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Validation study of assay method for DE-310, a novel polymer-bound camptothecin derivative, and the free drug in mouse plasma by liquid chromatography with fluorimetric detection

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Abstract

DE-310 is a macromolecular carrier conjugate containing an anti-tumor camptothecin derivative, DX-8951, which is conjugated to a water-soluble polymer via a peptide spacer. Assay methods have been developed for the determination of a polymer-bonded DX-8951 conjugate, DX-8951, and Glycyl-DX-8951 (G-DX-8951) in mouse plasma. Free DX-8951 and Glycyl-DX-8951 were extracted from plasma by protein precipitation and analyzed by HPLC (Method I). Conjugated DX-8951 was extracted by protein precipitation and digested by using a thermolysin. The productive compound was analyzed by HPLC (Method II). The lower limits of quantitation of DX-8951, Glycyl-DX-8951, and Conjugated DX-8951 were 0.60, and 0.77 ng/ml and 3.45 μg/ml (as DX-8951 equivalent). These two methods showed satisfactory sensitivity, precision, accuracy, recovery, and selectivity.

Keywords: DE-310; Camptothecin

1. Introduction

DE-310 is a macromolecular carrier conjugate containing DX-8951, the free form of DX-8951f (exatecan; monomethanesulfonate salt of (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), which is conjugated to a carboxymethyldextran polyalcohol via a glycyl-glycyl-phenylalanyl-glycyl-peptide spacer (Fig. 1). Several different drug delivery systems have been developed for camptothecin analogues to reduce their severe systemic toxicities and to enhance the anti-tumor effects. DE-310 is designed to permit specific distribution to tumor tissue and intra-tumoral cleavage by lysosomal enzymes owing to "enhanced permeability and retention effect"(EPR effect) [1–3]. This enzymatic cleavage is supposed to provide a desired active product. From a pre-clinical study, it is suggested that cleavage of this linkage releases free DX-8951 and Glycyl-DX-8951 (G-DX-8951) into the blood stream [4]. To study the pharmacokinetics of DE-310, it would be of great help to assay the polymer-bonded DX-8951 (Conjugated DX-8951) and its released drugs (DX-8951 and G-DX-8951) in plasma.

We have recently developed LC/MS/MS methods for determination of DX-8951 in human plasma and urine [5,6]. Furthermore, we have reported HPLC methods for measuring DX-8951 in mouse plasma and human plasma by using a fluorometric detector [7,8]. The LLOQs of DX-8951 were 3 ng/ml for 100 μl mouse plasma and 0.2 ng/ml for 500 μl human plasma. It is suggested that these HPLC methods with a fluorometric detector are applicable for analyzing for free drugs in mouse plasma. Furthermore, a method for analyzing Conjugated DX-8951 was required for pharmacokinetics studies of DE-310. Some researchers have developed analytical methods for studying polymer-bound anticancer drug. Previous work on a polymer-bound camptothecin derivative (MAG-CPT) has demonstrated that the total levels of CPT (free plus polymer-bound) are determined

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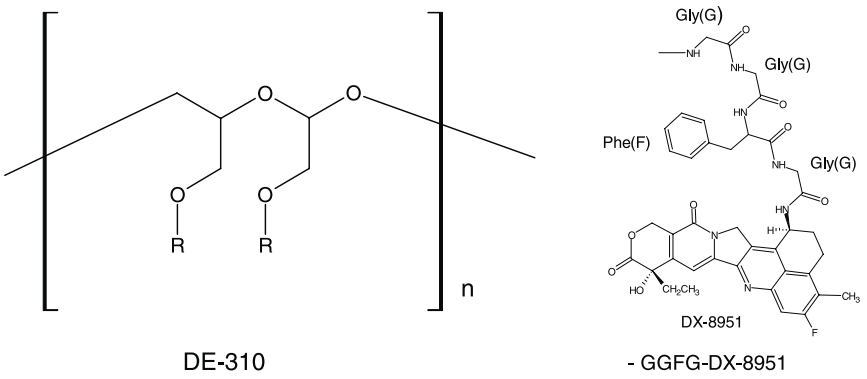

Fig. 1. Chemical structure of DE-310.

after alkaline-mediated hydrolysis of MAG-CPT [9,10]. The LLOQs of this method were 100 ng/ml of total and 1 ng/ml of free CPT for 50 and 250 μl human plasma, respectively. A sensitive HPLC method for a different water-soluble polymer-bound compound, doxorubicin (PK1), has been developed [11]. Total (polymer-bound plus free) doxorubicin was determined after acid hydrolysis to release doxorubicinone as aglycones from free or polymer-bound doxorubicin. The LLOQs of this method were 5.1 ng/ml of bound doxorubicin and 0.38 ng/ml of free doxorubicin for 1 ml human plasma, respectively.

