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Quantitative acid hydrolysis of DE-310, a macromolecular carrier system for the camptothecin analog DX-8951f

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Abstract

DE-310 is a novel macromolecular prodrug of the topoisomerase-I inhibitor DX-8951. DX-8951 is covalently linked to carboxymethyl dextran polyalcohol (CM-Dex-PA) via a Gly-Gly-Phe-Gly (GGFG) tetrapeptide spacer. The present study was conducted to identify the portions of DX-8951 linked to DE-310, as well as to quantify the number of DX-8951 molecules associated with DE-310. Two different structures terminated with either glycolaldehyde (CM-GA-GGFG-DX-8951) or glycerol (CM-Glr-GGFG-DX-8951) are obtained when the polymer backbone is fragmented with 1 M HCl. The two products, i.e., CM-GA-GGFG-DX-8951 and CM-Glr-GGFG-DX-8951, indicate linkage of GGFG-DX-8951 with carboxymethyl (CM) group at C-2 and C-4 position of the glucose units, respectively. In the present study, CM-GA-GGFG-DX-8951 was reduced to CM-ethyleneglycol (EG)-GGFG-DX-8951 in order to improve stability prior to HPLC analysis. Hydrolysis results revealed that the amount of CM-GA-GGFG-DX-8951 liberated was 84.7 nmol/mg DE-310 and the amount of CM-Glr-GGFG-DX-8951 was 71.8 nmol/mg DE-310. Considering the ratio of generation between CM-GA-GGFG-DX8951 and CM-Glr-GGFG-DX8951, it suggested that slightly larger amount of GGFG-DX-8951 was linked to carboxymethyl groups at the C-2 position of glucose units in DE-310. The sum of the amounts of CM-GA-GGFG-DX-8951 and CM-Glr-GGFG-DX-8951 agrees well with the amount of G-DX-8951 produced from DE-310 by α-chymotrypsin treatment (157.5 nmol/mg DE-310). The data indicate that the established hydrolysis give a quantitative evaluation of the DX-8951 linked to DE-310.

Keywords: Macromolecular prodrug; DE-310; Acid hydrolysis; Smith degradation; Camptothecin

1. Introduction

DE-310 is a novel macromolecular prodrug of the topoisomerase-I inhibitor DX-8951, i.e., (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. In DE-310, the drug moiety is covalently linked to carboxymethyl dextran polyalcohol (CM-Dex-PA) backbone through a Gly-Gly-Phe-Gly (GGFG) tetrapeptide spacer as shown in Fig. 1A [1]. DE-310 is designed to accumulate preferentially in solid tumors and retain a high level in the tumor for a long time, based on the well-known "Enhanced Permeability and Retention (EPR) effect" [2]. The parent drug is then by lysosomal enzymatic cleavage after being taken up into the interior of the tumor cells. Previous pharmacokinetic study using Meth A tumor-bearing mice revealed that the half-life ($t_{1/2}$) of conjugated DX-8951, released DX-8951, and G-DX-8951 in plasma, liver, and tumor tissue is 2–3 days following intravenous injection of DE-310, whereas that of DX-8951f (exatecan; DX-8951 monomethansulfonate salt) was 0.6 h [3]. Additionally, DE-310 decreases unexpected toxicity of DX-8951f while maintaining antitumor activity against several human and murine tumor models [4].

Despite these advantaged, exact determination of the amount of DX-8951 conjugated with DE-310 is an important issue. Without this information, prediction of maximum tolerated dose, the effective dose range, or the quality of the product is very difficult. Quantitation of conjugated drug molecules by measurement of UV or visible light absorbance of the polymer conjugate is simple and convenient, but not necessarily accurate because the extinction coefficients of free and polymer-bound drugs are not necessarily the same [5]. A better method would be to liberate active drug molecules from the polymer conjugate and purify them prior to determination. However, in DE-310, there are two

