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Comparative study of A HPLC–MS assay versus an UHPLC–MS/MS for anti-tumoral alkyl lysophospholipid edelfosine determination in both biological samples and in lipid nanoparticulate systems

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ABSTRACT

The anti-tumor agent edelfosine represents a promising option in the treatment of cancer due to its capacity of promoting apoptosis in tumor cells selectively, while sparing healthy ones. In the present study, a novel ultra high performance liquid chromatography–tandem mass spectrometry method (UHPLC–MS/MS) was developed to quantify edelfosine concentrations in biological matrices (plasma, tissues or tumor) and in lipid nanoparticles, and compared with a conventional high performance liquid chromatography–mass spectrometry method (HPLC–MS). Compared with the HPLC method, the UHPLC method offered a threefold decrease in retention time, and a twofold decrease in asymmetry USP factor. Both methods were validated. Calibration curves for the HPLC method (0.1–1 and 1–75 μg/mL range in the plasma samples, 1–75 μg/mL range in lipid nanoparticle samples and 0.2–31.75 μg/mL range in tissue homogenate samples), and UHPLC method (0.0075–75 μg/mL for all kind of samples) showed a linear range of detector response ($r > 0.999$). Intra-batch and inter-batch precision ranged from 1.66% to 7.77% for the HPLC method and from 3.72% to 12.23% for the UHPLC method. Accuracy of the HPLC and UHPLC assays, expressed as bias, ranged from −5.83% to 7.13% and from −6.84% to 6.49%, respectively. Matrix effects on edelfosine were similar in the HPLC and UHPLC methods. The assay methods developed were successfully applied to the quality control procedure of the manufacture of edelfosine lipid nanoparticles, and to evaluate the pharmacokinetic and *in vivo* tissue distribution in mice after oral administration of edelfosine-loaded lipid nanoparticles. A good correlation between both techniques was found ($r = 0.953$) when tissue samples were analyzed with both methods.

1. Introduction

Edelfosine is considered to be the prototype molecule of a promising family of anti-cancer compounds together known as synthetic alkyl-lysophospholipids, which also comprise clinically relevant drugs that can be administered orally, such as miltefosine and perifosine [1–4]. Unlike most currently available chemotherapeutic drugs that target the nuclear DNA, this class of synthetic anti-cancer agents acts at the level of the cell membrane and induces selective apoptosis in malignant cells, sparing normal ones [5–7]. In the last decade, edelfosine has been used as purging agent to rid remission marrows of residual leukemic cells, in autologous bone marrow transplantation and acute leukemia [8]. Besides, the use of edelfosine in clinical trials or treatments has been mainly hampered by its side-effects, which include gastrointestinal, lung, liver, renal and hemolytic toxicities found *in vivo*.

The availability of rapid, sensitive and accurate analytical methods for drug quantitation in biological matrices is a requirement to carry out preclinical pharmacokinetic studies. The lack of a reliable methodology to quantify edelfosine is a major problem in ongoing and scheduled preclinical and clinical trials with this drug. A critical fact in the development of chromatographic methods for the quantitation of edelfosine is that UV-detection cannot be employed to monitor the chromatographic separation of edelfosine because of the lack of chromophore groups in the chemical structure of edelfosine and alkylphospholipids in general. Several analytical procedures have been reported for the quantitation of edelfosine in biological fluid matrices. High performance liquid chromatography assays with radiochemical detection were initially employed for the quantitation of radiolabeled edelfosine in early distribution preclinical studies [9]. However, measurement of total radioactivity does not reflect the

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real pharmacokinetic behavior or the tissue distribution of the parent drug edelfosine since the radiolabel may be included in some metabolites. High performance thin layer chromatography [10] was also evaluated, resulting in the detection of edelfosine to a limit of quantitation (LOQ) of about 26 ng of injected sample (0.3 ng with 0.1 mL of sample), inadequate to perform a pharmacokinetic analysis. High performance liquid chromatography combined with light scattering detection was investigated as an alternative technique for the quantitation of edelfosine [11]. The drawback of this method is that it can only be used for studies of relatively high-concentration edelfosine samples, such as pharmaceutical samples. The possibility of using capillary gas chromatography for the analysis of edelfosine was also evaluated [12,13]. This technique appears to be very attractive because of its intrinsically high selectivity and sensitivity. Nevertheless, dephosphorylation of the molecule is required and this reaction is far from being quantitative.