In this study, enzyme digestion was used for the first time to treat samples for analysis of polymer-bound compound in Method II. We report here that enzymatic digestion with thermolysin is a very useful method for the determination of polymer-bound compounds such as DX-8951. Our data suggest that Phenylalanyl-Glycyl-DX-8951 is released from Conjugated DX-8951 after enzyme digestion. In addition, the peak areas derived from Phenylalanyl-Glycyl-DX-8951 are proportional to the concentrations of Conjugated DX-8951. This tendency permits the determination of Conjugated DX-8951 in plasma. The level of Phenylalanyl-Glycyl-DX-8951 in plasma is very low. Thus, it is not necessary to subtract the free drug level from the total drug level to estimate the polymer-bound drug. For the determination of free drug (not conjugated), it is suggested that two major compounds, DX-8951 and G-DX-8951, are released from Conjugated DX-8951 into the blood stream. The level of G-DX-8951 is higher than that of DX-8951 in mouse plasma from our preliminary study. Thus, the assay of Method I estimates DX-8951 and G-DX-8951 in plasma. In this paper, we describe methods for determining DX-8951 and G-DX-8951 (Method I), and Conjugated DX-8951 (Method II) levels in mouse plasma by reversed-phase HPLC with fluorimetric detection. The validations of these methods are also described.

2. Experimental

2.1. Chemicals

DE-310, DX-8951f, and G-DX-8951 were synthesized at Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). 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.). The methanol and acetonitrile used were of HPLC-grade (Kanto Chemical Co. Inc., Tokyo, Japan). All other chemicals were of analytical reagent grade and used without purification. Water was purified by the Milli-Q system (Millipore, Milford, MA, USA).

2.2. Equipment

The chromatographic system consisted of a Waters 600E multi-solvent pump system, a reverse-phase Inertsil ODS-2 column (4.6 mm i.d. × 250 mm for Method I, 6.0 mm i.d. × 150 mm for Method II), and a Waters 474 scanning fluorescence detector (all from 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 45 °C in a Model RCM-100 column oven (Millipore Co.). To the HPLC system equipped with a Waters 717 autosampler (Millipore Co.), 0.1 ml samples were injected for Method I, 0.01 ml samples were injected for Method II. Gradient HPLC was used for Method I with a mobile phase A consisting of 0.1 M sodium acetate buffer (pH 5 adjusted with 0.1 M acetate) and a mobile phase B consisting of acetonitrile. The elution timetable was set as follows: 18% of mobile phase B isocratically for 24 min, then up to 50% of mobile phase B from 24 to 27 min, and down to 18% of mobile phase B from 27 to 32 min, and finally from 32 to 40 min isocratic at 18% of mobile phase B. The mobile phase for Method II was a mixture (13:25:62, v/v/v) of methanol–acetonitrile–0.05 M

Table 1
Summary of assay methods

	Method I	Method II
Analytes	Released compounds	Conjugated DX-8951 (polymer-bounded DX-8951)
Detection	DX-8951, G-DX-8951	Phenylalanyl-Glycyl-DX-8951 (enzymatic production using thermolysin)

potassium dihydrogen phosphate (pH 3 adjusted with phosphoric acid). The flow rate was set at 1 ml/min. Peak areas for all components were integrated by Millennium^{TM} 2010J (version 2.1.) software. The peak-area ratios (area of DX-8951, G-DX-8951/area of DW-8579) in Method I were plotted versus concentration by weighted linear regression (1/concentration^2). The peak areas of Conjugated DX-8951 in Method II were plotted versus concentration by weighted linear regression (1/concentration^2) (Table 1).

2.3. Preparation of standard solutions

Standard solutions of DX-8951, G-DX-8951, and DW-8579 were prepared in 0.04 M Britton–Robinson buffer (pH 3) and diluted with the same buffer. The concentrations of these compounds were expressed as equivalents of their free bases. We analyzed the conjugated form to its carrier, Conjugated DX-8951, using standard solution of DE-310. Standard solutions of DE-310 were prepared in purified water and diluted with 0.1% Brij35 solution. This compound concentration was expressed as equivalents of DX-8951. These standard solutions were stored at 4 °C under dark conditions.