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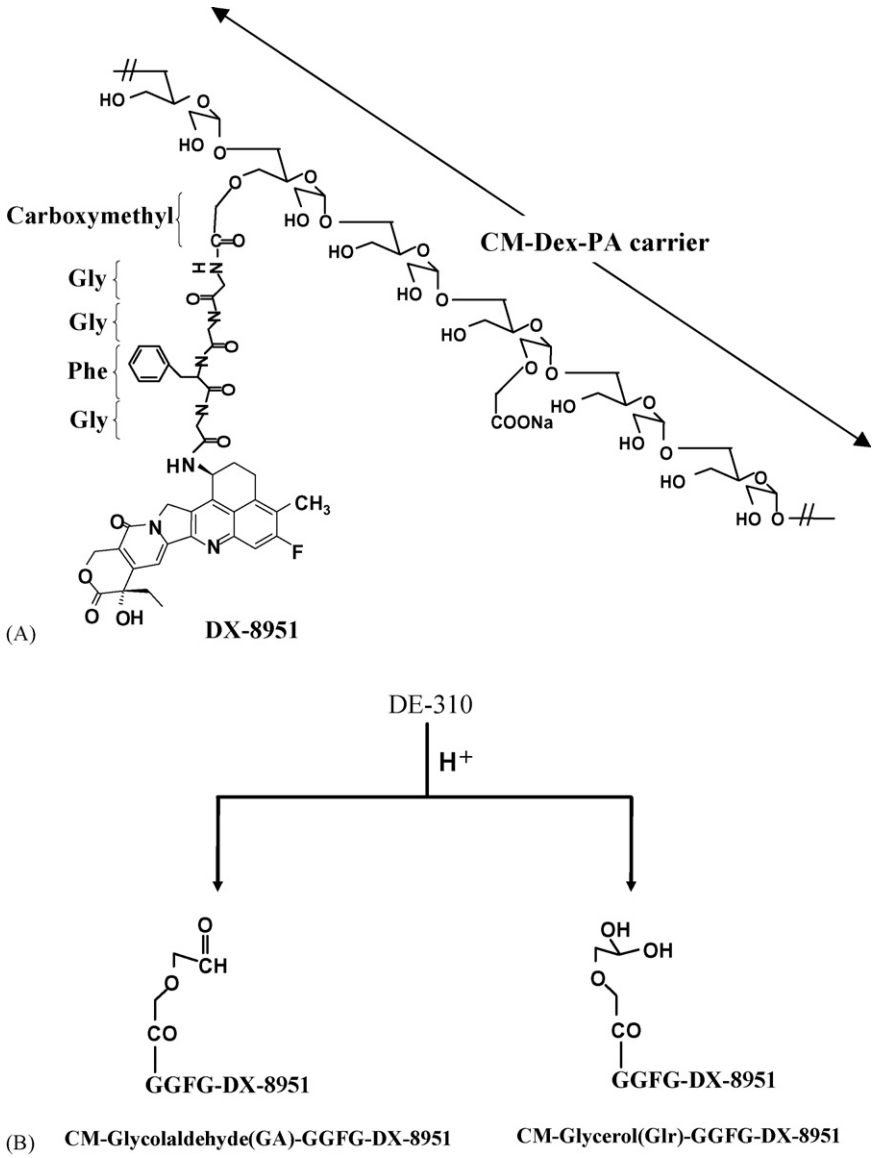

Fig. 1. Chemical structure of DE-310 (A) and major DX-8951 fragments (B) that are formed by acid hydrolysis of DE-310.

possible positions to which GGFG-DX-8951 can link, i.e., the carboxymethyl (CM) groups at either the C-2 or C-4 position of the glucose units. Differences in steric and electronic properties between these positions might affect the enzymatic kinetics of DX-8951 release. Therefore, it is important to clarify not just the amount of DX-8951, but also the position of GGFG-DX-8951 linked to the backbone.

The present study was conducted to identify the CM groups to which GGFG-DX-8951 is covalently linked and to quantify the total DX-8951 content of DE-310. Because two different structures, terminated with either glycolaldehyde (CM-GA-GGFG-DX-8951) or glycerol (CM-Glr-GGFG-DX-8951), were obtained when the polymer backbone was fragmented with 1 M HCl (see Fig. 1B). The optimal condition for detection of both species in terms of efficiency of polymer hydrolysis and stability of the products was determined. We found that reduction of CM-GA-GGFG-DX-8951 to CM-ethyleneglycol (EG)-GGFG-DX-8951 using sodium borohydride was effective for reproducible determination. Additionally, to validate this new analytic method, the content of DX-8951 in DE-310 was determined, and compared to the amount produced by enzymatic hydrolysis.

2. Experimental

2.1. Materials

DE-310; 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*-benzo[*de*]pyrano)[3′,4′,:6,7] indolizino[1,2-*b*]quinoline-10,13-dione); and the DX-8951 derivatives, CM-glycerol(Glr)-GGFG-DX-8951, CM-ethyleneglycol (EG)-GGFG-DX-8951, CM-glycolaldehyde (GA)-GGFG-DX-8951, and glycyl (G)-DX-8951 were provided by the Research Laboratory of Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). The average molecular weight (MW)

of DE-310 was approximately 340,000 with a narrow polydispersity (weight average MW/number average MW < 1.3). The DX-8951 content of DE-310 was designed from 5 to 7% (w/w). The degree of substitution of CM groups in the carrier was 0.4 per glucose moiety. HCl (1 M) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan), and sodium borohydride and α-chymotrypsin from Kanto Chemical, Inc. (Kyoto, Japan).

2.2. HPLC apparatus and conditions

The HPLC system consisted of a model CCPM-II pump with a PX-8020 controller (Tosoh, Tokyo, Japan), an AS-100 autosampler (Bio-Rad, Tokyo, Japan) and an F-1050 fluorescence detector (Hitachi, Tokyo, Japan). The excitation and emission wavelengths were 375 and 445 nm, respectively. HPLC data were compiled using a C-R7A Chromatopac (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on a 250 mm × 4.6 mm reversed-phase column packed with Symmetry C18 (Waters, Milford, MA). The mobile phase was methanol–acetonitrile–100 mM sodium acetate (11.3:22.7:66.0, v/v/v) at a flow rate of 1.0 ml/min.

