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Liquid chromatographic assays for DE-310, a novel camptothecin analog, and two major enzymatic products in human matrices

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Abstract

Assays were developed for determination of DE-310, a carboxymethyldextran polyalcohol conjugate of the topoisomerase I inhibitor DX-8951 (exatecan) and two enzymatic products (i.e. glycyl-DX-8951 and unconjugated DX-8951) in human whole blood, erythrocytes and saliva. Sample pretreatment involved a single protein-precipitation step, followed by a thermolysin-mediated deconjugation for the parent molecule. Separation of the compounds was achieved on an Inertsil ODS-80A column (150 mm × 4.6 mm i.d.; 5 μm PS), using isocratic elution. The column effluent was monitored at excitation and emission wavelengths of 375 and 445 nm, respectively. Validation results indicated that the methods are accurate and precise at lower limits of quantitation of 0.5–6.9 ng/ml. The methods were used to study the blood distribution and salivary concentrations in patients receiving DE-310.

Keywords: Camptothecin; DE-310

1. Introduction

DE-310 comprises the active moiety DX-8951 (exatecan; [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*]pyranol)-[3′,4′:6,7]-indolizino-[1,2-*b*]-quinoline-10,13-dione), a hexacyclic camptothecin derivative, linked to a biodegradable macromolecular carboxymethyldextran polyalcohol drug-delivery system via a glycyl-glycyl-phenylalanyl-glycyl-peptide spacer (Fig. 1) [1]. The biodegradable polymer portion of DE-310 is designed to provide preferential tumoural uptake and sustained release of the active moiety within the tumour, as a result of an enhanced permeability and retention effect [2–4]. After entering the tumour area, DE-310 is taken up by tumour cells through endocytosis. The breakdown of the carrier is mediated by lysosomal enzymes, i.e. cathepsins, which results in two enzymatic products, viz. glycyl-DX-8951 (G-DX-8951) and unconjugated DX-8951 (DX-8951).

Currently, DE-310 is being tested in clinical trials administered as a 3 h infusion given once every 4 or 6 weeks [5,6]. Preliminary pharmacokinetic analysis showed that the apparent plasma half-lifes of conjugated DX-8951, glycyl-DX-8951 (G-DX-8951) and unconjugated DX-8951 (DX-8951) in humans, were in the order of 10–13 days [5]. This pharmacokinetic behavior is very distinct from that of other known camptothecin analogs [7]. To allow more detailed studies on the distribution of DE-310 and to elucidate the basis for its unique pharmacokinetic behavior, information of dynamic changes of free drug in vivo in different body compartments is essential.

Previously, several physiologically based approaches have been put forward for the determination of the non-protein-bound fraction of drugs in dynamically functioning biological systems, including analysis of saliva [8], cerebrospinal fluid [9], and red blood cell partitioning [10]. Here, we report on the development and validation of HPLC-based analytical methods for the determination of