2.4. Sample preparation

For the analytical procedure of DX-8951 and G-DX-8951 (Method I), a 0.2 ml of standard, quality control (QC), unknown mouse plasma sample and 0.05 ml of internal standard (DW-8579: 30 μg/ml) were placed into a test tube (1.5 ml). A 1 ml volume of 1 M hydrochloric/methanol (1:99, v/v) was added to each tube and mixed thoroughly by vortexing for 3 s, followed by centrifugation at 9000 × *g* for 5 min at 4 °C. The supernatants were transferred into disposable test tubes (13 mm × 100 mm) and were dried by using a rotating evaporator (Model EC57C, Sakuma, Tokyo, Japan). The residues were reconstituted in 0.2 ml of acetonitrile–0.05 M potassium dihydrogen phosphate (pH 3 adjusted with phosphoric acid) with vortexing for 10 s. In general, camptothecin analogues contain a closed α-hydroxy-δ-lactone ring (lactone form) that can undergo reversible hydrolysis to form the open-ring form (carboxylate form). In this step of reconstitution, all the carboxylate form is converted into its lactone form under acidic condition. Therefore, this method estimates concentrations as the total of lactone and carboxylate forms. The solution was then transferred to an ULTRA FREE-MC (30000 NWWL Filter Unit, Millipore), and it was centrifuged at 6000 × *g* for 2 h at 4 °C. The filtrate solution, 0.1 ml, was injected onto the HPLC system.

To analyze Conjugated DX-8951 (Method II), 0.1 ml of standard, QC, unknown mouse plasma sample was placed into a test tube (1.5 ml). A 0.6 ml volume of methanol was added to each tube and mixed thoroughly by vortexing for 3 s, followed by centrifugation at 9000 × *g* for 5 min at 4 °C. The supernatants were transferred into disposable test tubes (13 mm × 100 mm) and were dried by using the rotating evaporator (Model EC57C, Sakuma, Tokyo, Japan). The residues were reconstituted in 0.1 ml of purified water with vortexing for 10 s, followed by addition to the tubes of 0.2 ml of 0.1 M Tris–hydrochloric buffer (pH 8.5) and 0.1 ml of thermolysin solution (thermolysin enzyme was dissolved in 0.1 M calcium chloride solution to give a concentration of 2 mg/ml). The enzymatic reaction was conducted in an incubator at 50 °C for 1 h. The enzymatic reaction was terminated by adding 2.5 ml of 0.5 M hydrochloric/methanol (1:1, v/v) followed by vortexing for 10 s. In this step, the carboxylate form is converted into its lactone form under acidic condition. Therefore, this method estimates concentrations as the total of lactone and carboxylate forms. Finally, 0.01 ml of processed solution was injected onto the HPLC system.

2.5. Validation procedures

The absolute recoveries of DX-8951, G-DX-8951, and Conjugated DX-8951 from mouse plasma were estimated by comparing the peak areas of extracted samples versus those of unextracted standard solutions. The selectivity of the assay was investigated by processing and analyzing six independent blank (drug-free) samples by the procedure described above. Calibration curves were constructed by plotting the peak-area ratio of the analytes to I.S. against the concentration of the analyte in Method I. Each calibration curve consisted of six calibration points ($n = 1$). The data were fitted to least squares linear regression with a weighting factor of 1/(concentration)^2. Calibration curves were constructed by plotting the peak area of the analyte against the concentration of the analyte in Method II. Each calibration curve consisted of five calibration points ($n = 1$). The data were fitted to least squares linear regression with a weighting factor of 1/(concentration)^2. Intra-day precision evaluated as relative standard deviation (RSD) and accuracy of the method were determined by replicate analyses ($n = 5$) of QC samples. Inter-day precision (RSD) and accuracy were determined by assaying the QC samples on five separate days. The lower limit of quantitation (LLOQ) was chosen as the concentration of the lowest calibration standard with an acceptable limit of variance (within 20% for both precision and accuracy). Stability during freezing and thawing was

assessed by using the QC samples after three freeze–thaw cycles. Stability in autosampler was assessed using the QC samples after the reconstituted solutions were prepared and placed into the autosampler for 36 h according to the method described above. Stability during storage at −80 °C was assessed by using the QC samples that had been placed in a storage freezer set at −80 ± 5 °C.

2.6. Application of the method

To demonstrate the reliability of these methods for the study of pharmacokinetics, these assays were used to determine concentrations of DX-8951, G-DX-8951, and Conjugated DX-8951 in male mouse plasma samples. Animals were administrated a single intravenous infusion of DE-310 at a dose of 7 mg/kg. Blood samples were collected from each animal at 4, 8, and 24 h, at 2, 4, 6, 8, 10, 12, and 14 days after the start of the infusion. Samples were stored at −80 °C until analyzed.