2.3. NMR and MS spectroscopies

The NMR spectra were recorded on a JEOL ECP500 NMR spectrometer (JEOL Ltd., Tokyo, Japan) in DMSO-d_6 using TMS as an internal standard. Coupling constants (J values) are given in Hertz. The MS spectra were measured on a JMS-HX110 mass spectrometer (JEOL Ltd., Tokyo, Japan).

2.4. Stability of CM-Glr-GGFG-DX-8951 and CM-GA-GGFG-DX-8951 in 1 M HCl

CM-Glr-GGFG-DX-8951 was dissolved in 1 M HCl to yield a concentration of 50 μg/ml, and a 400-μl aliquot of the solution was transferred to a glass test tube (Iwaki Glass, Tokyo, Japan). CM-GA-GGFG-DX-8951 was dissolved at a concentration of 10 μg/ml in 1 M HCl or CM-Dex-PA (2 mg/ml) containing 1 M HCl, and 500-μl aliquot of the solutions were transferred into a glass test tubes. All tubes were purged with nitrogen gas, the test tubes were sealed and incubated at 30 °C for 1, 3, 5, or 7 h. Acetonitrile (100 μl) was added to the CM-Glr-GGFG-DX-8951 hydrolysate, followed by HPLC measurement. For CM-GA-GGFG-DX-8951, after 100 μl of acetonitrile was added to the hydrolysate, 50 μl of the solution was removed from the tube. The aliquot was neutralized with 50 μl of 1 M NaOH, mixed with 900 μl of 0.1 M Tris–HCl buffer (pH 9.0) that contained 20% (v/v) acetonitrile, and left to stand at room temperature for 20 min. Then, 20 μl of 200 mM sodium borohydride was added to 180 μl of the reaction solution, followed by incubation at room temperature for 20 min. Finally, the excess borohydride was eliminated by addition of 600 μl of 0.5 M HCl containing 50% acetonitrile, and 10 μl of the solution was injected into the HPLC.

2.5. Stability of CM-Glr-GGFG-DX-8951 in sodium borohydride solution

CM-Glr-GGFG-DX-8951 was dissolved to a concentration of 1.26 μg/ml with 450 μl of borate buffer (0.05 M, pH 9.0) and was mixed with 50 μl of freshly made 20, 200 and 2000 mM sodium borohydride solution. After incubation (0, 30, 60 min) at 30 °C, the excess borohydride was eliminated by addition of 1 M HCl contained 40% acetonitrile, and 10 μl of the solution was injected into the HPLC.

2.6. Acid hydrolysis of DE-310

Two milligrams of DE-310 was dissolved in 1 ml of 1 M HCl, and a 500-μl aliquot of the solution was transferred to a glass test tube. After the tube air was purged with nitrogen gas, the tube was sealed and acid hydrolysis was conducted at 30 °C for 1, 3, 5, 8, 15, or 24 h. At the given times, 100 μl acetonitrile was added to 500 μl of DE-310 hydrolysate, then 50 μl of the solution was proceeded. Thus, 50 μl of 1 M NaOH and Tris–HCl buffer (0.1 M, pH 9.0) containing 20% (v/v) acetonitrile were added. After standing for 20 min at room temperature, the solution was treated with borohydride. Twenty minutes later, the excess borohydride was eliminated by the addition of 0.5 M HCl containing 50% (v/v) acetonitrile. Both CM-Glr-GGFG-DX-8951 and CM-EG-GGFG-DX-8951 produced by hydrolysis of DE-310 were analyzed by the HPLC.

2.7. Calibration curves for CM-Glr-GGFG-DX8951 and CM-EG-GGFG-DX8951

Standard solution of both CM-Glr-GGFG-DX8951 and CM-EG-GGFG-DX8951 were separately adjusted to 125 ng/ml and diluted sequentially (125 ng/ml to 400 pg/ml) with a 0.1 M acetic acid solution containing 50% (v/v) acetonitrile and were analyzed by the HPLC.

2.8. Drug release from DE-310 by enzymatic hydrolysis

DE-310 was dissolved in distilled water to yield a concentration of 400 μg/ml, and a 10-μl aliquot of the solution was mixed with 190 μl of 2 mg/ml α-chymotrypsin (Wako Pure Chemicals, Tokyo, Japan) solution prepared in 40 mM Britton Robinson buffer (pH 6.0). Enzymatic hydrolysis was conducted at 40 °C for 10, 30, 60, 120, and 180 min. At the given times, 200 μl of a 1:1 (v/v) mixture of acetonitrile and 0.5 M HCl was added, and the aliquot was centrifuged at 10,000 × g for 10 min at 4 °C. HPLC was used to determine the amount of G-DX-8951 in the supernatant.

