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Simultaneous determination of gemcitabine and gemcitabine-squalene by liquid chromatography–tandem mass spectrometry in human plasma

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Abstract

Gemcitabine-squalene is a new prodrug that self-organizes in water forming nanoassemblies. It exhibits better anti-cancer properties in vitro and in vivo than gemcitabine. A liquid chromatography/tandem mass spectrometry assay of gemcitabine-squalene and gemcitabine was developed in human plasma in order to quantitate gemcitabine and its squalene conjugate. After protein precipitation with acetonitrile/methanol (90/10, v/v), the compounds were analyzed by reversed-phase high performance liquid chromatography and detected by tandem mass spectrometry using multiple reaction monitoring. The method was linear over the concentration range of 10–10,000 ng/ml of human plasma for both compounds with an accuracy lower than 10.4% and a precision below 14.8%. The method showed a lower limit of quantitation of 10 ng/ml of human plasma for dFdC and dFdC-SQ. A preliminary in vivo study in mice was shown as application of the method as no significant difference between human and mice plasma for the analysis of dFdC and dFdC-SQ was demonstrated.

Keywords: Gemcitabine; Gemcitabine-squalene; Mass spectrometry; Cancer; Nucleosides analogues

1. Introduction

Gemcitabine (Gemzar®, 2′,2′-difluorodeoxycytidine, dFdC) is a known nucleoside analogue that displays anticancer activity both in vitro and in vivo [1,2]. It is used in the treatment of various types of cancer such as metastatic breast cancer, non-small cell lung cancer and pancreatic cancer. In order to exert its anticancer activity, dFdC is taken up into the cell by nucleoside transporters and then phosphorylated by deoxycytidine kinase into its 5′-monophosphate (dFdCMP) and subsequently into its active 5′-diphosphate (dFdCDP) and 5′-triphosphate (dFdCTP) nucleotides. This latter incorporates into DNA, leading to inhibition of DNA synthesis and apoptosis [3]. Besides, intracellular concentrations of dFdCTP in white blood cells as surrogate for tumour tissue are often determined for describing the pharmacokinetics and pharmacodynamics of gemcitabine [4].

However, dFdC is also metabolised by intracellular and extracellular deoxycytidine deaminase into the chemotherapeutically inactive uracil derivative (2′,2′-difluorodeoxyuridine (dFdU)), [5–7], leading to a short terminal half-life of dFdC (range: 11–26 min) [8,9]. Different approaches have been attempted to decrease deamination of dFdC to dFdU [10,11]. One of them is to link the 4-amino site of dFdC to a fatty acid derivative, thereby protecting it from deamination by cytidine deaminase [11]. This strategy of linking a long chain of fatty acids aims as well to improve the lipophilicity of dFdC, hence, increase its interactions with the lipidic membranes and its cellular uptake [11]. Herein, by linking long chain saturated and mono-unsaturated fatty acids to the 4-amino group of gemcitabine, its lipophilicity [11] and cytotoxicity increased [10]. Recently, Couvreur et al. have succeeded in coupling dFdC to an isoprenoid chain of squalene to obtain gemcitabine-squalene (dFdC-SQ) (Fig. 1) [2]. This new analogue has an amphiphilic character and is capable

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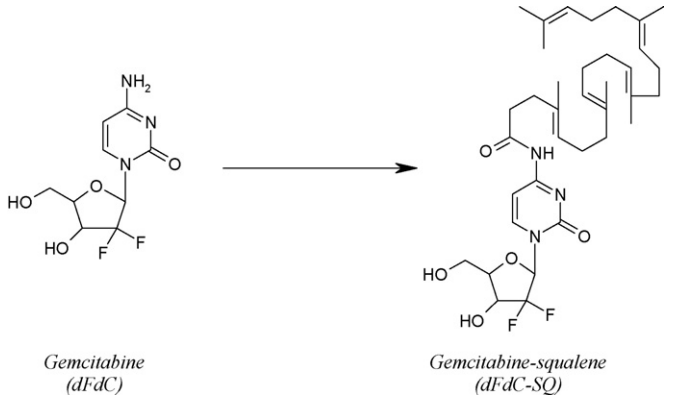

Fig. 1. Structure of gemcitabine (dFdC) and gemcitabine-squalene (dFdC-SQ).

of spontaneously forming nanoassemblies of 150 nm in water [2].

Interestingly, the squalenoylation of dFdC made dFdC-SQ more potent than dFdC in vitro and in vivo against different types of murine and human cancers, including resistant cancers [2]. Yet, until now, the mechanism of action of dFdC-SQ remains to be elucidated.

In order to study the release of dFdC from its prodrug (dFdC-SQ), a sensitive and rapid analytical method for the quantitation of both compounds was developed for human plasma. Nevertheless, the assay was first evaluated and applied to mice plasma in order to explore the dFdC-SQ behaviour, mainly its dFdC liberation, when injected intravenously in a preclinical study.