3. Results and discussion

Camptothecin derivatives, including DX-8951, contain a lactone ring in the molecule that can undergo reversible hydrolysis to form the carboxylate form at physiological pH. Furthermore, many conventional methods using HPLC have been developed and used routinely to determine the total lactone and carboxylate forms. CPT analogues have strong fluorescence characteristics. Thus, relatively low concentrations of these compounds could be measured in biological matrices without the need for laborious pre-column derivatization procedures. For these reasons, we attempted to develop a method that coupled HPLC with fluorometric detection.

3.1. Recoveries

Recoveries of DX-8951 and G-DX-8951 from spiked mouse plasma were determined at the concentrations of 0.60, 2.40, and 19.18 ng/ml in five replicates by Method I.

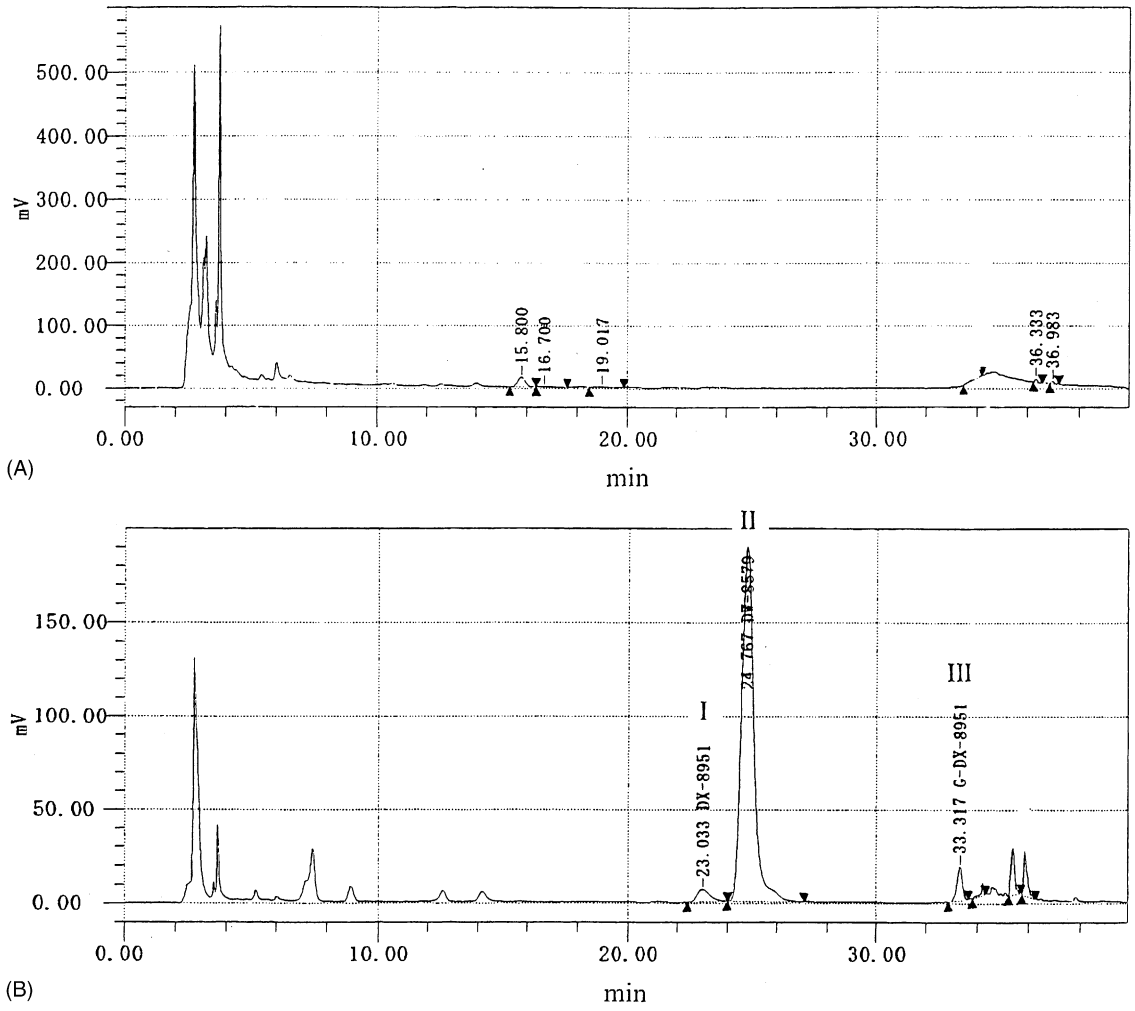

Fig. 2. Chromatograms of extracts of (A) blank plasma and (B) blank plasma spiked with DX-8951 (2.40 ng/ml), G-DX-8951 (3.08 ng/ml), and I.S. (33.92 ng/ml) (I: DX-8951; II: I.S.; and III: G-DX-8951).