2.9. Data analysis

The amounts of CM-Glr-GGFG-DX8951 and CM-EG-GGFG-DX8951 generated by DE-310 hydrolysis were estimated by a deconvolution method [6]. The decomposition rate constants (k_{dec}) for CM-Glr-GGFG-DX8951 and CM-EG-GGFG-DX8951 were preliminarily estimated by the linear

regression analysis of semi-logarithmic concentration–time profiles. Assuming that the time courses for CM-Glr-GGFG-DX8951 and CM-EG-GGFG-DX8951 obtained experimentally were the products of their generation and decomposition, the rates of generation of the products were estimated from the deconvolution method using k_{dec} as a weighting function.

3. Results

3.1. Stability of CM-Glr-GGFG-DX-8951 and CM-GA-GGFG-DX-8951 in acid

The stability of CM-Glr-GGFG-DX-8951 and CM-GA-GGFG-DX-8951, which are the products obtained from acid hydrolysis of DE-310, was examined (Figs. 2 and 3). CM-Glr-GGFG-DX-8951 decomposed very slowly in 1 M HCl at 30 °C, where about 90% of CM-Glr-GGFG-DX-8951 remained after a 5-h incubation (Fig. 2). The k_{dec} value of CM-Glr-GGFG-DX-8951 was $0.0247 \pm 0.0036\,h^{-1}$.

However, CM-GA-GGFG-DX8951 indicated lower stability, with only 53% remaining after a 5-h incubation in 1 M HCl. The k_{dec} value of CM-GA-GGFG-DX8951 under the conditions was $0.100 \pm 0.008\,h^{-1}$. In order to mimic the hydrolysis conditions of DE-310, the stability of CM-GA-GGFG-DX8951 in the presence of 2 mg/ml CM-Dex-PA was examined. As Fig. 3 shows, the amount of CM-GA-GGFG-DX-8951 remaining after 5 h was increased up to 90%, with the k_{dec} value of $0.0189 \pm 0.0080\,h^{-1}$. These data suggest that the CM-Dex-PA stabilizes the unbound CM-GA-GGFG-DX-8951 in acidic solution, although the mechanism is unclear.

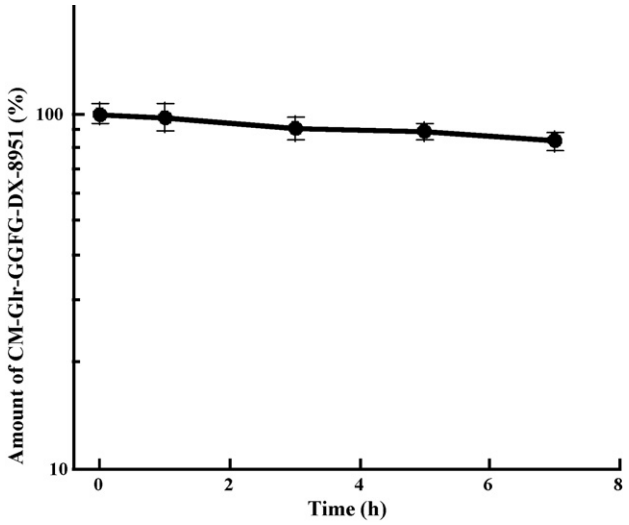

Fig. 2. Stability of CM-Glr-GGFG-DX-8951 in 1 M HCl at 30 °C. All data were normalized by an initial concentration of CM-Glr-GGFG-DX-8951. Each datum represents the mean ± S.D.

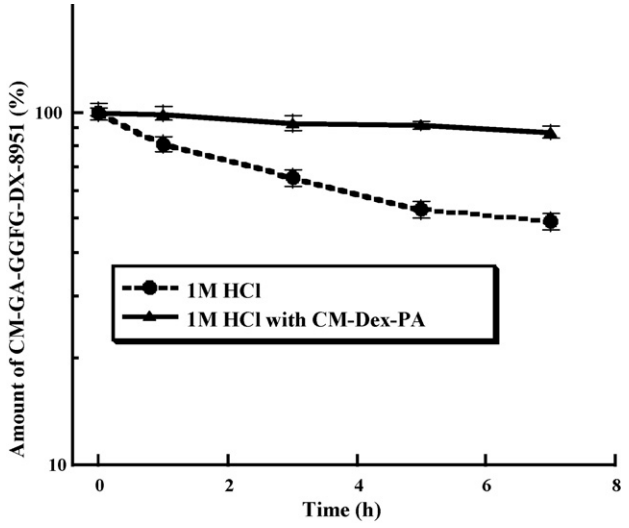

Fig. 3. Stability of CM-GA-GGFG-DX-8951 in 1 M HCl at 30 °C in the presence or absence of CM-Dex-PA. All data were normalized by an initial concentration of CM-GA-GGFG-DX-8951. Each datum represents the mean ± S.D.