Several quantitative methods of dFdC have been developed using either HPLC/UV [12–16] or sensitive LC/MS methods with negative electrospray mass spectrometry [17] or with positive electrospray and tandem mass spectrometry ESI-MS/MS [15,18] or with APCI tandem mass spectrometry [19]. None of these previous methods could be adapted to the determination of both dFdC and dFdC-SQ simultaneously. Thus, this study was aiming to develop a LC–MS/MS method for the simultaneous quantitation of dFdC-SQ and dFdC in human plasma over the range of concentrations from 10 to 10,000 ng/ml of human plasma. Deoxycytidine was used as an internal standard based on previously described methods for dFdC quantitation by HPLC/UV [12,20,21] and by LC–MS/MS [18].

2. Experimental

2.1. Chemicals

dFdC was purchased from Sequoia (Sequoia, UK) with 98% minimum purity. dFdC-SQ was synthesized according to Couvreur et al. [2]. Deoxycytidine (dC), used as internal standard, was obtained from Sigma–Aldrich (Steinheim, Germany) with 99% purity. HPLC grade methanol and acetonitrile were provided by Carlo Erba (Rodano, Italy), formic acid by Merck (Darmstadt, Germany). Ultra pure water was prepared using a Milli-Q^{TM} system (Millipore, St Quentin-en-Yvelines, France). Drug-free heparinised human plasma was obtained from EFS (Rungis, France). Tetrahydrouridine (THU) was provided from Calbiochem (La Jolla, CA, USA).

2.2. Stock solutions

Independent standard and quality control (QC) stock solutions were prepared and stored at −20 °C. Stock solutions of dFdC and dC were prepared separately at 1 mg/ml solution in methanol. For dFdC-SQ, a 1 mg/ml stock solution was prepared in ethanol. A stock solution of THU was prepared in ultra pure water at 10 mg/ml and was flushed under nitrogen flow after each use and then stored at −20 °C.

A working solution of dFdC and dFdC-SQ mixture (50/50, v/v) was prepared at 0.5 mg/ml in a mixture of methanol/ethanol (50/50, v/v); further dilutions were performed for calibration standards. Similarly, for QCs, a working solution was set at 0.5 mg/ml in methanol/ethanol (50/50, v/v). The working solution of the internal standard (dC) was prepared at 10 μg/ml in methanol.

2.3. Plasma preparation

2.3.1. Standards and QC sample preparation

Calibration standards and QC samples were prepared by adding 20 and 15 μl, respectively, of an appropriate working solution containing both dFdC and dFdC-SQ, and 20 μl of the internal standard dC (10 μg/ml) to 100 μl of THU-pretreated human plasma. The mixture was then vortexed for 5 s. Thus, 10 standard samples were prepared in duplicate containing both analytes at different final concentrations (10, 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10,000 ng/ml of human plasma) and the internal standard at final concentration of 2000 ng/ml of human plasma. Four QC samples were prepared at 10 (QC1), 150 (QC2), 750 (QC3) and 6000 ng/ml of human plasma (QC4) for both analytes. Then, standards and QC samples were extracted according to the procedure as described in Section 2.3.2.

2.3.2. Plasma sample preparation

Forty microlitres of THU (10 mg/ml) were added to 5 ml of human plasma in order to prevent ex vivo deamination of dFdC into dFdU [20]. The extraction method is adapted from Lin et al. [15]. Briefly, to a 100 μl of THU-treated unknown mice plasma or spiked human plasma (calibration standards and QC samples) containing 20 μl of internal standard (10 μg/ml), 1 ml of a mixture of acetonitrile/methanol (90/10, v/v) was added. The sample was vortexed 10 s and then centrifuged at 15,800 × *g* (*Eppendorf centrifuge 5415R*) for 20 min at 4 °C. The supernatant was transferred to a conic polypropylene tube and evaporated at room temperature to dryness under a nitrogen flow. The residues were then stored at −20 °C until analysis. Prior analysis, these residues were dissolved in 200 μl of a mixture of methanol/water containing 0.1% formic acid (95/5, v/v). Twenty microlitres were injected into the chromatographic system.

2.4. Chromatography and tandem mass spectrometry

Chromatographic analysis was performed using a 1100 series HPLC system (Agilent Technologies, Massy, France) including an autosampler, a binary pump and an Uptisphere® C18 column 3 μm, 2 mm i.d. × 50 mm length (Interchim, Montluçon,

France) at a flow rate of 0.2 ml/min. Gradient elution started with the initial step for 1 min of 100% of solvent A (methanol/water with 0.1% formic acid, 10/90, v/v), followed by 1 min linear gradient up to 100% solvent B (methanol/water with 0.2% formic acid, 95/5, v/v) and held for 6 min, then back again to 100% solvent A. The column was then equilibrated for 6 min at the initial conditions before the next injection. Prior to each sample injection, the autosampler was washed twice with 30 μl of solvent methanol/water with 0.1% formic acid (95/5, v/v). The total analysis time was 14 min per sample.