Previously, we have developed a simple, highly selective and sensitive HPLC–MS technique with a limit of quantitation of 0.3 ng of injected sample [14], which implies the benefit of the lack of need for using radiolabeled compounds. This method was used to quantify edelfosine in polymeric drug delivery systems and in uptake studies in cancer cells, and could be used with relevant modifications for the bioanalytical evaluation of the pharmacokinetic behavior of edelfosine.

Over the past 10 years there has been a constant efforts to increase analytical throughput and hence to make better use of the LC–MS/MS instrumentation. Ultra-performance liquid chromatography (UHPLC) is a significant advance in rapid, sensitive, and high-resolution liquid chromatography. This technology offers important advantages in resolution, speed and sensitivity for analytical determination, particularly when coupled with high-speed-acquisition mass spectrometers [15]. The application of UHPLC technology to clinical, toxicological, and forensic analysis has been reported in the literature in the last years. Since 2005 several authors have reported determination of different compounds in plasma and urine by UHPLC [16–23]. In most of these papers the developed UHPLC analytical procedures have been compared with conventional HPLC methods, which result in higher sensitivity, shorter analysis times, and narrow peaks, and minimization of matrix effects even after application of non-specific clean sample pre-treatment procedures.

The objective of this work is to present a comparison of chromatographic performance of two different chromatographic systems, UHPLC and conventional HPLC, for the bioanalytical determination of edelfosine (plasma, tissues or tumor) in preclinical pharmacokinetic studies developed in small rodents or in nanoparticulate lipid systems, as a quality control procedure of the manufacture of the formulation.

2. Experimental

2.1. Chemicals and reagents

Edelfosine was provided by APOINTECH (Salamanca, Spain). The internal standard, platelet activating factor (acetyl-glyceryl-ether-phosphorylcholine, PAF) and phosphate buffered saline (PBS) were obtained from Sigma–Aldrich (Madrid, Spain). Formic acid 99% was purchased from Fluka (Madrid, Spain) and methanol was obtained from Merck (Barcelona, Spain). All solvents employed for the analysis were analytical grade. Type I deionized water (18.2 MΩ resistivity) was obtained using a water purification system (Wasserlab, Pamplona, Spain). Nitrogen gas (ultra-pure, >99%) was produced by a Whatman model 75-72-K727 nitrogen generator (Haverhill, MA, USA) and by a Domnick Hunter LCMS series (Madrid, Spain). Argon gas (ultra-pure, >99.9%) was provided by Praxair (Madrid, Spain). Chemical structures of edelfosine and PAF are shown in Fig. 1.

2.2. Instruments and analysis conditions

2.2.1. HPLC–MS method

This method is based on a previous method developed in our group [14]. The apparatus used for the HPLC analysis was a Model 1100 series LC coupled with an atmospheric pressure–electrospray ionization (ESI) single quadrupole mass spectrometer equipped with a collision-induced dissociation cell (HP 1100 with MSD VL, Waldbronn, Germany). Separation was carried out at 50 °C on a reversed-phase, 150 mm × 3 mm column packed with C_{18}, 5 μm silica reversed-phase particles (Gemini®) obtained from Phenomenex® (Torrance, CA, USA). This column was preceded by a reversed-phase, C_{18}, 5 μm guard column (SecurityGuard™, 20 mm × 4 mm, Phenomenex®, Torrance, CA, USA). The mobile phase was a mixture of methanol–1% formic acid (95:5, v/v). Separation was achieved by isocratic solvent elution at a flow rate of 0.5 mL/min. The MS was operated in the positive ESI mode. The detection of edelfosine and the internal standard was performed by selected ionization monitoring (SIM) mode. ESI-MS conditions were as follows: source temperature 350 °C, capillary voltage 4 kV, and collision-induced dissociation voltage 140 V. Nitrogen was used as the desolvation gas with a flow rate of 12 L/min and a pressure of 30 psi (1 psi = 6894.76 Pa). Optimization of the interface variables, such as gas flows and voltages was done manually during direct infusion of 10 μg/mL of the target analyte dissolved in methanol.

The spectrometer was programmed to monitor both the ion of edelfosine at m/z 524.4 and platelet activating factor at m/z 574.4. Under these conditions, edelfosine and I.S. were eluted at 3.65 and 3.50 min, respectively. Data acquisition and analysis were performed with a Hewlett-Packard computer using the ChemStation G2171 AA program (Agilent, Palo Alto, CA, USA).

