1.9	92.4±3.3	110.3±2.7	101.2±5.2
	1 week	93.1±2.1	94.5±3.6	94.7±1.9	97.7±2.9
	3 weeks	116.8±7.2	108.3±1.5	115.3±3.9	106.2±1.9
	3 months	119.9±9.8	115.3±1.6	114.6±4.8	111.4±2.0
	6 months	117.8±8.0	103.0±2.0	114.4±9.5	97.8±2.5

[a] Total=the lactone plus hydroxy-acid forms.
[b] SD=standard deviation.

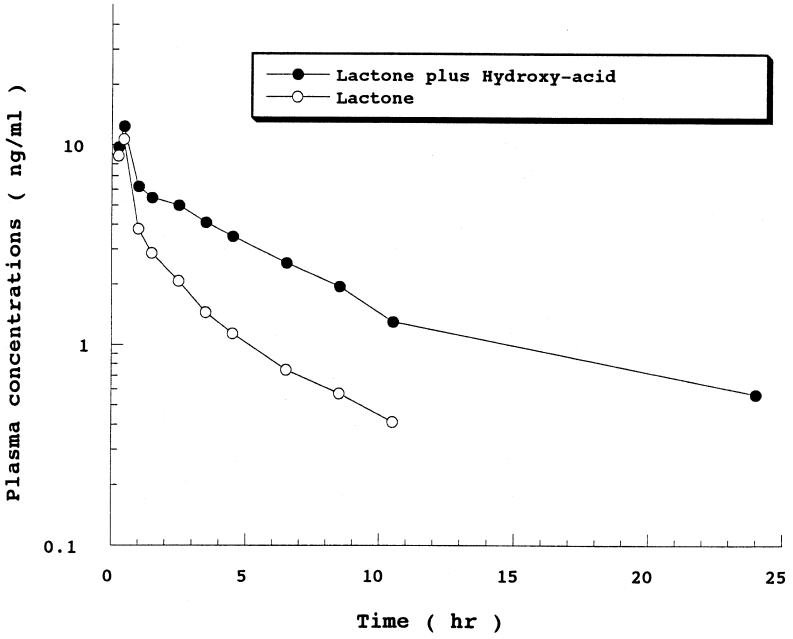

Fig. 4. The plasma concentration versus time profile of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 after the 30 min continuous infusion of DX-8951f (0.1 mg/m^2).

4. Conclusions

A sensitive and selective analytical method for the determination of the lactone form and the lactone plus hydroxy-acid forms of DX-8951 in human plasma was established by HPLC. This method proved to be suitable for clinical pharmacokinetic studies of DX-8951f.

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