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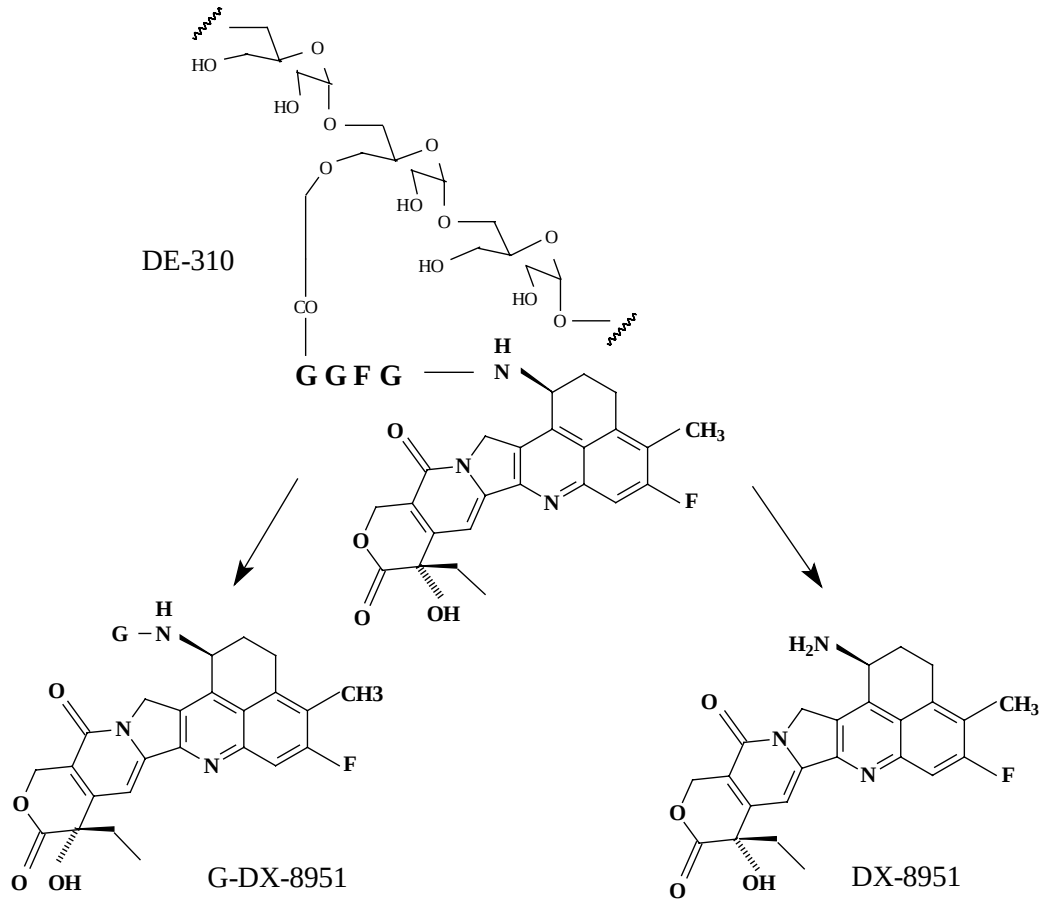

Fig. 1. The chemical structures of DE-310 (MW 3.4×10^5), DX-8951 (MW 435.45) and G-DX-8951.

conjugated DX-8951, DX-8951 and G-DX-8951 in human whole blood, erythrocytes and saliva.

2. Experimental

2.1. Chemicals and reagents

Reference standards of DE-310 (batch, P-IW114B; purity, 100%); DX-8951f (batch, 654-654-98-003; purity, 99.9%), which refers to the monomethanesulfonate dihydrate salt form of DX-8951; G-DX-8951 trifluoroacetate (batch, AR068103; purity, 98.8%) and the internal standard DW-8579 (batch, AR141301; purity, 98.2%) were supplied by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Acetonitrile was obtained from Biosolve (Valkenswaard, the Netherlands), Tris–hydrochloride buffer and thermolysin from Sigma (St. Louis, MO, USA), and zinc sulfate and calcium chloride from J.T. Baker (Deventer, the Netherlands). All other chemicals and HPLC solvents were of highest grade available commercially. Milli-Q-UF (Millipore, Bedford, MA, USA) water was used throughout. Blank human plasma, whole blood and saliva were obtained from healthy volunteers via the Central Laboratory of the Blood Transfusion Service (Amsterdam, the Netherlands). Unwashed erythrocytes were isolated from whole blood samples using MESED instruments (Kelmis, Belgium), as described previously [11].

2.2. Sample preparation

2.2.1. DX-8951 and G-DX-8951 in whole blood, erythrocytes and saliva

Aliquots of 100 μl blood were transferred to 4.5 ml glass tubes, followed by the addition of 900 μl of human plasma, 500 μl of acetonitrile, 100 μl of internal standard DW-8579 (100 ng/ml in acetonitrile–water (1:1 (v/v)), and 100 μl of zinc sulfate [70% (w/v) in water]). After vigorous vortex mixing for 5 min on a multi-tube vortex mixer (Dade S8215-1X, Miami, FL, USA), samples were centrifuged using a Hettig Universal 30RF centrifuge (Tüttlingen, Germany) for 5 min at $4000 \times g$. The supernatant was transferred to a clean 4.5 ml glass tube and partially evaporated under a gentle stream of nitrogen at 60 °C for exactly 30 min. An aliquot of 200 μl of the residue was transferred to a low-volume insert of glass, and 100 μl was injected into the HPLC system.

