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Journal of Chromatography B, 818 (2005) 249–256
JOURNAL OF CHROMATOGRAPHY B
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Validation study of a method for assaying DE-310, a macromolecular carrier conjugate containing an anti-tumor camptothecin derivative, and the free drug in human plasma by HPLC and LC/MS/MS

Toshihiro Oguma[a,*], Dino Cicci[b], Fleur Gaudette[b], Ginette Gauthier-Dubois[b], Rudolf Guilbaud[b], Ryo Atsumi[a]

[a] *Drug Metabolism and Physicochemical Property Research Laboratory, Daiichi Pharmaceutical Co. Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134-8630, Japan*
[b] *MDS Pharma Services, 2350 Cohen Street, Que., Saint-Laurent (Montreal), Canada H4R 2N6*

Received 22 June 2004; accepted 10 January 2005
Available online 26 January 2005

Abstract

DE-310 is a macromolecular carrier conjugate containing an anti-tumor camptothecin derivative, DX-8951, conjugated to a water-soluble polymer by means of a peptide spacer. New assay methods have been developed to determine the polymer-bonded DX-8951 conjugate, free DX-8951, and Glycyl-DX-8951 in human plasma. Solid-phase extraction was used to extract free DX-8951 and Glycyl-DX-8951 from plasma, and LC/MS/MS (Method I) was used to determine the amount of each analyte. Protein precipitation was used to extract Conjugated DX-8951, which was then digested with thermolysin. HPLC (Method II) was used to determine the productive compound (Phenylalanyl-Glycyl-DX-8951). The lower limit of quantitation of DX-8951 was 50 pg/ml, of Glycyl-DX-8951 was 80 pg/ml, and of Conjugated DX-8951 was 100 ng/ml (as DX-8951 equivalent). Both methods showed satisfactory sensitivity, precision, and accuracy.

Keywords: HPLC; LC/MS/MS; DE-310; Camptothecin

1. Introduction

Camptothecin and its analogues are potent anti-tumor agent. However, these agents are extremely cytotoxic, which sometimes limits their clinical usefulness. To get around this problem, several drug delivery systems have been developed for camptothecin analogues to reduce their severe systemic toxicities and to enhance their anti-tumor effects, thus increasing their clinical utility. One of these drugs, DE-310 is a macromolecular conjugate composed of DX-8951 (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 and a carboxymethyldextran polyalcohol carrier, which are covalently linked via a glycyl-glycyl-phenylalanyl-glycyl-peptide spacer (Fig. 1). DE-310 is designed to distribute itself primary to tumor tissue, where intra-tumoral cleavage by lysosomal enzymes owing to "enhanced permeability and retention effect" (EPR effect) [1–3]. This enzymatic cleavage is supposed to provide the desired active product. Results from a pre-clinical study show 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-bound DX-8951 (Conjugated DX-8951) and its released forms, DX-8951 and G-DX-8951, in human plasma.

HPLC methods to determine Conjugated DX-8951 and its released forms in human whole blood, erythrocytes and saliva have been developed recently [5]. The lower limit of

* Corresponding author. Tel.: +81 3 3680 0151; fax: +81 3 5696 8228.
E-mail address: ogumab0g@daiichipharm.co.jp (T. Oguma).

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doi:10.1016/j.jchromb.2005.01.005