Detection was performed with a Quattro®-LCZ triple quadrupole mass spectrometer equipped with the orthogonal electrospray source (Waters Micromass, Manchester, UK). Analytes were detected in the positive ion mode using tandem mass spectrometry with multiple reaction monitoring (MRM). The dwell time was set at 0.5 s for dFdC-SQ and 0.5 s for dFdC and dC. The capillary voltage was set at 3500 V. The source temperature and the nebulization gas temperature were set at 90 and 200 °C, respectively. Nitrogen gas flow was set at 450 l/h. Collision gas (argon) pressure was set at 1.3 mbar. Cone voltages were set at 35 V for dFdC-SQ, 20 V for dFdC and 15 V for dC. Collision energies and transitions ion pairs were optimized for each analyte by infusion at 10 μl/min with a Harvard syringe pump. MRM transitions for the detection of dC, dFdC and dFdC-SQ were carried out with a collision energy of 10, 15, 25 eV, respectively. Data were processed using MassLynx™ software (Waters Micromass, Manchester, UK).

2.5. Extraction recovery

In order to study the release of dFdC from its prodrug, dFdC-SQ, an appropriate extraction method was needed for both compounds simultaneously. For this purpose, we have determined and compared the extraction yields of both dFdC and dFdC-SQ after applying different methods described previously for dFdC. These methods can be divided in three major categories (i) protein precipitation [14,15], (ii) liquid–liquid extraction [12] and (iii) solid phase extraction [17,18]. Recovery values for both compounds and the internal standard were determined comparing the three QCs values (three replicates) obtained with the analysed human plasma extract values spiked with dFdC and dFdC-SQ at the same QCs levels.

2.6. Validation procedure of the LC–MS/MS assay

The quantitative LC–MS/MS assay was validated according to International Conference of Harmonization (ICH) guidelines [22] in terms of specificity, linearity, accuracy and precision.

2.6.1. Stability

The stability of dFdC and dFdC-SQ in human plasma was studied by spiking THU pretreated plasma with 750 ng/ml of dFdC and dFdC-SQ. The mixture was then stored at −20 °C for 2 months until analysis. The analytes, dFdC-SQ and dFdC, were considered stable when the measured concentrations were at less than 15% bias from the freshly spiked plasma at that same concentration.

2.6.2. Specificity

The specificity was studied by preparing and analyzing a 10 ng/ml of human plasma (LLOQ) standard compared to blank human plasma. Six different batches of human plasma and one batch of pooled mice plasma were studied. The analytical method was considered selective when there is no detectable interference from blank extracts with the analytes.

2.6.3. Linearity and sensitivity (LLOQ/LOD)

Calibration curves were obtained by plotting the peak area ratio of each analyte and the internal standard versus the analyte concentration and were fitted by linear least-squares regression with $1/x^2$ weighting over the range from 10–10,000 ng/ml of human plasma. Each standard was prepared in duplicate each day and over three different days. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of each analyte that could be determined with accuracy (bias lower than 20%) and precision less than 20%. The limit of detection (LOD) was defined as the concentration that gives a chromatographic peak higher than three-fold signal to noise ratio.

2.6.4. Accuracy and precision

Accuracy was measured by the deviation or bias (%) of the mean found concentration from the actual concentration in standards and in QCs. Repeatability and intermediate precision were studied. Repeatability, expressed through its coefficient of variation (CV_r), was investigated for the four levels of QCs (five replicates prepared the same day). Intermediate precision, expressed through its coefficient of variation (CV_i), was evaluated for each QC concentration over three different days (15 replicates). The acceptable criteria for precision (intermediate and repeatability) and accuracy were set lower than 15%, except for the LLOQ was set at less than 20%.

2.7. Application of the analytical method

QC samples were prepared in mice plasma at the four concentrations (10, 150, 750 and 6000 ng/ml) for dFdC and dFdC-SQ and were analyzed by LC–MS/MS in order to evaluate the accuracy (bias) and precision (CV%). They were compared to QC values in human plasma. (bias and CV% should be lower than 15%, except 20% for LLOQ).

To test the applicability of the validated method, we have analysed dFdC and dFdC-SQ concentrations in plasma of DBA/2 female mice ($n = 4$), aged between 5 weeks purchased from Janvier (Le-Genest-Saint-Isle, France). The administered dose was 36.6 mg/kg of dFdC-SQ nanoassemblies equivalent to 15 mg/kg of dFdC. dFdC-SQ was administered by retro-orbital route in the right eye. Blood samples (about 0.5 ml) were collected into tubes containing 5 μl of 3% EDTA, by retro-orbital puncture in the left eye 60 min after administration. Plasma was prepared, without delay, by centrifugation at 3000 × *g* for 10 min at 4 °C. 2 μl of THU (10 mg/ml in water) was added to 100 μl of collected mice plasma in order to avoid ex vivo deamination of dFdC into dFdU. As a result, the amount of THU added to mice plasma (20 μg per 100 μl) was 2.5-fold higher than the amount of THU added to human plasma (8 μg per 100 μl) so that cyti-

dine deaminase in the freshly collected mice plasma was most likely inhibited. In addition, we realize that it would have been preferred to add THU immediately to whole blood instead of to the plasma after centrifugation. However, this experiment was designed for verifying the applicability of the method. Accordingly, we intend to add THU in freshly collected blood before centrifugation in further experiments in order to prevent a potential over-estimation of dFdU.