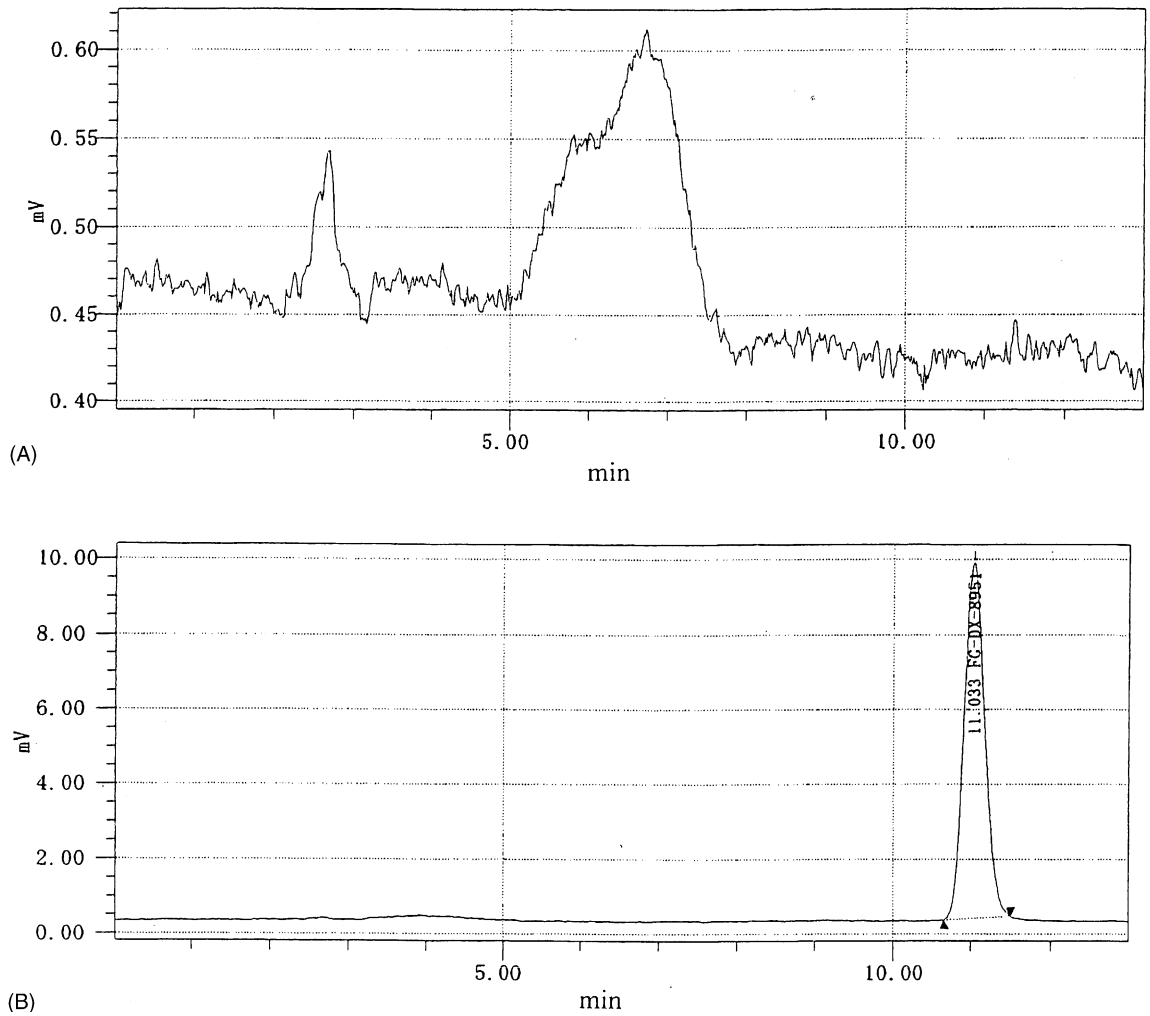

Fig. 3. Chromatograms of extracts of (A) blank plasma and (B) blank plasma spiked with Conjugated DX-8951 (3.48 μg/ml).

The mean recoveries of DX-8951 ranged from 72.0 to 84.0%. The mean recoveries of G-DX-8951 ranged from 77.5 to 95.8%, and that of DW-8579 was 82.7%.

Recoveries of Conjugated DX-8951 from spiked mouse plasma were determined at the concentrations of 3.45, 31.04, and 279.36 μg/ml (DX-8951 equivalent) in five replicates by Method II. The mean recoveries of Conjugated DX-8951 ranged from 88.6 to 90.6%. These extraction methods have been successfully applied to the extraction of DX-8951, G-DX-8951, and Conjugated DX-8951 from mouse plasma.