Prior to extraction of erythrocyte samples, 100 μl volumes of erythrocytes were diluted with 150 μl of human plasma,

followed by vortex mixing on an MS2 minishaker (IKA Works, Inc., Wilmington, NL, USA). This procedure was performed to achieve a uniform assay in order to interpolate the chromatographic data on a single standard measure line in the matrix (plasma). Aliquots of 100 μl of plasma-diluted erythrocytes were further prepared as blood (see above).

Sample pretreatment for DX-8951 and G-DX-8951 in saliva was identical to that described for whole blood, using saliva aliquots of 250 μl diluted with 750 μl of human plasma. This procedure was performed to achieve an uniform assay in order to interpolate the chromatographic data on a single standard measure line in the matrix (plasma). At the end of the evaporation period, 250 μl of the residue was transferred into a low volume insert of glass, and 200 μl were subjected to chromatography.

2.2.2. Conjugated DX-8951 in whole blood, erythrocytes and saliva

Aliquots of 100 μl of blood, plasma-diluted erythrocytes, or saliva were transferred to 1.5 ml polypropylene microtubes (Eppendorf, Hamburg, Germany), followed by the addition of 100 μl of plasma and 600 μl of methanol. Following vigorous mixing for 1 min on the multi-tube mixer, samples were centrifuged at ambient temperature for 5 min at 10,000 × *g*. Next, 600 μl of the upper layer were transferred to a 4.5 ml glass tube and then evaporated to dryness under nitrogen at 60 °C. The residue was reconstituted in 100 μl of water, 200 μl Tris–hydrochloride buffer (0.1 M; pH 8.5), and 100 μl of a thermolysin solution (2.0 mg/ml in 0.1 M calcium chloride). This mixture was incubated for 1 h at 50 °C in a water bath to release phenylalanylglycyl-DX-8951 (FG-DX-8951) from conjugated DX-8951. The reaction was stopped by adding 500 μl of a 0.5 M hydrochloric acid–water mixture (1:1 (v/v)). The resulting solution was centrifuged for 5 min at 4000 × *g*, and 100 μl of the supernatant were diluted with 100 μl of phosphate-buffered saline (pH 7.2), from which 100 μl were injected into the HPLC system.

2.3. Equipment and chromatographic conditions

Chromatographic analyses were performed using a constaMetric 3200 pump (LDC Analytical, Riviera Beach, FL, USA), a 717plus autosampler (Waters, Milford, MA, USA) and a Jasco FP-920 fluorescence detector (Hachioji City, Japan). The analytical column used was packed with Inertsil ODS-80A material (150 mm × 4.6 mm i.d.; 5 μm PS) from GL Science (Tokyo, Japan). The column temperature was maintained at 60 °C by using a Spark Mistral column heater (Meppel, the Netherlands). The fluorescence detector operated at excitation and emission wavelengths of 375 and 445 nm, respectively, with the emission bandwidth set at 18 nm.

The mobile phase for determination of DX-8951 and G-DX-8951 consisted of a 0.1 M sodium acetate (pH 5.0, acetic acid)–tetrahydrofuran mixture (15:2 (v/v)), whereas that for conjugated DX-8951 was composed of 0.1 M sodium acetate (pH 5.0, acetic acid)–methanol (10:12 (v/v)). The mobile phases were prepared daily, filtered and degassed before use, and delivered isocratically at a flow rate of 1.0 ml/min.