3.2. Stability of CM-Glr-GGFG-DX-8951 against sodium borohydride

Since the aldehyde moiety of CM-GA-GGFG-DX-8951 is not chemically stable, it was converted to the stable compound CM-EG-GGFG-DX-8951 using sodium borohydride before HPLC analysis. Thus, assuming that CM-Glr-GGFG-DX-8951 co-exists in the DE-310 hydrolysate, its stability against sodium borohydride is also a concern. As Fig. 4 shows CM-Glr-GGFG-DX-8951 seems to be stable in solutions containing ≤20 mM sodium borohydride for up to 1 h.

3.3. Sodium borohydride reduction of DE-310 hydrolysate

The protocol being developed called for DE-310 to be hydrolyzed in 1 M HCl at 30 °C for 5 h, and, after neutralization, the hydrolysate to be reduced with 20 mM sodium borohydride. Fig. 5 shows chromatograms of DE-310 hydrolysate sample analyzed before or after reduction with sodium borohydride. CM-Glr-GGFG-DX-8951, CM-EG-GGFG-DX-8951, and CM-GA-GGFG-DX-8951 were detected at about 17, 21, and 24 min, respectively. The fraction corresponding to the

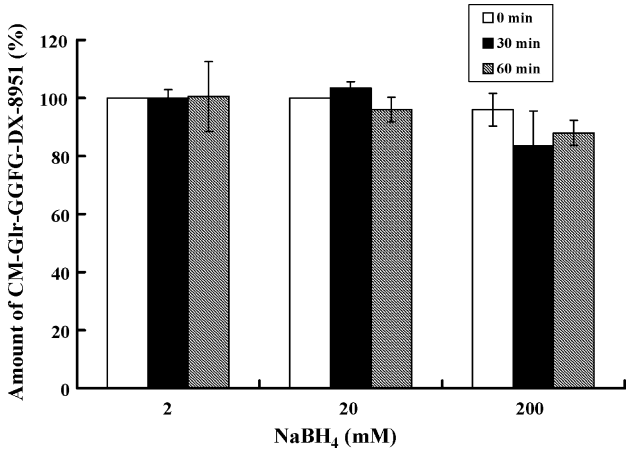

Fig. 4. Stability of CM-Glr-GGFG-DX-8951 at 30 °C in the presence of 2–200 mM sodium borohydride. All data were normalized by an initial concentration of CM-Glr-GGFG-DX-8951. Each datum represents the mean ± S.D.

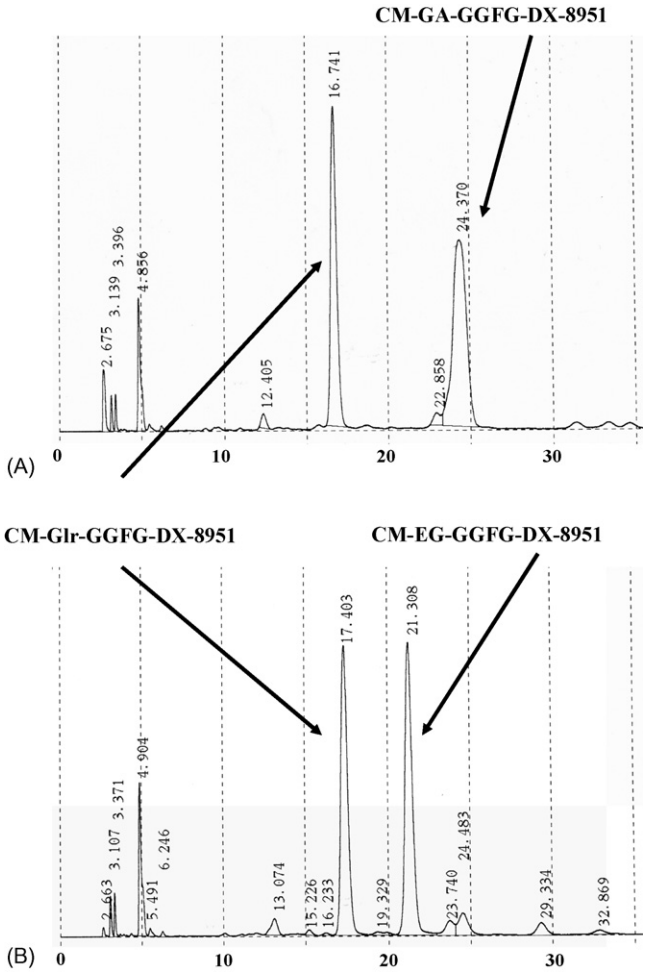

Fig. 5. HPLC chromatograms of the unreduced DE-310 hydrolysate (A), and the hydrolysate reduced with sodium borohydrate (B).

peak at 24 min in Fig. 5B was collected and subjected to MS spectroscopy analysis. The FAB-MS indicated *m/z* 928 and 950, which corresponded to $[M+H]^+$, $[M+Na]^+$, respectively, demonstrating that the peak in Fig. 5B was not due to CM-GA-GGFG-DX-8951 (*m/z* 853). Thus, CM-GA-GGFG-DX-8951 was completely converted to CM-EG-GGFG-DX-8951 by the reducing agent, whereas CM-Glr-GGFG-DX-8951 was unchanged.