3.2. Chromatography and selectivity

Chromatograms of plasma extracts obtained with (A) blank mouse plasma and (B) blank mouse plasma spiked with DX-8951 (2.40 ng/ml), G-DX-8951 (3.08 ng/ml), and I.S. (33.92 ng/ml) using Method I are shown in Fig. 2. The retention times of DX-8951, G-DX-8951, and I.S. were approximately 24, 33, and 25 min, respectively. The overall chromatographic run times were within ca. 40 min.

Chromatograms of plasma extracts obtained from (A) blank mouse plasma and (B) blank mouse plasma spiked with Conjugated DX-8951 (3.45 μg/ml) using Method II are given in Fig. 3. The retention time of Conjugated DX-8951 was approximately 10.5 min. The overall chromatographic run time was within ca. 12 min. These chromatograms indicated that no endogenous compounds interfered with the detection of DX-8951, G-DX-8951, and Conjugated DX-8951 at their retention times.

3.3. Calibration curves

Calibration curves of plasma for DX-8951 and G-DX-8951 obtained on five separate days were linear over the concentration ranges of 0.60–19.18 ng/ml (DX-8951) and 0.77–24.65 ng/ml (G-DX-8951). The means of coefficients of determination (r^2) for DX-8951 and G-DX-8951 were 0.996 and 0.981 ($n = 5$), respectively.

Calibration curves of plasma for Conjugated DX-8951 obtained on five separate days were linear over the concentration ranges of 3.45–279.36 μg/ml (DX-8951 equivalent).

Table 2
Intra- and inter-assay precision and accuracy for DX-8951 and G-DX-8951 in mouse plasma using Method I

Analytes	Nominal concentration (ng/ml)	Intra-assay precision and accuracy			Inter-assay precision and accuracy		
		Mean, $n = 5$ (ng/ml)	RSD (%)	Mean accuracy, $n = 5$ (%)	Mean, $n = 5$ (ng/ml)	RSD (%)	Mean accuracy, $n = 5$ (%)
DX-8951	0.60	0.70	4.61	16.00	0.63	12.53	5.33
	2.40	2.53	1.74	5.25	2.44	3.53	1.75
	19.18	21.32	1.80	11.17	20.53	6.87	7.03
G-DX-8951	0.77	0.87	0.81	12.99	0.86	4.83	11.43
	3.08	2.81	3.59	−8.70	2.92	7.41	−5.26
	24.65	27.84	4.41	12.94	27.37	2.37	11.03

Table 3
Intra- and inter-assay precision and accuracy for Conjugated DX-8951 in mouse plasma using Method II

Analyte	Nominal concentration[a] (μg/ml)	Intra-assay precision and accuracy			Inter-assay precision and accuracy		
		Mean, $n = 5$ (ng/ml)	RSD (%)	Mean accuracy, $n = 5$ (%)	Mean, $n = 5$ (ng/ml)	RSD (%)	Mean accuracy, $n = 5$ (%)
Conjugated DX-8951	3.45	3.42	0.88	−0.87	3.31	3.02	−4.06
	31.04	32.04	3.12	3.22	30.32	4.39	−2.32
	279.36	284.97	1.77	2.01	279.99	5.22	0.23

[a] Equivalent as DX-8951.

The mean of coefficients of determination (r^2) was 0.999 ($n = 5$).

3.4. Precision and accuracy

The results of intra- and inter-assay precision and accuracy for DX-8951 and G-DX-8951 are shown in Table 2. The intra-assay precisions (RSD) of DX-8951 and G-DX-8951 estimates for QC samples were less than 4.61 and 4.41%, respectively. The intra-assay accuracies of DX-8951 and G-DX-8951 were within 16.00 and 12.99%, respectively. The intra-assay accuracy of DX-8951 for 0.6 ng/ml (LLOQ level) was 16.00%, but the accuracies of other concentrations were shown to be within 15%. The inter-assay precisions

Table 4
Stability of DX-8951 and G-DX-8951 in mouse plasma

Analytes	Nominal concentration (ng/ml)	After three cycles of freeze/thaw		Nominal concentration (ng/ml)	After storage at −80 °C for 9 weeks	
		Mean, $n = 3$ (ng/ml)	Mean accuracy, $n = 3$ (%)		Mean, $n = 3$ (ng/ml)	Mean difference[a], $n = 3$ (%)
DX-8951	0.60	0.66	9.4	0.96	0.95	−7.8
	2.40	2.39	−0.6	3.84	3.76	3.0
	19.18	21.74	13.3	15.34	15.90	−6.0
G-DX-8951	0.77	0.81	5.2	1.23	1.05	−12.8
	3.08	2.88	−6.5	4.93	4.59	9.1
	24.65	28.10	14.0	19.72	20.00	−3.8

[a] Difference from the concentrations on the first day.