3. Results and discussion

3.1. Mass spectrometry study

In order to characterize each analyte with its specific transition ion, a scan (MS) and daughter scan (MS/MS) analyses have been achieved by direct infusion of each standard diluted in methanol/water with 0.2% formic acid (95/5, v/v). dFdC analysis showed a well known transition from *m/z* 264 → *m/z* 112, as described earlier [17–19]. The product ion mass spectrum of dFdC-SQ showed the parent ion at *m/z* 646.5 and three daughter ions detected at *m/z* 494, *m/z* 264 and *m/z* 112 (Fig. 2). The daughter ion *m/z* 494 corresponded to the loss of the sugar ring of dFdC-SQ. The same loss of 152 u was observed during dFdC fragmentation [19]. A break in the amide covalent bond induced the formation of *m/z* 264 daughter ion corresponding to the dFdC part of dFdC-SQ. Finally, the observed *m/z* 112 daughter ion corresponded to cytosine (M_r = 111), after the loss of the squalenoyl and of the sugar ring.

The analytes dC, dFdC and dFdC-SQ have different molecular masses. Nevertheless, their specific transitions, *m/z* 228.0 → 112.0, *m/z* 264.0 → 112.0 and *m/z* 646.5 → 112.0 for dC, dFdC and dFdC-SQ, respectively, showed that they share the same daughter ion (*m/z* 112) corresponding to cytosine after the loss of the sugar ring (152 u) for dC and dFdC and after the loss of squalenoyl and sugar ring for dFdC-SQ (Fig. 2). In order to avoid signal interferences, this latter daughter ion was used as the specific transition for the quantification of dFdC-SQ. These data allow optimizing the MS/MS parameters in order to obtain the best sensitivity for each compound in MRM mode of the quantitation method.

3.2. LC–MS/MS analysis

The quantitative LC–MS/MS method was developed using MRM for selective detection of dC, dFdC and dFdC-SQ with molecular ions *m/z* 228.0, 264.0 and 646.5, respectively (Fig. 3A). dFdC and dFdC-SQ could be eluted with a fast gradient of elution and detected by tandem mass spectrometry with positive electrospray ionization (ESI-MS/MS). The composition of methanol in the mobile phase with 0.2% formic acid was optimized to get reproducible HPLC analysis and sensitive detection of dFdC-SQ in positive ESI mode. In order to overcome the challenge of the difference of the physical properties between dFdC and dFdC-SQ, a gradient of two different mobile phases was required. The LC–MS/MS gradient run allowed the separation of dFdC and dFdC-SQ with retention times of 0.95 and 7.15 min, respectively (Fig. 3A). However, although dFdC and dC were eluted at 0.95 and 0.98 min, respectively, the separation was achieved by means of their specific MRM transitions (*m/z* 264 → 112 and *m/z* 228 → 112, respectively). In addition, we found that dFdU (*m/z* 265), by using this developed analytical method based on specific MRM detection, co-eluted with dC and dFdC (data not shown). Knowing that the isotopic ion *m/z* 265 of dFdC (*m/z* 264) might interfere with the detected ion of dFdU (*m/z* 265) leading to its overestimation, thus dFdU could not be analysed with this method described for the simultaneous determination of dFdC-SQ and dFdC. In fact, the aim of the method was the quantitation of the prodrug dFdC-SQ and the dFdC liberated from dFdC-SQ after administration to mice of dFdC-SQ nanoassemblies.