2.2.2. UHPLC–MS/MS method

The UHPLC system was composed of an Acquity UPLC™ system (Waters Corp., Milford, MA, USA) with thermostatized autosampler and column compartment. Separation was carried out on an Acquity UPLC™ BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) with isocratic elution using a mobile phase composed of 5% of a 1% formic acid aqueous solution and 95% of methanol. Column temperature was maintained at 50 °C. The flow rate was set at 0.5 mL/min. The autosampler was conditioned at 4 °C and the injection volume was 2 μL using partial loop mode for sample injection.

Triple-quadrupole tandem mass spectrometric detection was performed on an Acquity™ TQD mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The mass spectrometer operated in positive mode was set up for multiple reaction monitoring (MRM) to monitor the transition of m/z 524.3 → 104.2 for edelfosine and the transition of m/z 552.3 → 184.2 for the internal standard, with the dwell time of 0.1 s per transition. To optimize the MS parameters, standard solutions of both the analyte and internal standard were infused into the mass spectrometer. The following optimized MS parameters were employed: 4 kV capillary voltage, 60 V cone voltage for edelfosine and 30 V for the internal standard, 150 °C source temperature and 350 °C desolvation temperature. Nitrogen was used for the desolvation and as cone gas at a flow rate of 650 and 50 L/h, respectively. Argon was used as the collision gas. The optimized collision energy for edelfosine was 30 and 20 eV for the internal standard. Under these conditions, edelfosine and I.S. were eluted at 1.23 ± 0.01 and 1.19 ± 0.02 min, respectively. Data acquisition and analysis were performed using the MassLynx™

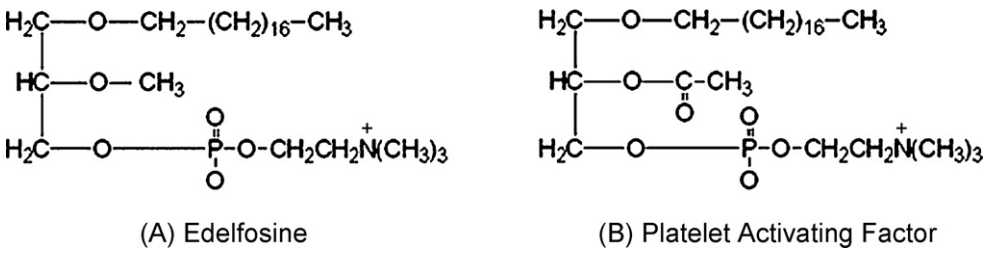

Fig. 1. Chemical structure of (A) Edelfosine and (B) platelet activation factor.

NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standard and quality control (QC) samples

2.3.1. Standard solutions and QC samples for plasma

Stock solutions of edelfosine were prepared in methanol. The stock solution of PAF was prepared in methanol at a concentration of 0.2 mg/mL. For the HPLC, two calibration ranges had to be established for sample quantitation between 0.1 and 75 μg/mL. The calibration curves for concentration range from 0.1 to 1 μg/mL were prepared by adding 50 μL of the standard solutions of 0.2, 1 and 2 μg/mL to 100 μL of blank mouse plasma. Effective concentrations of edelfosine were 0.1, 0.5 and 1 μg/mL. Calibration curves for a concentration range from 1 to 75 μg/mL were prepared by adding 50 μL of the standard solutions of 2, 30, 60 and 150 μg/mL to mouse plasma. Effective concentrations of edelfosine were 1, 15, 30 and 75 μg/mL. The QC samples were pooled at concentrations of 0.1, 5, 10 and 50 μg/mL. For the UHPLC, calibration curves for a concentration range from 0.0075 to 75 μg/mL were prepared by adding 50 μL of the standard solutions of 0.015, 6, 60 and 150 μg/mL to 100 μL of blank mouse plasma. Effective concentrations of edelfosine were