2.4. Calibration

Separate standard stock solutions of DX-8951, G-DX-8951 and DW-8579 were prepared in triplicate at 1.0 mg/ml in dimethylsulfoxide and were stored at –80 °C. The amount of compounds in the triplicate solutions was measured by injection aliquots of 50 μl of 2500-fold diluted stock solutions in acetonitrile–water (1:1 (v/v)), followed by a 50-fold dilution in the mobile phase (all in triplicate) and injection into the HPLC system. The mean value of the individual stock solutions was within 2.0–4.6% of each other. Stock solutions of DE-310 were prepared at 1.0 mg/ml (equivalent to 69.0 μg/ml of DX-8951) in water, and were stored at −80 °C. The amount of compound in the triplicate solutions was measured by incubation of aliquots of DE-310 with thermolysin for 1 h in a water bath at 50 °C, followed by injection of 10 μl aliquots after stopping the enzyme reaction.

Acquisition and integration of HPLC data was performed with Chrom-Card data analysis system (Fisons, Milan, Italy), running on an ICW chromatographic workstation. Six-point calibration curves were prepared daily in a blank matrix by serial dilution from the standard stock solution. For DX-8951 and G-DX-8951, weighted $1/x^2$ linear-regression analysis of the peak–height ratio of the compounds and the internal standard versus the concentration were used for the calculation of the calibration curves. For conjugated DX-8951, weighted $1/x^2$ linear-regression analysis of the peak height versus the concentration was used.

2.5. Method validation

The selectivity of the methods was assessed using a set of five independent blank (drug-free) human blood and saliva samples, which were handled as described above. The presence of potentially interfering endogenous compounds with retention times similar to that of DX-8951, G-DX-8951, conjugated DX-8951 and/or the internal standard was investigated by visual inspection of the chromatograms.

The precision and accuracy of the analytical procedures was evaluated by repeat analysis of quality control (QC) samples, prepared in batch at various concentrations in each matrix and stored at −80 °C, on separate occasions. QC samples of unwashed erythrocytes were prepared by incubation of 30 ml of whole blood for a period of 30 min at 37 °C with DX-8951 and G-DX-8951 (each at a final concentration of 50 ng/ml). Subsequently, 1.5 ml aliquots of whole blood were transferred to 20 MESED instruments for isolation of unwashed erythrocytes. The remaining blood was used for the determination of DX-8951 and G-DX-8951 concentrations, and for hematocrit measurement.

The between-run precision (BRP) was evaluated prospectively as the percentage relative standard deviation obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable, calculating between-groups mean square (B), the within-groups mean square (W) and the grand mean (M) of the observed concentrations across run days. BRP was defined as

$$\mathrm{BRP} = 100 \times \frac{\left[(B - W)/n\right]^{0.5}}{M}$$

The within-run precision (WRP) was estimated in a similar manner using the one-way ANOVA as

$$\mathrm{WRP} = 100 \times \frac{W^{0.5}}{M}$$

In case where W is greater than B, the resulting variance is negative, implying that no significant additional variation was observed on performing the assay in different runs. The average accuracy of the determinations were calculated as percentage deviation from nominal values (%DEV) and was defined as

$$\%\mathrm{DEV} = 100 \times \left(\frac{M}{\text{nominal concentration}}\right)$$

For determination of the lowest standard concentration with a definite level of certainty, i.e. the lower limit of quantitation (LLQ), blank samples were spiked with each of the analytes and subjected to repeat analysis as described for the QC samples. Stability of the analytes in the tested matrices was evaluated following (1) three freeze–thaw cycles of 15 min each, (2) at 37 °C for 24 h, and (3) after extraction at room temperature (autosampler stability). The extraction efficiency was established by comparing peak areas of samples prepared in plasma with those for non-processed samples prepared in the mobile phase. All statistical analyses were performed using the software package Number Cruncher Statistical System v5.0 (Dr. J.L. Hintze, Kaysville, UT, USA; 1992).

2.6. Patient samples

The patients studied participated in a clinical Phase I and pharmacokinetic study of DE-310 in various nonhematological malignancies [5]. The DE-310 doses of 6.0 or 7.5 mg/m^2 were delivered as a 3 h intravenous infusion, with treatment cycles repeated every 6 weeks. The protocol was approved by the Institutional Review Boards of Erasmus University Medical Center (Rotterdam, the Netherlands) and written informed consent was obtained prior to treatment.