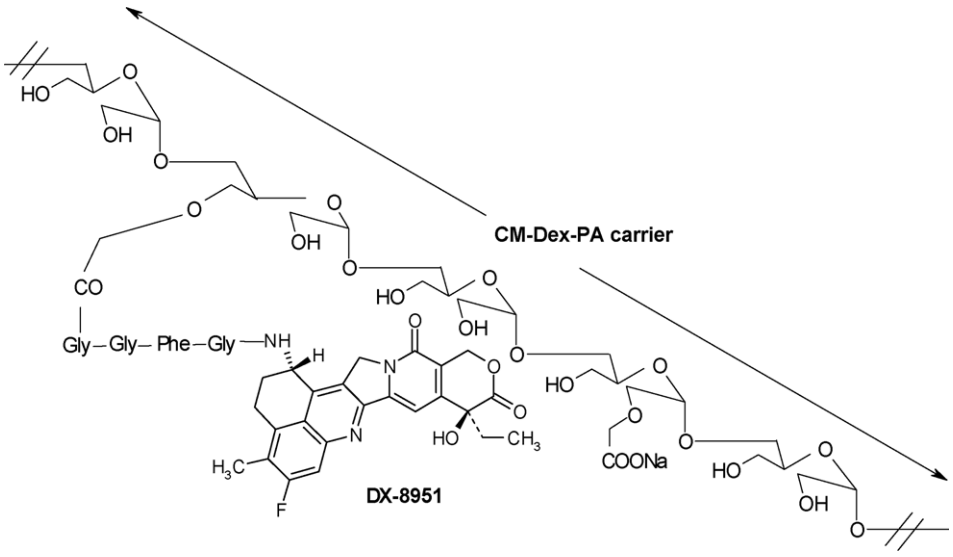

Fig. 1. Chemical structure of DE-310.

quantitations (LLOQs) of DX-8951 and G-DX-8951 in human saliva were 0.5 ng/ml. LC/MS/MS and HPLC methods to determine DX-8951 in human plasma have been developed [6,7]. The LLOQs of DX-8951 were 0.1 ng/ml for 300 μl human plasma using LC/MS/MS, and 0.2 ng/ml for 500 μl human plasma using HPLC. The sensitivity of HPLC was not enough to analyze clinical samples in starting dose level. It is suggested that the use of LC/MS/MS method enables to reduce the chromatographic run time, and applicable for analyzing very small quantity of DX-8951 and G-DX-8951 in human plasma.

However, a method for analyzing Conjugated DX-8951 is required for complete pharmacokinetics studies of DE-310. Analytical methods to study a polymer-bound camptothecin derivative (MAG-CPT) have demonstrated that the total levels of CPT (free plus polymer-bound) can be determined after alkaline-mediated hydrolysis of MAG-CPT [8,9]. The LLOQs of this method were 100 ng/ml of total CPT for 50 μl of human plasma, and 1 ng/ml of free CPT for 250 μl human plasma. Additionally, HPLC method for a different water-soluble polymer-bound compound, doxorubicin (PK1), has been developed [10]. Total (polymer-bound plus free) doxorubicin was determined after acid hydrolysis to release doxorubicinone as aglycones from free or polymer-bound doxorubicin. The LLOQs for this method were 5.1 ng/ml of bound doxorubicin and 0.38 ng/ml of free doxorubicin. Consequently, it might be possible to use a similar enzymatic treatment to release Phenylalanyl-Glycyl-DX-8951 from DE-310.

This paper describes methods to assay human plasma samples for DX-8951 and G-DX-8951 using LC/MS/MS (Method I), and Conjugated DX-8951 using thermolysin treatment to release Phenylalanyl-Glycyl-DX-8951 followed by reversed-phase HPLC with fluorimetric detection (Method II). Validation procedures and results for these methods are also described.

2. Experimental

2.1. Chemicals

DE-310, DX-8951, and G-DX-8951 were synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). D91-7117, (9*S*)-9-ethyl-2,3-dihydro-9-hydroxy-4-[(isopentylamino)-methyl]-1*H*,12*H*-benzo[*de*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione, was synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo) and used as an internal standard (I.S.). The acetonitrile used was of HPLC grade (BDH, Chicoutimi, Canada), and the methanol used was of HPLC grade (EM Science, Gibbstown, NJ, USA). All other chemicals were of analytical reagent grade or reagent grade and used without further purification.