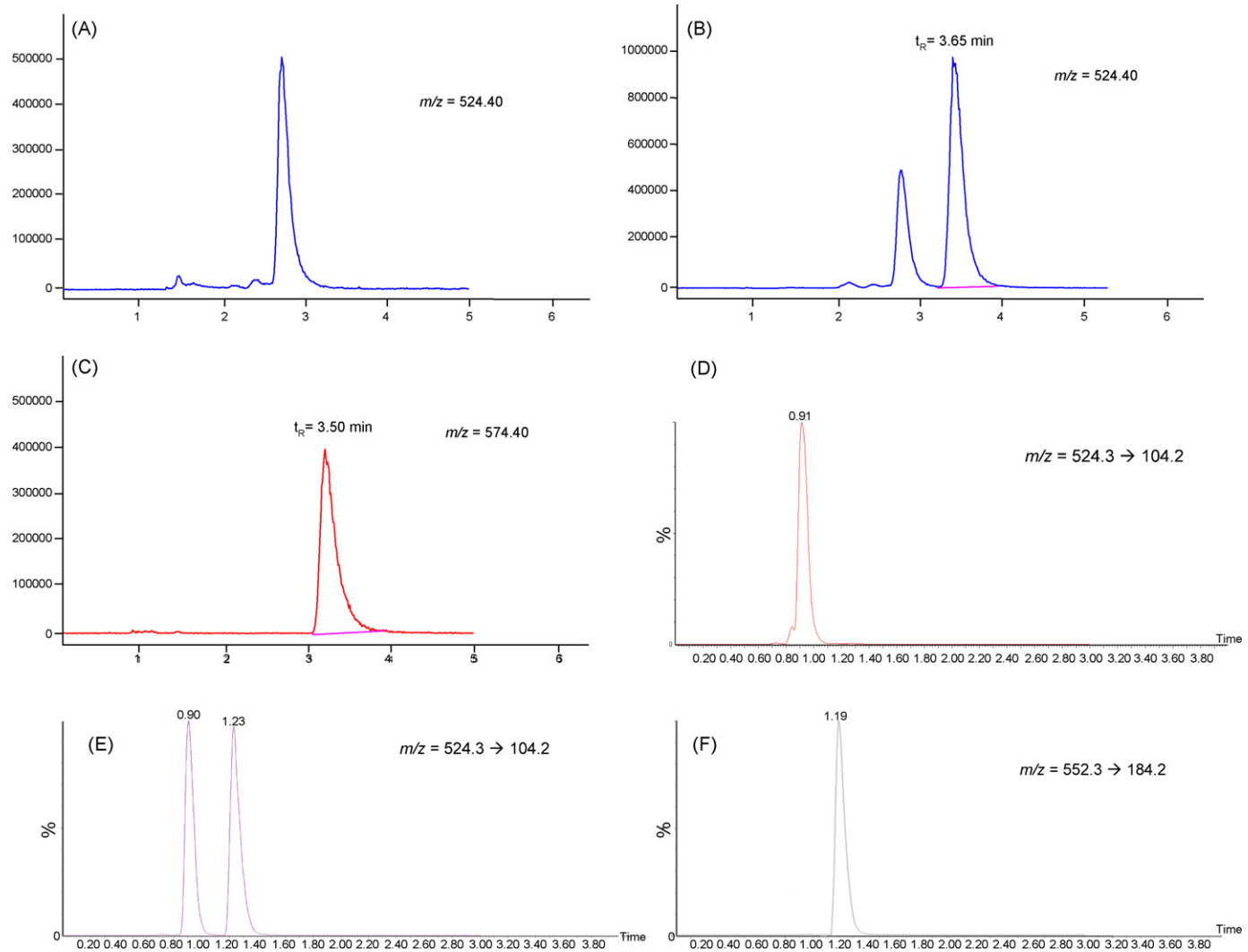

Fig. 2. Representative chromatograms of: (A) blank mouse plasma sample; (B) edelfosine resulting from the analysis of mouse plasma obtained at 1 min after intravenous administration of 200 μg of edelfosine; (C) Platelet Activating Factor (PAF) resulting from the analysis of mouse plasma after intravenous administration of 200 μg of edelfosine (PAF concentration = 0.2 mg/mL); (D) blank mouse plasma sample; (E) plasma sample after an intravenous administration of 200 μg of edelfosine to a BALB/c mouse and (F) Platelet Activating Factor (PAF) resulting from the analysis of mouse plasma after intravenous administration of 200 μg of edelfosine (PAF concentration = 0.2 mg/mL).

0.0075, 3, 30 and 75 μg/mL. The QC samples were pooled at concentrations of 0.075, 0.75, 15 and 60 μg/mL. The spiked plasma samples (standard and QC) were then processed following the application of the extraction procedure.