Blood samples were collected in sodium heparinized coated tubes before and at 1, 2, 4, 6, 8, 24, 48, 72, 168, 240, 336, 504, 672 and 840 h after the end of the DE-310 infusion. After sampling, the blood specimens were immediately put in an ice-water bath (4 °C) until centrifuged at 2000 rpm for 15 min at 4 °C. Plasma was transferred into plastic specimen storage vials and the red blood cell fraction into Falcon vials, and both were stored frozen at −20 °C until analysis. Saliva samples were collected in plastic specimen storage vials before and at 3, 7, 27, 51, 72, 168, 240, 336, 504, 672 and 840 h after the end of the DE-310 infusion and stored frozen at −20 °C until analysis as described above.

3. Results and discussion

3.1. Chromatography and detection

Because of the pH-dependent instability of the α-hydroxy-δ-lactone moiety in the core structure of most camptothecin analogs [12], including DX-8951, resulting in the ring-opened carboxylate form at high pH and the ring-closed lactone form at low pH, we decided to focus only on measurement of total concentrations in the present study, since disruption of the carboxymethyldextran polyalcohol side chain while maintaining the physiologic lactone-to-carboxylate ratio may not be feasible. In addition, for most camptothecin analogs it has been shown that total drug monitoring serves as an appropriate surrogate of the lactone forms [13]. The choice of the internal standard DW-8579, which lacks the C4-methyl substituent as compared to DX-8951 (Fig. 1), and the optimal fluorescence wavelength couple of the analytes (365/445 nm) was based on earlier work described for determination of DX-8951 in human plasma [14] and mouse plasma [15]. Furthermore, no other metabolites were expected around the retention time of the internal standard [14].

The choice of the Inertsil ODS-80A analytical column was based on previous experience during the development of an assay for total drug forms (i.e. lactone plus carboxylate) of the related agent topotecan in human whole blood and unwashed erythrocytes [16], and resulted in optimal selectivity factors and resolution. The composition and pH of the mobile phase was selected in order to optimize separation factors and peak shapes of the analytes. To ensure sufficient selectivity in our assays, tetrahydrofuran was added to the mobile phase as an organic modifier.

In order to allow for convenient and inexpensive sample-pretreatment procedure of the biological samples, we opted for a solvent extraction, rather than taking recourse to solid-phase cartridges [13]. This was achieved by the use of a protein-precipitation step in the presence of aqueous zinc sulfate [17]. This sample handling was also chosen for its optimal elimination of endogenous interferences, while maintaining a high extraction efficiency for the analytes in all matrices.

3.2. Method validation

3.2.1. Whole blood and erythrocytes

Chromatograms of blank and spiked human whole blood and erythrocyte samples are shown in Fig. 2A and B,