2.2. Equipment

The chromatographic system for Method I consisted of HP1090 (Hewlett Packard, Wald brounn, Germany) and a Puresil C18 column (150 mm × 4.6 mm i.d.; Waters, Milford, MA, USA). The column temperature was kept at 50 °C. The mobile phase for Method I was a solution of methanol–0.1%(v/v) trifluoroacetic acid (11:9, v/v). The flow rate was 1 ml/min. Seventy microliters of aliquot of each sample were loaded onto this LC/MS/MS system. An API-3000 mass spectrometer (Sciex, Concord, Canada), equipped with an atmospheric pressure chemical ionization (APCI) source, was used. We selected the APCI mode, for the ion suppression was lower than that of using electrospray ionization mode

in our previous study [6]. The mass spectrometer was operated in the positive-ion detection mode. The temperature of the nebulizer was set at 480 °C. Nitrogen gas was used for collision-induced detection (CID). The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Detection of the ions was performed by monitoring the decay of the *m*/*z* 436 precursor ion to the *m*/*z* 419 product ion for DX-8951, the decay of the *m*/*z* 493 precursor ion to the *m*/*z* 419 product ion for G-DX-8951, and the decay of the *m*/*z* 488 precursor ion to the *m*/*z* 401 product ion for D91-7117. Peak-area ratios (area of analytes/area of I.S.) were plotted against the concentration, and weighted quadratic regression (1/concentration^2) was used to analyze the results.

The chromatographic system for Method II consisted of a Spectra Series P2000 (Spectra-Physics, Riviera Beach, FL, USA) and an Inertsil ODS-2 column (150 mm × 6 mm i.d.; Metachem Technologies, Torrance, CA, USA). A Waters 474 scanning fluorescence detector (Millipore Co., Milford, MA, USA) set at an excitation wavelength of 375 nm and an emission wavelength of 445 nm was used to detect the analyte. The column temperature was kept at 45 °C in a CH-30 TC-50 column oven (Eppendorf, Westbury, NY, USA). A 10 μl aliquot of each prepared sample was loaded onto the HPLC system from a Spectra AS-300 autosampler (Spectra-Physics). The mobile phase for Method II was a mixture (16:32:52, v/v/v) of methanol–acetonitrile–0.05 M potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid). The flow rate was set at 1 ml/min. A ChromJet Integrator (Spectra-Physics) was used to integrate the peak areas. Peak-areas were plotted against the concentration, and weighted linear regression (1/concentration) was used to analyze the results.

2.3. Preparation of standard solutions and quality control samples

Standard solutions of DX-8951, G-DX-8951, and D91-7117 were prepared in 0.04 M Britton–Robinson buffer (pH 3) and diluted with the same buffer. The concentrations of these solutions were expressed as mass equivalents of their free bases. The nominal plasma concentrations (eight points) of calibration standards were 50.0, 100.0, 280.0, 799.9, 2599.7, 3799.5, 4399.5, and 4999.4 pg/ml for DX-8951 and 80.2, 160.4, 280.7, 802.0, 2606.4, 3809.4, 4410.9, and 50124 pg/ml for G-DX-8951. Quality control (QC) levels were chosen at 50.0, 150.0, 2000.2, and 4000.4 pg/ml for DX-8951 and 80.2, 240.0, 2000.2, and 4000.4 pg/ml for G-DX-8951.

We analyzed the conjugated form to its carrier, Conjugated DX-8951, using standard solution of DE-310. The standard solution of DE-310 was prepared in purified water and diluted with a 0.1% (w/v) Brij35 solution. The concentration was expressed in terms of the DX-8951 mass equivalents. These standard solutions were stored at 4 °C protected from light. The nominal plasma concentrations (eight points) of calibration standards were 99.8, 199.5, 707.3, 1511.4, 2539.2, 3506.4, 4534.2, and 5017.8 ng/ml. QC levels were chosen at 99.8, 299.7, 1998.1, and 3814.5 ng/ml.

2.4. Sample preparation

For the DX-8951 and G-DX-8951 analytical procedure (Method I), 1 ml of calibration, quality control (QC), unknown human plasma sample, and 0.2 ml of the internal standard solution (D91-7117: 3.5 ng/ml) were placed into a test tube. A 1.5 ml volume of 0.05 M phosphate buffer (pH 2) was added to each tube, and a vortex mixer was used to mix each sample thoroughly. Each sample was loaded onto a solid extraction cartridge CBA (3 ml, 500 mg; Varian, Harbor City, CA, USA), pre-conditioned first with 2 ml of methanol, and then with 2 ml of purified water. After loading, each cartridge was washed first with 2 ml of purified water, and then with 2 ml of methanol. The analytes were then eluted with 2 ml of a 1 mol/l hydrochloric acid–methanol solution (1:99, v/v) and collected in separate test tubes. The eluates were evaporated to dryness under a stream of nitrogen gas at 40 °C. The residues were reconstituted in 0.1 ml of a solution of methanol–water–trifluoroacetic acid (30:70:0.1, v/v/v) and mixed thoroughly. 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 solutions were transferred to Eppendorf micro vials and centrifuged at 15,300 × *g* for 15 min. A 80 μl aliquot of each supernatant was then loaded onto the LC/MS/MS system.