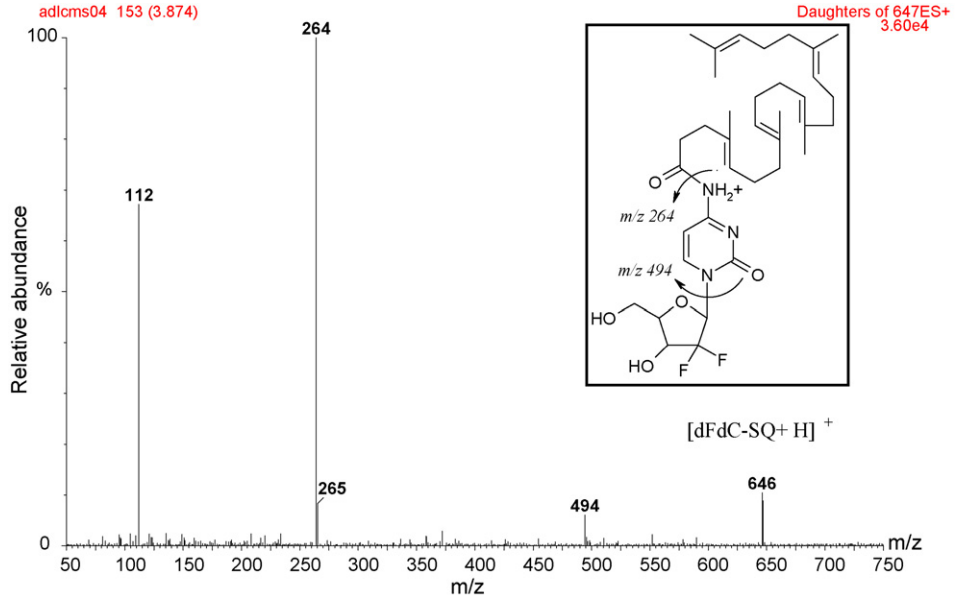

Fig. 2. Product mass spectrum of dFdC-SQ. Insert: proposed fragmentation scheme for dFdC-SQ.

3.3. Validation study of the LC–MS/MS method

3.3.1. Stability

After the analysis of THU treated human plasma spiked with 750 ng/ml of dFdC or dFdC-SQ and stored at −20 °C, dFdC-SQ and dFdC were stable at least 2 months (bias < −12%). In addition, the stability of dFdC and dFdC-SQ in the solvent mixture ethanol/methanol was at least 3 months at −20 °C. Furthermore, after extraction, the compounds were stable in the injection solvent at least 48 h at room temperature.

3.3.2. Specificity

The detection method was selective using the combination of specific MRM transitions and retention times of each analyte. The specificity was demonstrated by comparing analyses of LLOQ standard (Fig. 3A) and extracted blank human plasma (Fig. 3B). As illustrated on Fig. 3B, no significant interference in the different blank human plasma ($n = 6$) was found at the retention times of dFdC, dFdC-SQ and dC for each MRM transition, respectively. A lack of interference was also observed in the blank mice plasma (data not shown).

3.3.3. Calibration curves and sensitivity (LLOQ/LOD)

The calibration curves were determined over the range 10–10000 ng/ml of human plasma for dFdC and dFdC-SQ. Linear regressions were preformed with $1/x^2$ weighting factor. The mean linear relationships were $y = 0.00206x - 0.00421$ for dFdC and $y = 0.00147x + 0.00542$ for dFdC-SQ. The regres-