3.4. Estimation of generation of CM-GA-GGFG-DX-8951 and CM-Glr-GGFG-DX-8951 by deconvolution analysis

The kinetics of DE-310 hydrolysis in 1 M HCl at 30 °C was evaluated (Fig. 6). The hydrolysis was stopped, and the lactone ring of DX-8951 was opened by the addition of an alkaline solution of 1 M NaOH and 0.1 M Tris–HCl buffer containing 20% (v/v) acetonitrile. The reducing agent, sodium borohydride, was added to transform CM-GA-GGFG-DX-8951 into the stable compound CM-EG-GGFG-DX-8951. Then, the DX-8951 lactone ring was closed by the addition of 0.5 M HCl containing acetonitrile before HPLC analysis. The yields of the two hydrolyzates, CM-Glr-GGFG-DX8951 and CM-GA-GGFG-DX8951, achieved a maximum level between 5 and 8 h, and decreased gradually thereafter (Fig. 6). Fig. 7 shows the rates of generation of the two products from DE-310 estimated by deconvolution analysis using k_{dec} values as a weighting function. Generation of CM-Glr-GGFG-DX8951 and CM-GA-GGFG-DX8951 appeared to be completed within 8 h. Finally, it was found that CM-Glr-GGFG-DX8951 and CM-GA-GGFG-DX8951 produced from DE-310 were 71.8 and 84.7 nmol/mg DE-310, respectively. Considering that CM-Glr-GGFG-DX8951 and CM-GA-GGFG-DX8951 indicate bonding of GGFG-DX-8951 to carboxymethyl group at the C-4 position of a glucose unit, and GA derivatives to that at the C-2 position, it suggested that slightly larger amount of GGFG-DX-8951 was linked to the carboxymethyl groups at the C-2 position in DE-310.

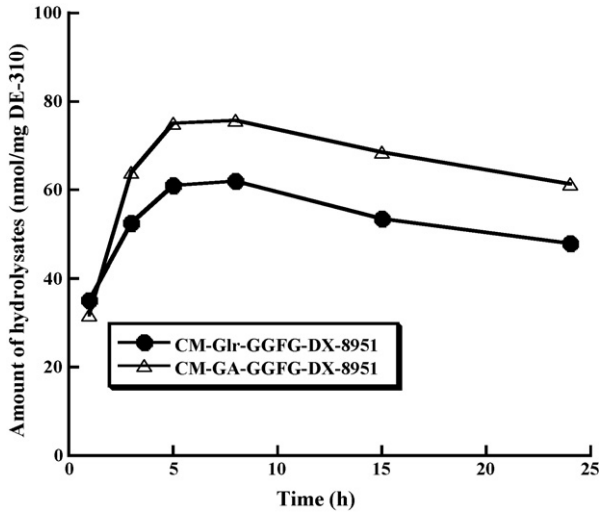

Fig. 6. Kinetics of CM-Glr-GGFG-DX-8951 and CM-GA-GGFG-DX-8951 produced from DE-310 in 1 M HCl at 30 °C and reduction with sodium borohydrate to DE-310.

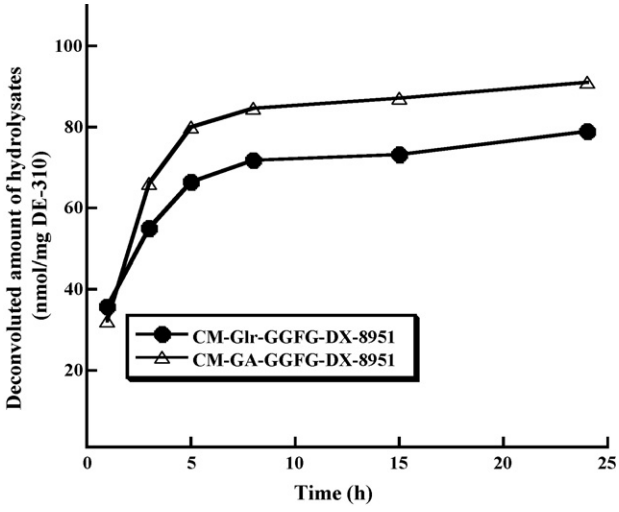

Fig. 7. Production of CM-Glr-GGFG-DX-8951 and CM-GA-GGFG-DX-8951 from DE-310 estimated by deconvolution analysis.