To analyze Conjugated DX-8951 (Method II), 0.1 ml of calibration, QC, unknown human plasma sample was placed into a test tube. A 0.1 ml volume of purified water was added to each tube and mixed thoroughly by vortexing for 3 s, followed by addition to each tube of 0.6 ml volume of methanol was added, and mixed thoroughly by vortexing for 3 s. The samples were centrifuged at 9100 × *g* for 5 min at 4 °C. Each supernatant was transferred to a separate disposable test tube (16 mm × 100 mm), and the supernatants were then evaporated to dryness under a stream of nitrogen gas at 40 °C. One hundred microliters of purified water was added to each tube, and mixing with a vortex mixer for 3 s was used to reconstitute the residues. Two hundred micro liters of 0.1 M Tris–hydrochloric buffer (pH 8.5) and 100 μl of thermolysin solution (a 2 mg/ml thermolysin solution in 0.1 M calcium chloride) were then added. The enzymatic reaction was conducted in an incubator at 50 °C for 1 h. The enzymatic reaction was terminated by adding 0.5 ml of 0.5 M hydrochloric acid–methanol (1:1, v/v), followed by vigorous mixing with a vortex mixer for 10 s. Finally, 10 μl of each processed solution was loaded onto the HPLC system.

2.5. Validation procedures

The absolute recoveries of DX-8951, G-DX-8951, and Conjugated DX-8951 from human plasma were estimated by comparing the peak areas of extracted samples with those of unextracted standard solutions. The selectivity of the assay was investigated by processing and analyzing ten independent blank (drug-free) samples using the previously described procedures. Each calibration curve consisted of eight calibration points ($n = 1$). The intra-day precision, evaluated as relative standard deviation (R.S.D.), and the accuracy of each method were determined from results for replicate analyses ($n = 6$) of QC samples. Similarly, the inter-day precision (R.S.D.) and accuracy were determined replicate analyses ($n = 6$) of the QC samples performed on three separate days. The 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 after freezing and thawing was assessed by analyzing QC samples ($n = 6$) over three freeze/thaw cycles. Stability at room temperature was assessed using QC samples ($n = 6$) that had been stored at room temperature for more than 11 h. Stability during storage at −20 °C was assessed by using QC samples ($n = 6$) that had been placed in a storage freezer for 340 days for Method I, or for 152 days for Method II.

2.6. Application of the method

To demonstrate that these methods are reliable enough to study the pharmacokinetics of DE-310 in human, these assays were used to determine concentrations of DX-8951, G-DX-8951, and Conjugated DX-8951 in human plasma. They were administrated a single 3 h intravenous infusion of DE-310 at a dose of 1 mg/m^2. Blood samples were collected from each subject at 1, 2, 3, 4, 5, 7, 9, 11, 27, 51, 75, 99, 123, 147, 171, 195, 243, and 315 h after the end of the infusion. Samples were stored at −20 °C until analyzed. Informed consents were obtained from all patients.

3. Results and discussion

3.1. Recovery of analytes

Recoveries of DX-8951 and G-DX-8951 from human plasma were determined at concentrations of 150, 2000, 4000 pg/ml for DX-8951 and 240, 2000, 4000 pg/ml for G-DX-8951 in six replicates. The mean recovery of DX-8951 ranged from 68.3 to 73.1%, the mean recovery of G-DX-8951 ranged from 69.6 to 72.0%, and that of D91-7117 was 54.9%.

Similarly, Method II was used to assess recovery of Conjugated DX-8951 from human plasma at concentrations of 300, 1998, and 3815 ng/ml (DX-8951 equivalent) in six replicates. The mean recovery of Conjugated DX-8951 ranged from 62.4 to 67.9%. These data shown that these extraction methods can be used to extract DX-8951, G-DX-8951, and Conjugated DX-8951 from human plasma.