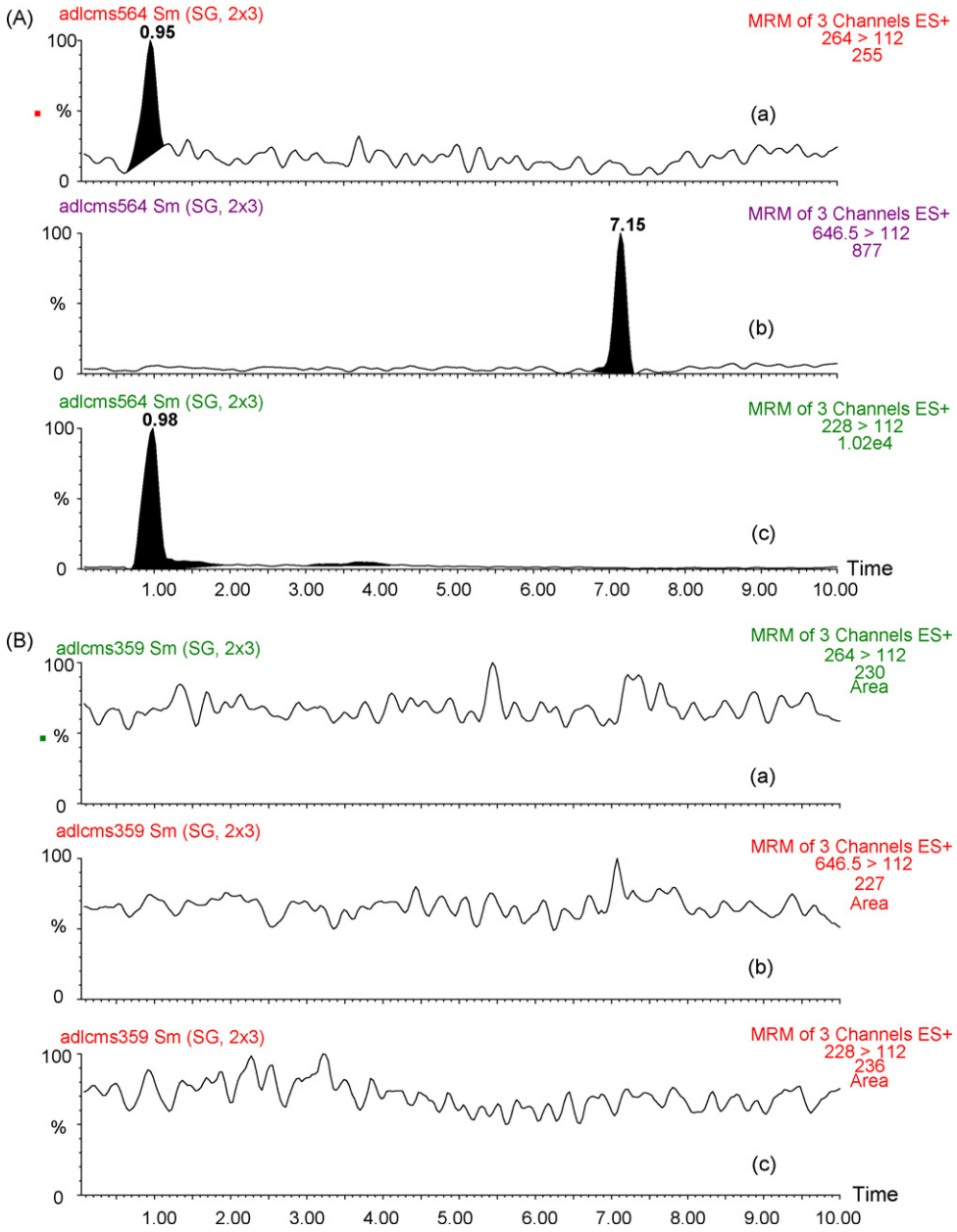

Fig. 3. (A) Mass chromatogram of LLOQ human plasma sample spiked with 10 ng/ml of (a) dFdC, (b) 10 ng/ml dFdC-SQ and with 2000 ng/ml of (c) dC (IS). (B) Mass chromatogram of blank human plasma sample. (C) Mass chromatogram of QC human plasma sample spiked with 750 ng/ml of (a) dFdC, (b) with 750 ng/ml of dFdC-SQ and (c) with 2000 ng/ml dC (IS).

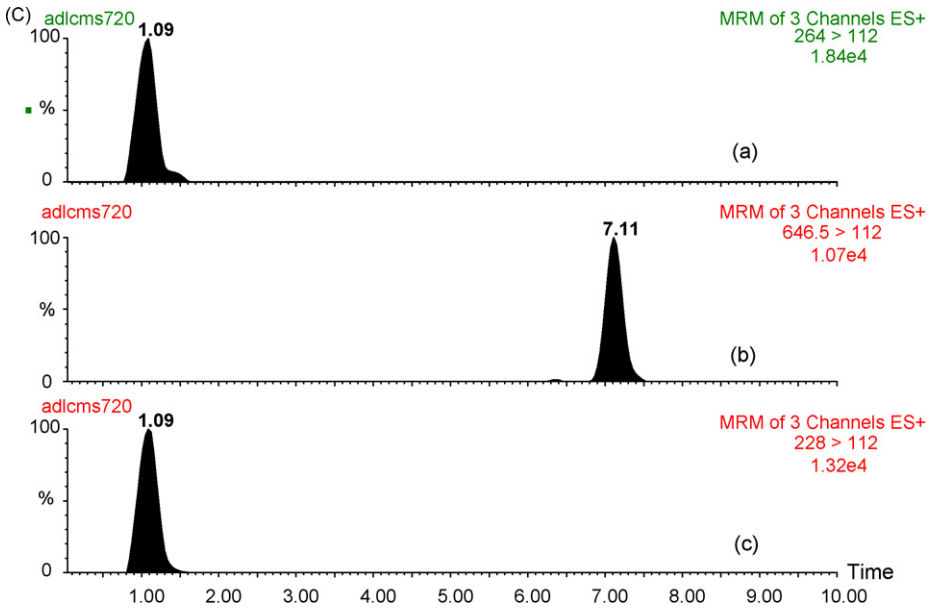

Fig. 3. (*Continued*)

sion coefficients (r^2) were 0.994 for dFdC and 0.998 for dFdC-SQ. The experimental LLOQ was found at 10 ng/ml of human plasma for both dFdC and dFdC-SQ according criteria of accuracy and precision lower than 20% (Table 1). LOD was evaluated to 3 ng/ml of human plasma for dFdC and 1 ng/ml of human plasma for dFdC-SQ with a signal/noise ratio of 3. The observed sensitivity of the method was in agreement with other LC/MS methods [19]. This LC–MS/MS method offers advantages over the LC–UV methods in terms of sensitivity, selectivity and shorter run times [12–15,16]

Compared to previously described methods for the analysis of dFdC by LC–MS/MS [15–19], our method allowed the accurate and precise determination of dFdC release in human plasma from dFdC-SQ with a LLOQ of 10 ng/ml (38 nM) and an upper LOQ of 10,000 ng/ml (38 μM) for dFdC, with accuracy and precision. Among these methods [15–19], Honeywell et al. developed an analytical method with a LLOQ of dFdC at 19 nM [17,19]. Nevertheless, as we mentioned previously, our goal was to develop a simultaneous quantitation of dFdC-SQ and dFdC.