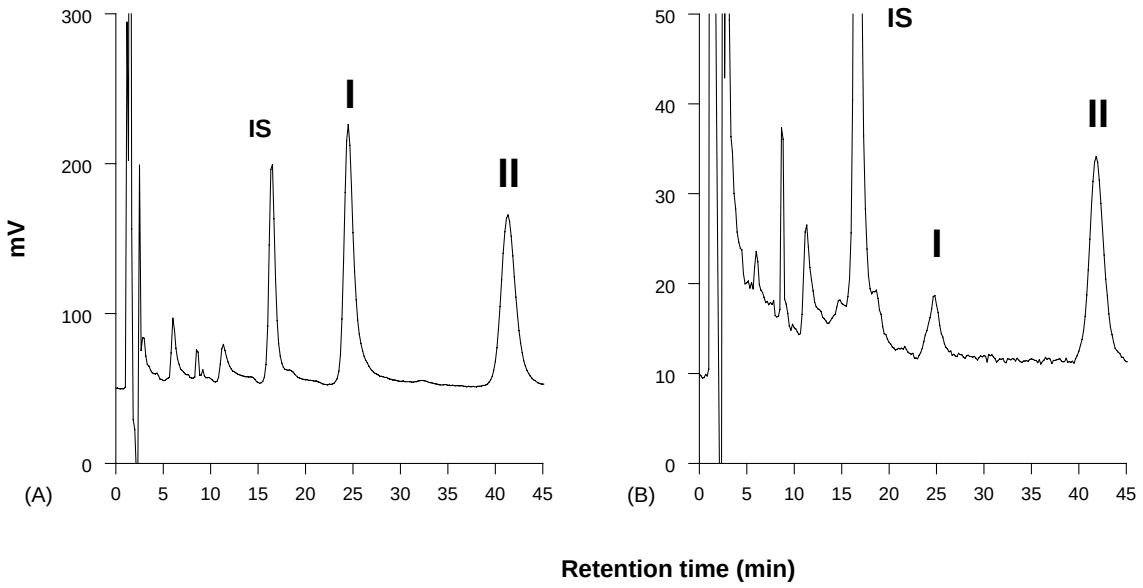

Fig. 2. HPLC chromatograms of internal standard (IS) DW-8579, (I) DX-8951 and (II) G-DX-8951 in (A) whole blood and (B) erythrocytes.

respectively. The selectivity for the analytes is shown by the sharp and symmetrical resolution of the peaks, with no significant interfering peak for all compounds in drug-free specimens, obtained from five different individuals. The retention times of the internal standard DW-8579, unconjugated DX-8951 and G-DX-8951 were 16, 24 and 42 min, respectively, with an overall chromatographic run time of 45 min. This long run time was chosen to obtain optimal chromatographic resolution and to eluate potential interfering substances previous to injection of a new sample. In addition, there was no need for a high-throughput assay because a limited number of saliva and erythrocyte samples per patient were expected. The calibration curves of assays of DX-8951 and G-DX-8951 in human blood were found to be linear in the range of 5.0–200 ng/ml when applying a weight factor of $1/x^2$, with correlation coefficients of at least 0.995 and 0.997 for DX-8951 and G-DX-8951, respectively.

QC samples in whole blood spiked with DX-8951 and G-DX-8951 (50 ng/ml, final concentration) were assayed in duplicate in 4 days, whereas QC samples in erythrocytes were assayed in quintuplicate in 4 days. Validation data of the analytical runs in terms of accuracy and precision indicated that all of the samples were within the acceptable range (Table 1). The mean overall extraction recoveries were 118.0 and 101.0% for DX-8951 and G-DX-8951, respectively. Stability data for DX-8951 and G-DX-8951 in human blood indicated that both compounds were stable following three freeze–thaw cycles as well as after incubation at 37 °C for 24 h (Table 2).

Following incubation of human blood spiked with DE-310 at a concentration of 5000 ng/ml (i.e. equivalent to DX-8951 at a concentration of 345.0 ng/ml) at 37 °C for 30 min, no conjugated DX-8951 could be detected in the erythrocytes following their isolation in the MESED instruments. For this reason, no further validation characteristics could be made for conjugated DX-8951 in erythrocytes (data not shown). This is consistent with the high molecular weight of the conjugated analyte, precluding (extensive) red blood cell partitioning.

3.2.2. Saliva

No chromatographic interference was noted from endogenous compounds in blank human saliva specimens (Fig. 3A). The calibration curves for DX-8951 and G-DX-8951 in

Table 1
Validation characteristics of DX-8951 and G-DX-8951 in human blood

Spiked concentration (ng/ml)	Observed concentration (ng/ml)		Precision (%)		No. of replicate observations
	Whole blood	Erythrocytes	Within-run	Between-run	
DX-8951					
50	–	7.26	7.8	4.6	5
50	50.6	–	1.3	0.65	2
G-DX-8951					
50	–	35.1	2.6	5.3	5
50	47.6	–	0.64	3.9	2