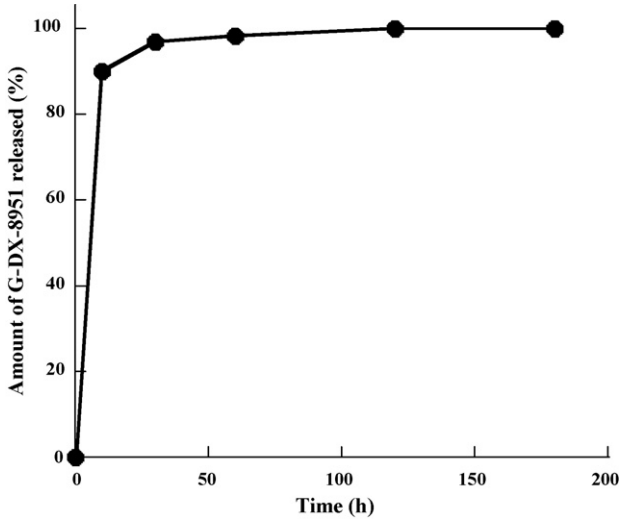

Fig. 8. Kinetics of G-DX-8951 released from DE-310 treated with α-chymotrypsin at 40 °C in pH 6.0.

3.5. Determination of DX-8951 content in DE-310 by enzymatic hydrolysis

It was found that G-DX-8951 is produced when DE-310 is treated with α-chymotrypsin. The amount of produced G-DX-8951 reached a plateau within 60 min, while it was approximately 90% of the maximum level by 10 min (Fig. 8). Additionally, GPC analysis indicated that after treatment with α-chymotrypsin for 2 h the macromolecular fraction did not possess UV absorbance derived from the DX-8951 moiety (data not shown). The amount of G-DX-8951 produced was finally 157.5 ± 0.9 nmol/mg DE-310 (mean ± S.D.), which was almost equal to the sum of CM-Glr-GGFG-DX-8951 and CM-EG-GGFG-DX-8951 (71.8 + 84.7 = 156.5 nmol/mg DE-310) mentioned above.

4. Discussion

Camptothecin (CPT) derived from the Oriental Tree, *Camptotheca acuminata*, is an inhibitor of topoisomerase-I and exhibits promising antitumor activity against various experimental tumors [7,8]. However, CPT is extremely water-insoluble, and this feature has severely restricted its clinical application [9,10]. Recently, a novel camptothecin analog, DX-8951f, has improved water solubility and exhibits antitumor activity against both murine tumors and human tumor xenografts in mice [11–13]. High efficiency of DX-8951f in clinical tests is expected, although toxicity is a concern as with other camptothecin analogs [14].

The main adverse clinical effects of anticancer drugs are myelosuppression and gastrointestinal toxicity, which are closely related to its pharmacokinetic properties. One possible way to enhance the therapeutic efficacy of these drugs while reducing their toxicity is to attach them to a macromolecular carrier [15–20]. DE-310 was designed to distribute preferentially to and retain itself for longer time in solid tumors than in healthy tissues, based on the well-known "Enhanced Permeability and Retention (EPR) effect" [2].

Although the structure of DE-310 and the DX-8951 content are closely associated with drug efficacy and toxicity, any reliable method for its analysis, have not yet been developed. This problem was associated with multiplicity of bonding sites of GGFG-DX-8951 in DE-310. In the present study, a method of analyzing the structure of DE-310 based on "Smith degradation" [21] was developed. Smith degradation is a series of chemical reactions to degrade polysaccharides; that is, periodic acid oxidation, reduction with borohydride, and hydrolysis with acids. Dextran, which is a material of CM-Dex-PA, consists of glucose units linked with an α(1 → 6) linkage. Periodic acid oxidation of dextran liberates the C-3 carbon of a glucose unit as formic acid and forms aldehydes at C-2 and C-4 portions. When α(1 → 6) linkages are hydrolyzed following reduction of the aldehyde groups to alcohols with sodium borohydride, glycolaldehyde (GA) and glycerol (Glr) are generated from the fragment comprising C-1 and C-2 and the other comprising C-4, C-5, and C-6, respectively. The first two steps of DE-310 synthesis, i.e., periodic acid oxidation and sodium borohydride reduction, are identical to the Smith degradation. In DE-310, GGFG-DX-8951 groups are covalently linked to carboxymethyl groups associated with C-2 or C-4 carbons. Therefore, the products obtained by hydrolysis, i.e., CM-GA-GGFG-DX-8951 and CM-Glr-GGFG-DX-8951, indicate C-2 and C-4 positions, respectively. Generation of CM-GA-GGFG-DX-8951 and CM-Glr-GGFG-DX-8951 after hydrolysis of DE-310 was confirmed in HPLC separation followed by NMR and MS spectroscopy (Fig. 5 and Table 1).