3.3.4. Accuracy and precision

The results of the accuracy study performed in human plasma for each quality control level over 3 days are summarized in Table 1. Studies of repeatability and of intermediate precision of the four quality controls were performed for dFdC and dFdC-SQ. The method was accurate for both compounds, since all bias values were within −10.4% and +0.8%. The method was precise for both compounds with CV_r less than 12.6% for dFdC and 14.8% for dFdC-SQ, while CV_i were below 11.7% and 12.8%, respectively (Table 1).

3.4. Evaluation of the extraction recovery

In order to evaluate simultaneous recoveries of dFdC-SQ and dFdC from human plasma, we have compared the recoveries of dFdC-SQ obtained by using different methods described for dFdC extraction [12,14,15,17,18]. The liquid–liquid extraction method described by Freeman et al. [12] and Yilmaz et al. [16], when applied to the extraction of dFdC-SQ and dFdC gave extraction yields of 39 and 95% ($n = 6$), respectively. Indeed, this latter value is in agreement with the reported values [12,16].

Table 1
Accuracy (bias), repeatability (CV_r) and intermediate precision (CV_i) of dFdC-SQ and dFdC (QC) samples

Analyte	QC	QC1	QC2	QC3	QC4
dFdC	Theoretical (ng/ml)	10	150	750	6000
	Mean ± SD (ng/ml)	10.3 ± 1.3	139.4 ± 7.7	712.2 ± 42.0	6327.0 ± 312.6
	CV_r (%)	12.6	5.6	5.9	4.9
	CV_i (%)	11.7	3.8	5.2	8.8
	Bias (%)	−0.8	−6.9	−6.9	0.8
dFdC-SQ	Mean ± S.D. (ng/ml)	9.5 ± 1.4	162.2 ± 19.8	683.8 ± 48.6	5496.9 ± 588.7
	CV_r (%)	14.8	12.2	7.1	10.7
	CV_i (%)	12.8	12.1	8.4	9.0
	Bias (%)	−1.1	0.3	−4.0	−10.4

Mean values were based on five different measurements for CV_r. Bias and CV_i were based on fifteen different measurements.

The low recovery of dFdC-SQ might be due to its amphiphilic properties. On the other hand, the solid-phase extraction method described by Xu et al. [17] and Sottani et al. [18] provided 90% yield of dFdC-SQ and 35% yield of dFdC ($n = 3$). In our experimental conditions, the poor solubility of dFdC-SQ in aqueous solvents necessitates the use of ethanol. This might explain the poor recovery of dFdC compared to the previously reported values [17,18]. These preliminary experiments helped eliminating these extraction method described for dFdC. Furthermore, we have tested two different protein precipitation methods [13,15] on the spiked human plasma. Protein precipitation by acetonitrile described by Keith et al. [13], gave very low % recoveries for both dFdC-SQ and dFdC (44 and 59%, respectively, $n = 3$). On the other hand, compared to acetonitrile protein precipitation, the mixture acetonitrile/methanol, described by Lin et al. [15], provided good extraction yields for the simultaneous extraction of dFdC-SQ and dFdC (Fig. 3C). Accordingly, this protein precipitation method was chosen since it provided good extraction yields compared to the other described extraction techniques [12,14–18].

Table 2
Extraction recoveries of dC, dFdC and dFdC-SQ in human plasma after protein precipitation by acetonitrile/methanol (9/1 v/v)

	dC	dFdC			dFdC-SQ		
Concentration (ng/ml)	2000	150	750	6000	150	750	6000
Recovery (%)	90 ± 6	94 ± 1	94 ± 3	109 ± 7	97 ± 9	103 ± 15	102 ± 20

Mean values were based on three different measurements performed at each level of QC.

For both compounds (dFdC and dFdC-SQ), three determinations were performed for the three QCs levels and three for dC (at 2000 ng/ml of human plasma). Fig. 3C illustrates an analysis

Table 3
Accuracy of dFdC-SQ and dFdC in human and mice plasma

Concentrations (ng/ml)	dFdC-SQ				Δ^a (%)
	Human plasma	Bias (%)	Mice plasma	Bias (%)	
10	9.5 ± 1.4	−5	10.4 ± 1.2	+4	9
150	162.2 ± 19.8	+8	172.1 ± 7.0	+15	6
750	683.8 ± 48.6	−9	796.3 ± 117.5	+6	16
6000	5496.9 ± 588.7	−8	5264.0 ± 277.4	−12	−4
	dFdC				
10	10.3 ± 1.3	+3	9.9 ± 1.4	−1	4
150	139.4 ± 7.7	−7	132.8 ± 7.5	−11	5
750	712.2 ± 42.0	−5	633.0 ± 18.5	−16	11
6000	6327.0 ± 312.6	+5	6138.6 ± 468.1	+2	3

Mean values were based on the five different measurements for human plasma and three different measurements for mice plasma.
[a] Deviation (Δ) was defined as the absolute difference of the measured concentrations between human and mice plasma.