3.2. Chromatography and selectivity

Method I chromatograms of plasma extracts obtained with blank human plasma and blank human plasma spiked with DX-8951 (50.0 ng/ml), G-DX-8951 (80.2 ng/ml), and I.S. are shown in Figs. 2 and 3. The retention time of DX-8951 was 2 min, of G-DX-8951 was about 3 min, and of I.S. was about 2.5 min. The overall chromatographic run times were within approximately 4 min.

Method II chromatograms of plasma extracts obtained from (A) blank human plasma and (B) blank human plasma spiked with Conjugated DX-8951 (99.8 ng/ml) are shown in Fig. 4. The retention time of Conjugated DX-8951 was approximately 5 min. The overall chromatographic run time was within approximately 7 min. These chromatograms indicate that no endogenous compounds interfere with the detection of DX-8951, G-DX-8951, or Conjugated DX-8951.

3.3. Calibration curves

Calibration curves for DX-8951 and G-DX-8951 obtained on three separate days were linear over the concentration ranges of 50.0–4999.4 pg DX-8951/ml and 80.2–5012.4 pg G-DX-8951/ml. The coefficients of determination (r^2) for DX-8951 was greater than 0.995, and for G-DX-8951 was greater than 0.999.

Calibration curves for Conjugated DX-8951 obtained on three separate days were linear over the concentration ranges of 99.8–5017.8 ng/ml (DX-8951 equivalent). The mean of r^2 was greater than 0.995.

3.4. Precision and accuracy

The intra- and inter-assay precision and accuracy values for DX-8951 and G-DX-8951 are shown in Table 1. The intra-assay precision (R.S.D.) values of DX-8951 for QC samples were less than or equal to 8.1%, and for G-DX-8951 were less than or equal to 16.2%. The R.S.D. value of G-DX-8951 for 80.2 pg/ml (LLOQ level) was 16.2%, but the R.S.D. values of other concentrations were shown to be within 15%. The intra-assay accuracies of DX-8951 were within 8.0%, and for G-DX-8951 were within 13.6%. The inter-assay precision (R.S.D.) values of DX-8951 were less than or equal to 10.6%, and for G-DX-8951 were less than or equal to 8.3%. The inter-assay accuracies of DX-8951 were within 12.3%, and for G-DX-8951 were within 7.0%.

The intra- and inter-assay precision and accuracy for Conjugated DX-8951 are shown in Table 2. The intra-assay precision (R.S.D.) values of Conjugated DX-8951 for QC samples were less than or equal to 4.1%. The intra-assay accuracy of Conjugated DX-8951 was within 11.8%. The inter-assay precision (R.S.D.) values of Conjugated DX-8951 for QC samples were less than or equal to 10.5%. The intra-assay accuracy of Conjugated DX-8951 was within 9.1%.