Table 5
Stability of Conjugated DX-8951 in mouse plasma

Analyte	Nominal concentration[a] (μg/ml)	After three cycles of freeze/thaw		Nominal concentration[a] (μg/ml)	After storage at −80 °C for 9 weeks	
		Mean, $n = 3$ (μg/ml)	Mean accuracy, $n = 3$ (%)		Mean, $n = 3$ (μg/ml)	Mean difference[b], $n = 3$ (%)
Conjugated DX-8951	3.45	3.59	4.1	3.45	3.62	7.2
	31.04	30.56	−1.5	31.04	21.78	−11.8
	279.36	297.08	6.3	279.36	282.32	3.3

[a] Equivalent as DX-8951.
[b] Difference from the concentrations on the first day.

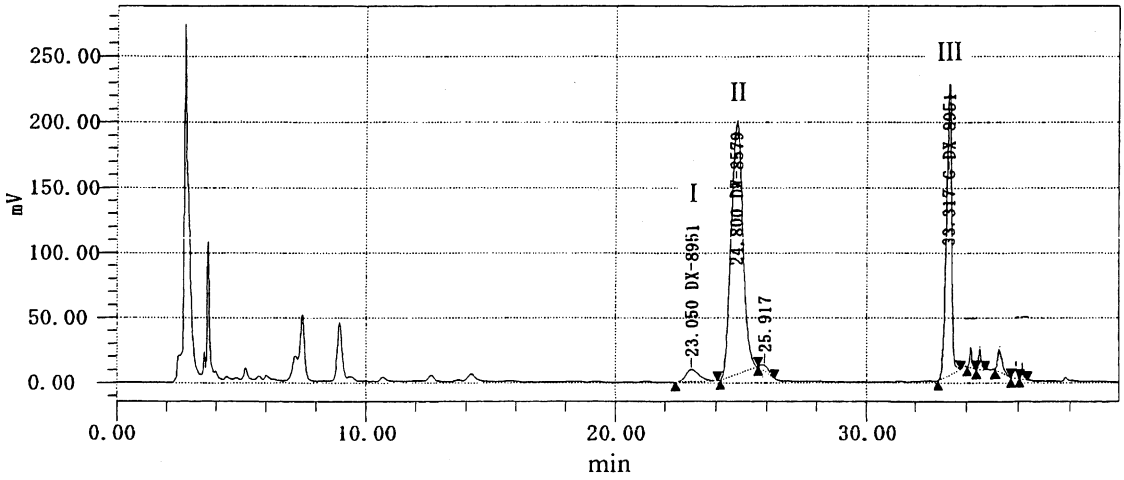

Fig. 4. Chromatogram of DX-8951 and G-DX-8951 in a mouse plasma sample obtained at 8 h after intravenous infusion of DE-310 (7 mg/kg) (I: DX-8951; II: I.S.; and III: G-DX-8951).

(RSD) of DX-8951 and G-DX-8951 were less than 12.53 and 7.41%, respectively. The inter-assay accuracies of DX-8951 and G-DX-8951 were within 7.03 and 11.43%, respectively.

The results of intra- and inter-assay precision and accuracy for Conjugated DX-8951 are shown in Table 3. The intra-assay precision (RSD) of Conjugated DX-8951 estimates for QC samples was less than 3.12%. The intra-assay accuracy of Conjugated DX-8951 was within 3.22%. The inter-assay precision (RSD) of Conjugated DX-8951 estimates for QC samples was less than 5.22%. The intra-assay accuracy of Conjugated DX-8951 was within −4.06%.

These results indicated that our described method has satisfactory precision and accuracy.

3.5. Stability

The freeze–thaw stabilities of DX-8951 and G-DX-8951 were evaluated (Table 4). The mean accuracies of DX-8951 and G-DX-8951 were less than 13.3 and 14.0%, respectively. After storage at −80 °C for 9 weeks, the concentrations of QCs of DX-8951 and G-DX-8951 were comparable to those of freshly prepared QCs (Table 4). The mean differences of DX-8951 and G-DX-8951 between the stored and fresh samples were within −7.8 and −12.8%, respectively.

The freeze–thaw stability of Conjugated DX-8951 was evaluated (Table 5). The mean accuracy of Conjugated DX-8951 was less than 6.3%. After storage at −80 °C for 9 weeks, the concentrations of QCs of Conjugated DX-8951 were comparable to those of freshly prepared QCs (Table 5). The mean differences of Conjugated DX-8951 between the stored and fresh samples were within −11.8%.