2.3.2. Standard solutions and QC samples for tissues

Stock solutions of edelfosine were prepared in methanol. The stock solution of PAF was prepared in methanol at a concentration of 0.2 mg/mL. For the HPLC, calibration curves were prepared by adding 50 μL of the standard solutions of 0.4, 4, 8, 16 and 63.5 μg/mL to tissue homogenate in buffer (see Section 2.6). Effective concentrations of edelfosine were 0.2, 2, 4, 8 and 31.75 μg/mL. The QC samples were pooled at concentrations of 0.1, 1.72, 17.19 and 31.75 μg/mL. For the UHPLC, calibration curves for a concentration range from 0.0075 to 75 μg/mL were prepared by adding 50 μL of the standard solutions of 0.015, 6, 60 and 150 μg/mL to the tissue homogenate. Effective concentrations of edelfosine were 0.0075, 3, 30 and 75 μg/mL. The QC samples were pooled at concentrations of 0.075, 0.75, 15 and 60 μg/mL. The spiked tissue samples (standard and QC) were then processed after application of extraction procedure.

2.3.3. Standard solutions and QC samples for lipid nanoparticles

The stock solution of PAF was prepared in methanol at a concentration of 0.2 mg/mL. Stock solutions of edelfosine were prepared in methanol. For the HPLC, effective concentrations of edelfosine were 1, 15, 30 and 75 μg/mL. The QC samples were pooled at concentrations of 1, 5, 10 and 50 μg/mL. For the UHPLC, effective concentrations of edelfosine were 0.0075, 3, 30 and 75 μg/mL. The QC samples were pooled at concentrations of 0.075, 0.75, 15 and 60 μg/mL. The edelfosine standards and QCs were then injected into the chromatographic system.

2.4. Sample preparation

2.4.1. Plasma samples

Blood was collected in EDTA surface-coated tubes and then centrifuged at 2000 × *g* for 10 min (4 °C) to separate the plasma. A portion of 100 μL of mouse plasma was transferred to a 1.5-mL tube and then 10 μL of PAF (0.2 mg/mL), used as internal standard (I.S.) were spiked to the samples. Then, 190 μL of mobile phase (methanol–1% formic acid (95:5, v/v)) were added and the mixture was vortex-mixed at room temperature for 1 min for precipitation. After centrifuging at 20,000 × *g* for 10 min, 200 μL of the supernatant were mixed with 800 μL of methanol and then, 5 and 2 μL aliquots were injected into the HPLC and UHPLC systems, respectively.

2.4.2. Tissue samples

A portion of 100 μL of tissue homogenate in buffer (see Section 2.6) was transferred to a 1.5-mL tube and then, 10 μL of I.S. (0.2 mg/mL) were spiked to the samples and 10 μL of 10% TCA were added to the mixture and vortex-mixed for 10 s for protein precipitation. Finally, 180 μL of mobile phase were added to the mixture. After vortex mixing for 1 min at room temperature and centrifuging at 20,000 × *g* for 10 min, 200 μL of the supernatant were mixed with 800 μL of methanol and then, 5 μL and 2 μL aliquots were injected into the HPLC and UHPLC systems, respectively.

2.4.3. Lipid nanoparticles

A sample of 10 mg of lyophilized nanoparticles was weighed in a 10-mL tube and then, 1 mL of chloroform was added in order to dissolve the nanoparticles. 10 μL of I.S. (0.2 mg/mL) were spiked to the samples. Then, 3 mL of mobile phase were added to the mixture. After vortex mixing for 1 min at room temperature and centrifuging at 20,000 × *g* for 10 min, 200 μL of the supernatant were mixed with 800 μL of methanol and then, 5 and 2 μL aliquots were injected into the HPLC and UHPLC systems, respectively.

2.5. Method validation

The selectivity of the assay was determined by the individual analysis of blank samples. The retention times of endogenous compounds in the matrix were compared with those of edelfosine and PAF.

LOD was defined as the sample concentration resulting in a peak area of three times the noise level. LOQ was defined as the lowest drug concentration, which can be determined with an accuracy and precision < 20%. In this work LOD of the assay method was determined by analysis of the peak baseline noise in ten blank samples.

Plasma samples were quantified using the internal standard method. Standard curves were calculated using linear least squares regression between theoretical edelfosine concentration on calibrator samples and the chromatographic peak area ratios of edelfosine to that of the internal standard. To evaluate linearity, calibrator samples were prepared and analyzed in duplicate on 3 separate days.