Table 2
Stability data of DX-8951 and G-DX-8951 in human blood

Spiked concentration (ng/ml)	Freeze–thaw (three cycles)		24 h, 37 °C		No. of replicate observations
	Observed concentration (ng/ml)	Difference (%)	Observed concentration (ng/ml)	Difference (%)	
DX-8951					
20.3	20.6	1.8	19.1	−5.8	3
74.1	72.0	−2.8	72.8	−1.7	3
158.8	155.1	−2.3	152.2	−4.2	3
G-DX-8951					
17.7	18.4	4.0	16.5	−7.0	3
65.1	64.7	−0.7	64.8	−0.6	3
144.2	138.8	−3.8	136.6	−6.0	3

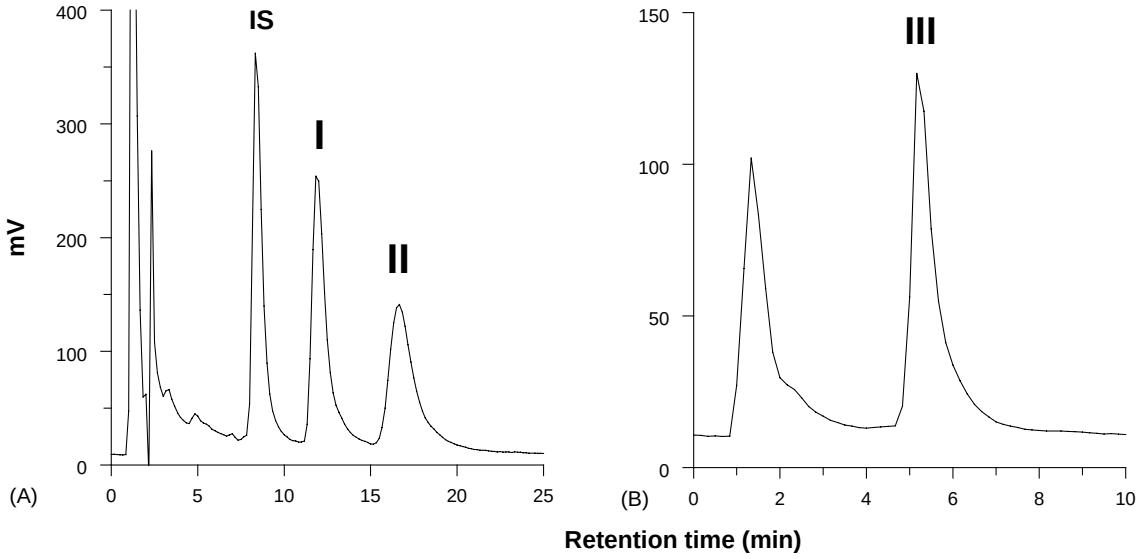

Fig. 3. HPLC chromatograms of (A) internal standard (IS) DW-8579, (I) DX-8951 and (II) G-DX-8951; and (B) (III) FG-DX-8951 in human saliva.

saliva were linear in the range of 0.5–20.0 ng/ml, with correlation coefficients of at least 0.990. The retention times for DW-8579, DX-8951 and G-DX-8951 were 9, 12 and 17 min, respectively, with an overall chromatographic run time of 45 min. A representative chromatogram of a QC sample containing DX-8951 and G-DX-8951 spiked at a concentration of 7.5 ng/ml is also shown in Fig. 3. The LLQ samples were assayed in quintuplicate on four separate occasions, and indicated that 95 and 80% of the analyzed samples containing DX-8951 and G-DX-8951, respectively, were within the acceptable range for accuracy of 80–120%. Likewise, QC samples were analyzed in quintuplicate on four consecutive days, and accuracy and precision data were within acceptable limits (Table 3). Stability data of DX-8951 and G-DX-8951 in human saliva after three freeze–thaw cycles (−80 °C) and after 24 h at 37 °C are given in Table 4. These data suggest a minor loss of material due to instability, necessitating rapid freezing of clinical samples after blood collection.