These results showed that no significant degradation occurred during three freeze–thaw cycles, and that these analytes were stable for at least 9 weeks at −80 °C in mouse plasma.

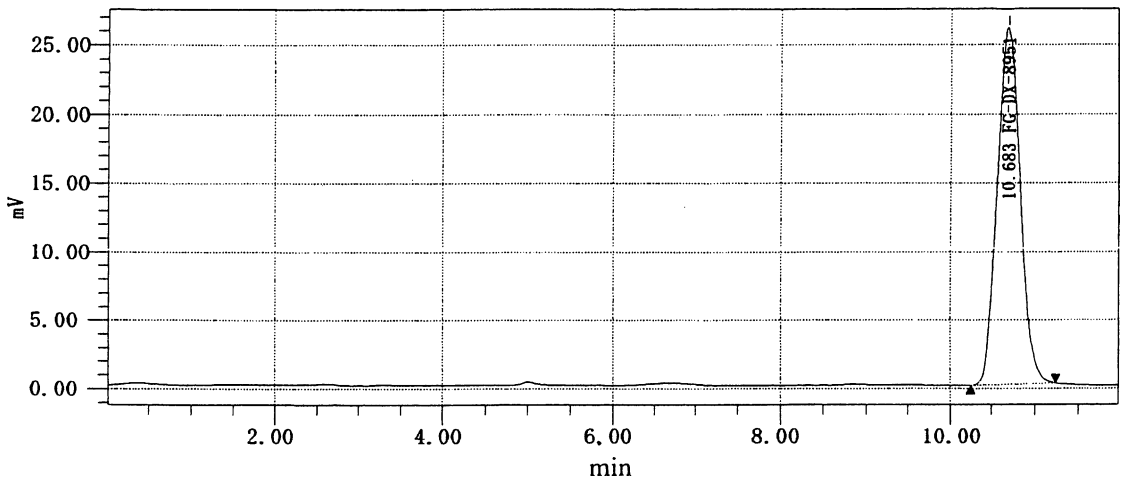

Fig. 5. Chromatogram of Conjugated DX-8951 in a mouse sample obtained at 8 h after intravenous infusion of DE-310 (7 mg/kg).

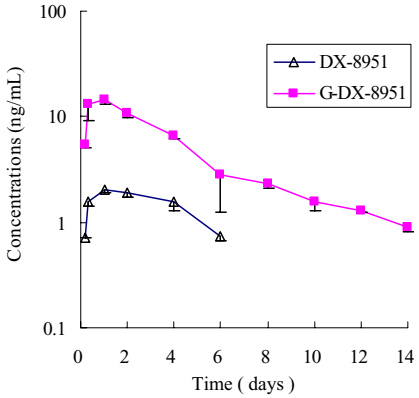

Fig. 6. Plasma concentration–time profiles of DX-8951, G-DX-8951 after intravenous infusion of DE-310 to male mice (7 mg/kg). Each point represents mean ± S.D. of three mice.

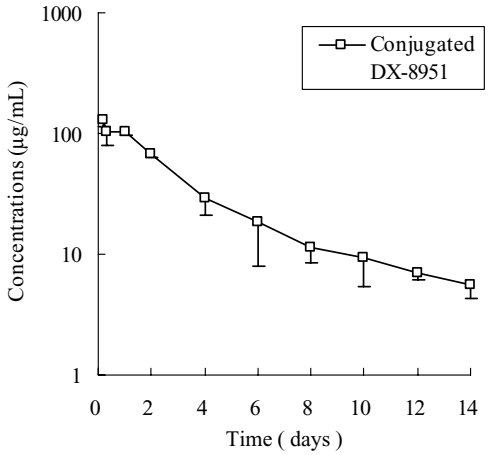

Fig. 7. Plasma concentration–time profile of Conjugated DX-8951 after intravenous infusion of DE-310 to male mice (7 mg/kg). Each point represents mean ± S.D. of three mice.

3.6. Analysis of samples

The suitability of these analytical methods for pharmacokinetics samples was demonstrated by the determination of DX-8951, G-DX-8951, and Conjugated DX-8951 in blood samples after the start of infusion (7 mg/kg) of DE-310. The chromatograms of the mouse plasma samples are shown in Figs. 4 and 5. The plasma concentration–time profiles of DX-8951, G-DX-8951, and Conjugated DX-8951 are shown in Figs. 6 and 7. These two methods are suitable to measure the concentrations of the compounds in pharmacokinetics study.

4. Conclusions

Sensitive and selective analytical methods for the determination of DX-8951, G-DX-8951, and Conjugated DX-8951 in mouse plasma were established by HPLC. These methods proved to be useful for pre-clinical pharmacokinetics studies of DE-310.

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