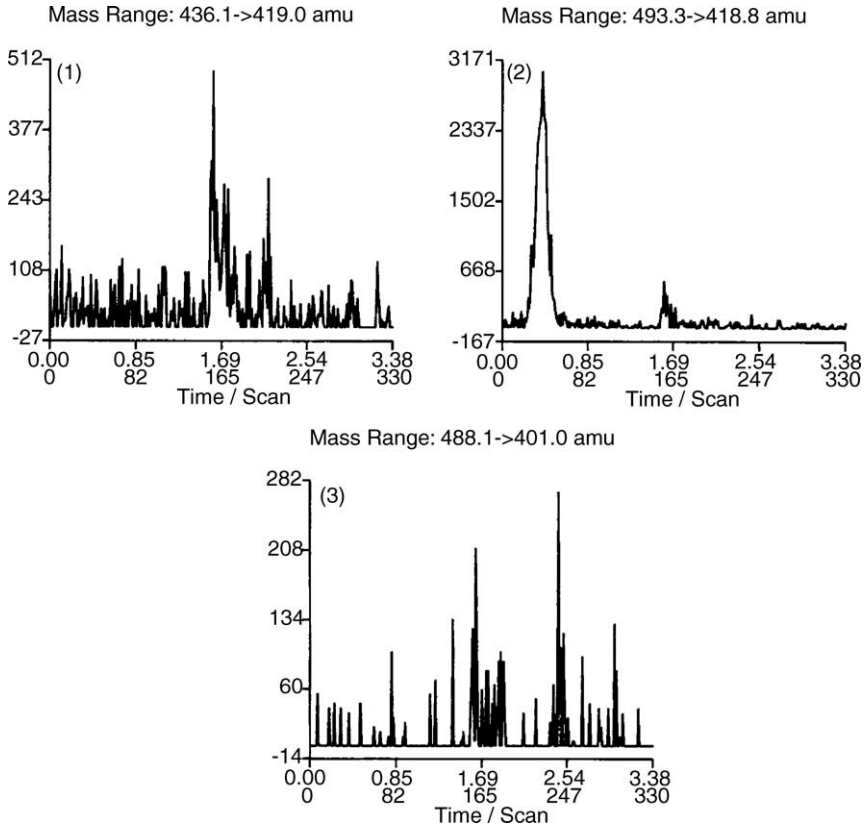

Fig. 2. MRM chromatograms of extract of blank human plasma using Method I. (1), DX-8951; (2), G-DX-8951; and (3), I.S.

Table 1
Intra- and inter-assay precision and accuracy for DX-8951 and G-DX-8951 in human plasma using Method I

Analytes	Nominal concentration (pg/ml)	Intra-assay precision and accuracy			Inter-assay precision and accuracy		
		Mean ($n = 6$) (pg/ml)	R.S.D. (%)	Mean accuracy (%, $n = 6$)	Mean ($n = 18$) (pg/ml)	R.S.D. (%)	Mean accuracy (%, $n = 18$, 3 days)
DX-8951	50.0	52.8	6.5	5.5	43.9	10.6	12.3
	150.0	154.3	8.1	2.9	151.2	8.0	0.8
	2000.2	2159.8	6.1	8.0	2013.5	7.1	0.7
	4000.4	4127.2	7.3	3.2	3848.1	6.5	3.8
G-DX-8951	80.2	69.3	16.2	−13.6	85.8	7.7	7.0
	240.0	235.4	8.6	−1.9	248.7	8.3	3.6
	2000.2	2149.4	9.9	7.5	1912.1	6.2	−4.4
	4000.4	4197.2	9.7	4.9	3998.5	7.0	0.0

Table 2
Intra- and inter-assay precision and accuracy for Conjugated DX-8951 in human plasma using Method II

Analytes	Nominal concentration (ng/ml)[a]	Intra-assay precision and accuracy			Inter-assay precision and accuracy		
		Mean ($n = 6$) (ng/ml)	R.S.D. (%)	Mean accuracy (%, $n = 6$)	Mean ($n = 18$) (ng/ml)	R.S.D. (%)	Mean accuracy (%, $n = 18$, 3 days)
Conjugated DX-8951	99.8	111.6	4.1	11.8	92.3	10.5	−7.5
	299.7	301.2	2.9	0.5	286.9	5.2	−4.3
	1998.1	1880.9	3.6	−5.9	1816.2	4.7	−9.1
	3814.5	3569.9	1.7	−6.4	3520.0	3.2	−7.7

[a] Equivalent as DX-8951.