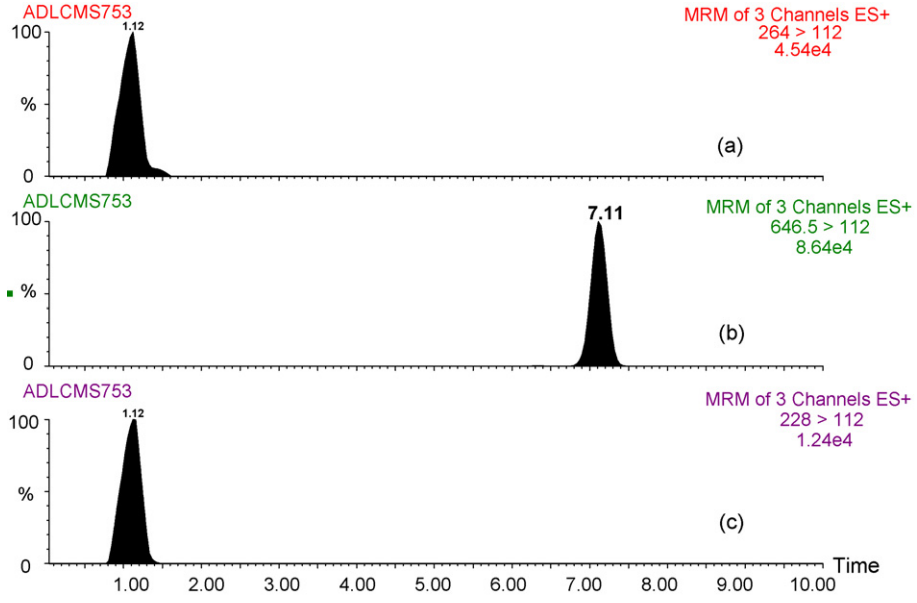

Fig. 4. Mass chromatogram of (a) dFdC, (b) dFdC-SQ in mice plasma after retro-orbital injection of dFdC-SQ nanoassemblies, spiked with (c) dC (2000 ng/ml). The concentrations of dFdC-SQ and dFdC were 3376 and 9168 ng/ml of mice plasma, respectively.

of QC sample of human plasma spiked with 750 ng/ml of dFdC, of dFdC-SQ and with 2000 ng/ml of dC (IS).

Mean extraction recoveries in human plasma were 103 and 99% for dFdC and dFdC-SQ, respectively (see Table 2). Extraction yield for the internal standard was 90%. Similar extraction recoveries for dFdC and dFdC-SQ were obtained with mice plasma. Thus, in agreement with the already published data concerning dFdC [15], this extraction method showed a good recovery for dFdC as well for dFdC-SQ. Because of the great differences in physico-chemical properties of dFdC and dFdC-SQ, a protein precipitation method adapted form Lin et al. [15] was chosen rather than liquid-liquid or solid-phase extraction.

3.5. Plasma analysis of treated mice

For QC mice plasma ($n = 3$), mean accuracy (bias) were calculated for the two analytes at the four QC concentration levels (Table 3). Bias values ranged from −16 to 2% for dFdC and from −4 to 15% for dFdC-SQ. Deviation (Δ) has been defined as the absolute difference of the measured concentrations in human and mice plasma. As the mean Δ values were within 16% at all concentration levels, and the accuracies were not significantly different from QC values obtained in human plasma, the assay was applied to mice plasma.

Herein, this LC–MS/MS assay was applied successfully to study the plasma content after intravenous administration to mice of 36.6 mg/kg (15 mg/kg in equivalence of dFdC) of dFdC-SQ nanoassemblies. Mass chromatogram of a mouse plasma sample showed the quantitative analysis of dFdC-SQ and dFdC (Fig. 4). It demonstrated that, 60 min after injection of dFdC-SQ nanoassemblies, dFdC-SQ was still present in plasma with an average concentration 10466 ± 1980 ng/ml of plasma. Moreover, it was shown that dFdC was released in part from its prodrug with an average concentration 2466 ± 690 ng/ml of plasma.

4. Conclusion

A LC–MS/MS method has been developed for the simultaneous determination of dFdC and dFdC-SQ in plasma over the concentration range of 10–10,000 ng/ml of human plasma, after a rapid and efficient sample preparation. The method was sensitive, precise and accurate over this extended range. The LLOQ in human plasma was similar to those obtained with previously described LC–MS/MS methods [18,19]. Finally, this assay could be applied for the analysis of dFdC-SQ and dFdC in samples from mice treated intravenous with dFdC-SQ nanoassemblies.

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