Another step integral to the success of the method was conversion of the GA derivative to the EG form by borohydride

Table 1
FAB MS and ^{1}H NMR spectral data of CM-Glr-GGFG-DX-8951 and CM-EG-GGFG-DX-8951

Compound (MW)	FAB MS, *m/z*	Chemical shift by ^{1}H NMR (DMSO-d_6), δ[a]
CM-Glr-GGFG-DX-8951 (884.90)	886 $[M+H]^+$	8.43–8.47 (1H, m), 7.80 (1H, d, *J* = 10.8 Hz), 7.31 (1H, s), 7.14–7.24 (5H, m), 6.52–6.54 (1H, tar), 5.55–5.59 (1H, m), 5.41 (2H, d, *J* = 7.8 Hz), 5.25 (2H, s), 4.38–4.43 (1H, m), 3.91 (2H, s), 3.25–3.80 (13H, m), 3.15–3.25 (2H, m), 2.98 (1H, dd, *J* = 4.5, 14.0 Hz), 2.76 (1H, dd, *J* = 9.5, 14.0 Hz), 2.40 (3H, s), 2.19–2.22 (1H, m), 2.09–2.14 (1H, m), 1.80–1.90 (2H, m), 0.88 (3H, t, *J* = 1A Hz)
CM-EG-GGFG-DX-8951 (854.88)	856 $[M+H]^+$	8.43–8.47 (1H, m), 7.80 (1H, d, *J* = 10.8 Hz), 7.31 (1H, s), 7.14–7.24 (5H, m), 6.52–6.54 (1H, br), 5.55–5.59 (1H, m), 5.41 (2H, d, *J* = 7.8 Hz), 5.25 (2H, s), 4.38–4.43 (1H, m), 3.91 (2H, s), 3.25–3.80 (11H, m), 3.15–3.25 (2H, m), 2.98 (1H, dd, *J* = 4.5, 14.0 Hz), 2.76 (1H, dd, *J* = 9.5, 14.0 Hz), 2.40 (3H, s), 2.19–2.22 (1H, m), 2.09–2.14 (1H, m), 1.80–1.90 (2H, m), 0.88 (3H, t, *J* = 7.4 Hz).

[a] Chemical shifts are referenced to TMS at 0 ppm for ^{1}H.

reduction, because the GA derivative was unstable. However, lactone ring of DX-8951 is unstable to the reduction condition. Here, in order to prevent the lactone ring of DX-8951 being reduced, it was opened with alkaline prior to treatment with borohydride. This method was validated during a preliminary experiment to assess the stability of CM-Glr-GGFG-DX-8151 against sodium borohydride. It seems to be stable in $\leq$20 mM sodium borohydride solution for up to 1 h (Fig. 4).

Deconvolution analysis was used to correct for the acid degradation of CM-Glr-GGFG-DX-8951 and CM-GA-GGFG-DX-8951 produced by the hydrolysis of DE-310. Assuming that the average molecular weight (MW) of DE-310 was approximately 340,000, the results show the 24.5 molecules of CM-Glr-GGFG-DX-8951 and 28.9 molecules of CM-EG-GGFG-DX-8951 can be produced from one molecule of DE-310. When DE-310 was hydrolyzed with α-chymotrypsin, only G-DX-8951 was produced (Fig. 8). The amount of G-DX-8951 produced was almost the same as the sum of CM-Glr-GGFG-DX-8951 and CM-GA-GGFG-DX-8951, confirming quantitability of the proposed method. It should be noted that the present method also enables the drug-backbone bonding site to be determined, as well as the molar amount of DX-8951 in DE-310, considering that the Glr derivative indicates bonding of GGFG-DX-8951 to the C-4 position, and GA derivatives to the C-2 position of a glucose unit.

The content of DX-8951 in DE-310 was determined by 360-nm UV absorption measurement for comparison. When DX-8951f was used as a reference substance, the estimated content of DX-8951 in DE-310 was 15% lower than that determined by acid hydrolysis (data not shown). On the other hand, it was identical when CM-Glr-GGFG-DX-8951 was used as a reference. Thus, difference in the extinction coefficient between DX-8951f and conjugated-DX-8951 should be careful in determining the content of DX-8951 in DE-310 by simple UV absorption measurement.

Chromatograms of the DE-310 hydrolysate included a few minor components that fluoresce at the 445 nm emission of DX-8951 (Fig. 5). One of these is supposed a glucose related-compound. Dex contains 4% of Glc*p*-(1 → 3,6) as a branched chain, and this linkage is not oxidized by periodic acid [22]. Therefore, 4% of Glc residue was evaluated in CM-Dex-PA, and it was predicted that one of the minor fragments observed in DE-310 hydrolyzate was GGFG-DX-8951 linked through the CM group of branched Glc.

In pharmacokinetic studies of polymer conjugate, the amount of drug released is usually measured but not that of a drug linked to the polymer conjugates, being primarily due to the difficulty in HPLC determination. The present method fragments DE-310 into small molecules, CM-Glr-GGFG-DX-8951 or CM-EG-GGFG-DX-8951, can easily be detected by HPLC, when toxicology samples such as tissue and serum were treated properly. It would provide information on tissue distribution and the stability of the macromolecular conjugate. Thus, the method developed during this study can help to clarify the pharmacokinetics of DE-310, in addition to structural analysis of the conjugate.

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