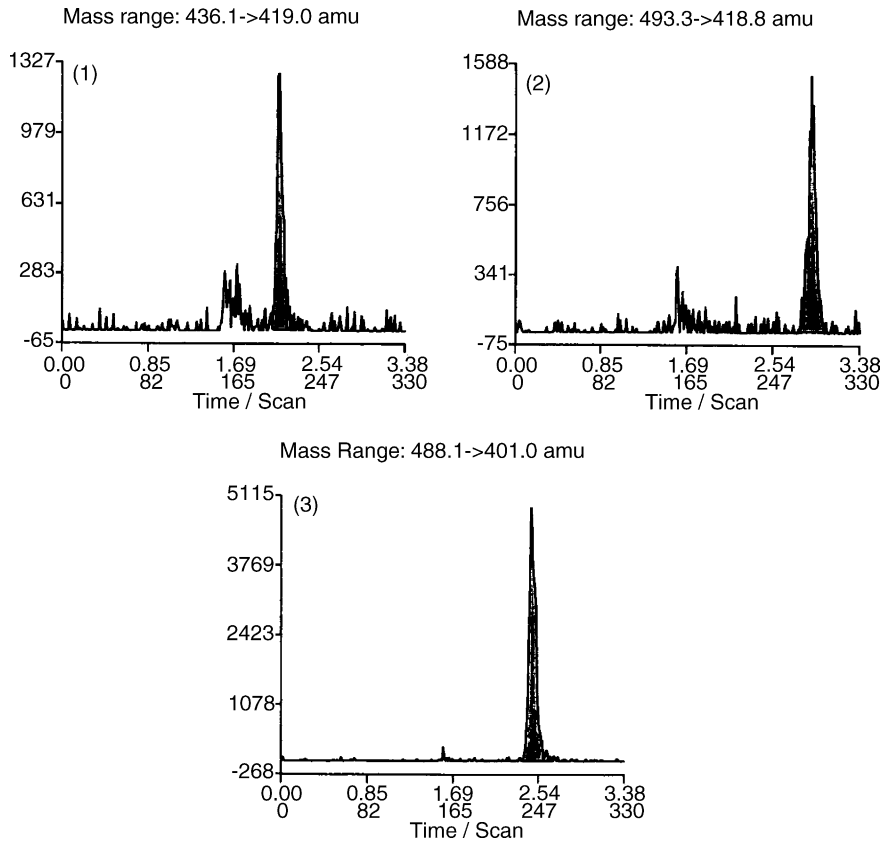

Fig. 3. MRM chromatograms of extracts of blank human plasma spiked with DX-8951 (50 pg/ml), G-DX-8951 (80.2 pg/ml), and I.S using Method I. (1), DX-8951; (2), G-DX-8951; and (3): I.S.

These results indicate that our methods have satisfactory precision and accuracy.

3.5. Stability

The freeze/thaw stability of DX-8951 and G-DX-8951 were evaluated (Table 3). The mean difference in measured DX-8951 concentration was 9.4% or less between freeze/thaw and freshly prepared samples, for G-DX-8951 was 2.0% or less. The concentrations of DX-8951 and G-DX-8951 in QCs stored at room temperature for 11 h were comparable to those of freshly prepared QCs (Table 3). The mean difference in measured DX-8951 concentration was 11.4% or less between the stored and fresh samples, for G-DX-8951 was 9.4% or less. The concentrations of DX-8951 and G-DX-8951 in QCs stored at −20 °C for 340 days were comparable to those of freshly prepared QCs (Table 3). The mean difference in measured DX-8951 concentration was 7.3% or less between the stored and fresh samples, for G-DX-8951 was 7.6% or less.

The freeze/thaw stability of Conjugated DX-8951 were evaluated (Table 4). The mean difference in measured Con-

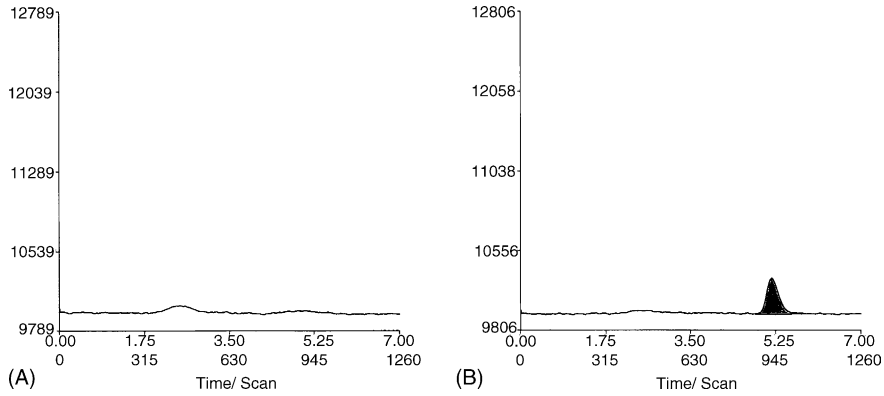

Fig. 4. HPLC chromatograms of extracts of (A) blank plasma and (B) blank plasma spiked with Conjugated DX-8951 (99.8 ng/ml) using Method II.