The calibration curves of the assay for conjugated DX-8951 in human saliva were linear in the range 100.0–5000 ng/ml (equivalent to 6.9–345 ng/ml of DX-8951), with correlation coefficients of at least 0.996. The retention time of conjugated DX-8951 was 5.2 min, and the total run time was set at 10 min. A representative chromatogram of a QC sample containing DE-310 spiked at a concentration of

Table 3
Validation characteristics of DX-8951 and G-DX-8951 in human saliva

Concentration (ng/ml)		Precision (%)		Accuracy (%)
Spiked	Observed	Within-run	Between-run	
DX-8951				
0.5 (LLQ[a])	0.51	6.1	3.0	101.6
2.5	2.43	2.7	3.1	97.2
7.5	7.19	2.1	3.6	95.8
15.0	14.4	2.3	2.6	95.9
G-DX-8951				
0.5 (LLQ)	0.48	11.5	[b]	95.4
2.5	2.25	3.7	[b]	90.1
7.5	6.67	2.8	3.8	90.2
15.0	13.8	2.4	3.6	91.7

[a] LLQ: lower limit of quantitation.
[b] No additional variation was observed on performing the assay in different runs.

Table 4
Stability data of DX-8951, G-DX-8951 and DE-310 in human saliva

Spiked concentration (ng/ml)	Freeze–thaw (three cycles)		24 h, 37 °C		No. of replicate observations
	Observed concentration (ng/ml)	Difference (%)	Observed concentration (ng/ml)	Difference (%)	
DX-8951					
2.5	2.2	−12.0	2.1	−16.0	2
7.1	6.4	−9.8	5.9	−16.9	2
14.3	12.7	−11.2	11.8	−17.5	2
G-DX-8951					
2.5	2.0	−20.0	1.7	−32.0	2
7.4	5.8	−21.6	5.0	−32.4	2
15.4	12.4	−19.5	10.4	−32.5	2
DE-310 (DX-8951 eq.)					
11.86	12.06	1.7	9.11	−23.2	3
129.8	129.5	−0.3	106.7	−17.8	3
303.3	291.5	−3.9	236.2	−22.1	3

Table 5
Validation characteristics of DE-310 in human saliva

Concentration (ng/ml)		Precision (%)		Accuracy (%)
Spiked	Observed	Within-run	Between-run	
100.0 (6.9 ng eq. DX-8951)	98.4	7.5	2.2	98.3
200.0 (13.8 ng eq. DX-8951)	204.2	3.8	4.2	102.0
1000.0 (69.0 ng eq. DX-8951)	940.0	3.4	3.1	94.6
2000.0 (138 ng eq. DX-8951)	1879.6	3.8	5.1	93.6

200.0 ng/ml (13.0 ng/ml of DX-8951) is shown in Fig. 3B. All of the analyzed LLQ and QC samples fell within the range of accuracy of 80–120%. Because of the fact that 100% of the analyzed LLQ and QC samples were within the acceptable range of accuracy, only three analytical runs were performed (Table 5). Stability experiments showed that conjugated DX-8951 was stable during the chromatographic run when using plastic inserts and at 4 °C in the dark, but not after three freeze–thaw cycles (−80 °C) and upon storage for 24 h at 37 °C (Table 4).

3.3. Pharmacokinetics

The suitability of the developed methods for the clinical use was demonstrated by the determination of DE-310 and its enzymatic products in human erythrocytes and saliva obtained from eight patients with different advanced solid tumours, treated with DE-310 at a dose level 6.0 or 7.5 mg/m^2 once every 6 weeks. In all analyzed samples of erythrocytes and saliva, the concentration of DE-310, G-DX-8951 and DX-8951 were below the lower limit of quantitation (<0.5 ng/ml). This would fit with the hypothesis of preferential drug uptake in tumour tissue, but not in other tissues.

In conclusion, we have developed and validated HPLC methods with fluorescence detection for DE-310 and two major metabolites in human whole blood, erythrocytes and saliva. The methods were shown to meet the current requirements as to the validation of bioanalytical methods, providing good accuracy and precision. The results obtained from the analysis of patient samples revealed that DE-310, G-DX-8951 and DX-8951 are neither distributed to erythrocytes nor excreted in saliva, which makes the biodistribution of this topoisomerase I inhibitor substantially different from that of topotecan [11], or irinotecan and its metabolite, SN-38 [18].

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