Table 3
Stability of DX-8951 and G-DX-8951 in human plasma

Analytes	Nominal concentration (pg/ml)	Difference (%, $n = 6$)[a]		
		After three cycles of freeze/thaw	After storage at room temperature for 11 h	After storage at −20 °C for 340 days
DX-8951	150.0	9.4	11.4	2.0
	4000.4	−8.2	−2.9	7.3
G-DX-8951	240.0	2.0	9.4	−3.0
	4000.4	0.2	−9.0	7.6

[a] Difference from the concentrations of freshly spiked QCs.

Table 4
Stability of Conjugated DX-8951 in human plasma

Analyte	Nominal concentration (ng/ml)	Difference[a] (%, $n = 6$)		
		After three cycles of freeze/thaw	After storage at room temperature for 5 h	After storage at −20 °C for 152 days
Conjugated DX-8951	299.7	1.9	−7.1	−12.1
	3814.5	0.9	−2.8	−4.8

[a] Difference from the concentrations of freshly spiked QCs.

jugated DX-8951 concentration was 1.9% or less between freeze/thaw and freshly prepared samples. The concentrations of Conjugated DX-8951 in QCs stored at room temperature for 5 h were comparable to those of freshly prepared QCs (Table 4). The mean difference in measured Conjugated DX-8951 concentration was −7.1% or less between the stored and fresh samples. The concentrations of Conjugated DX-8951 in QCs stored at −20 °C for 152 days were comparable to those of freshly prepared QCs (Table 4). The mean difference in measured Conjugated DX-8951 concentration was −12.1% or less between the stored and fresh samples.

These results show that no significant degradation occurs after three freeze/thaw cycles. The results also show that DX-8951 and G-DX-8951 are stable in human plasma for at least 11 h at room temperature, and at least 340 days at −20 °C. Conjugated DX-8951 was stable for at least 5 h at room temperature, and at least 152 days at −20 °C in human plasma.

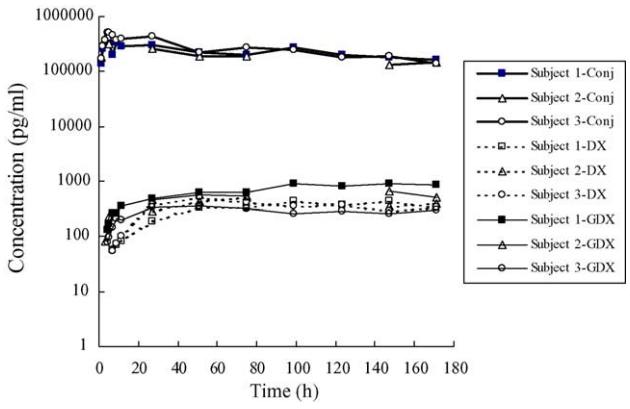

Fig. 5. The plasma concentration-time profiles of DX-8951, G-DX-8951, and Conjugated DX-8951 after single 3 h intravenous infusion of DE-310 (1 mg/m^2) (Conj, Conjugated DX-8951; DX, DX-8951; and GDX, G-DX-8951).

3.6. Analysis of samples

The suitability of these analytical methods for clinical analysis was demonstrated by the analysis of DX-8951, G-DX-8951, and Conjugated DX-8951 in plasma samples after the intravenous infusion (1 mg/m^2) of DE-310. The plasma concentration-time profiles of DX-8951, G-DX-8951, and Conjugated DX-8951 are shown in Fig. 5. The results indicate that the analytical methods are suitable to measure the concentrations of the compounds expected to be present in patients during clinical pharmacokinetics studies.

4. Conclusions

LC/MS/MS and HPLC analytical methods for the determination of DX-8951, G-DX-8951, and Conjugated DX-8951 in human plasma have been established. These two methods show satisfactory sensitivity, precision, and accuracy. The methods established may prove useful during clinical pharmacokinetics studies on DE-310.

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