Accuracy and precision were also determined by replicate measurements ($n = 6$) of quality control samples at four concentration levels on five different validation days. The accuracy was expressed as (real concentration − theoretical concentration)/(theoretical concentration) × 100 and the precision by the CV (%) of the measured concentration values obtained after analysis of the quality control samples with different nominal concentration values.

The absolute extraction recoveries of edelfosine at three QC levels were evaluated by measuring the samples as described above and comparing the peak areas of the edelfosine and the I.S., and then comparing with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank biological samples (plasma or the different tissues).

The matrix effect was evaluated by comparing the peak area of the analyte dissolved in the reconstituted residues of processed blank plasma with the standard solutions at the same concentration dissolved in mobile phase. The matrix effect was evaluated at three different concentration levels, with three samples analyzed in each set. The matrix effect of the internal standard was evaluated at the concentration in plasma samples using the same method.

2.6. Application of the method

The present method has been successfully applied to the quantitation of edelfosine in biological matrices and lipid nanocarriers. To demonstrate the reliability of this method for the study of edelfosine pharmacokinetics, this assay was applied to the quantitation of edelfosine in plasma samples obtained from 6 BALB/c mice treated with an intravenous dose of 200 μg of an edelfosine solution. Tissue distribution of edelfosine was also studied. Blood samples were withdrawn at 0, 1, 2, 5, 8 and 24 h postadministration in EDTA surface-coated tubes and then centrifuged at 2000 × *g* for 10 min (4 °C) to separate the plasma (100 μL). Plasma was stored frozen (−80 °C) until analysis. Then, the animals were sacrificed and the spleen, liver, lungs, kidneys, heart, brain, stomach and intestine were collected and weighed. Tissue samples were homogenized in 1 mL of Phosphate Buffered Saline (PBS) using a Mini-bead Beater (BioSpect Products, Inc., Bartelsville, OK, USA) and centrifuged at 10,000 × *g* for 10 min. Supernatant was separated and stored frozen (−80 °C) until analysis.

This method was also employed for the assessment of encapsulation efficiency in lipid nanoparticles. Compritol® 888 ATO is the lipid employed for the formulation of nanoparticles. The method for

Table 1
Standard calibration curves of edelfosine in plasma, tissue and particulate homogenate samples calculated by the HPLC method.

	Range (μg/mL)	Regression equation	r
Plasma	0.1–1	$y = 0.1342x + 0.0001$	0.999
		$y = 0.1013x + 0.0051$	1.000
		$y = 0.1159x + 0.0018$	0.999
	1–75	$y = 0.0221x - 0.0471$	0.999
		$y = 0.0345x + 0.0972$	0.999
		$y = 0.0267x - 0.0474$	0.999
Kidney	0.2–31.75	$y = 0.1387x - 0.0149$	0.999
Liver	0.2–31.75	$y = 0.1327x - 0.0059$	0.999
Lung	0.2–31.75	$y = 0.1410x - 0.0603$	0.999
Heart	0.2–31.75	$y = 0.1862x - 0.0038$	1.000
Spleen	0.2–31.75	$y = 0.1721x - 0.0662$	1.000
Brain	0.2–31.75	$y = 0.1519x - 0.0715$	0.999
Stomach	0.2–31.75	$y = 0.1251x + 0.0069$	0.999
Intestine	0.2–31.75	$y = 0.1244x + 0.0008$	1.000
Particulate homogenate	1–75	$y = 0.1276x + 0.0096$	0.999

r: correlation coefficient.

Table 2
Standard calibration curves of edelfosine in plasma, tissue and particulate homogenate samples calculated by the UHPLC method.

	Range (μg/mL)	Regression equation	R
Plasma	0.0075–75	$y = 0.03831x - 0.0004$	0.999
		$y = 0.04029x + 0.0005$	0.999
		$y = 0.03551x - 0.0007$	0.999
Kidney	0.0075–75	$y = 0.0321x + 0.0004$	0.999
Liver	0.0075–75	$y = 0.0413x + 0.0002$	0.999
Lung	0.0075–75	$y = 0.0429x - 0.0005$	0.999
Heart	0.0075–75	$y = 0.0526x + 0.0001$	0.999
Spleen	0.0075–75	$y = 0.0520x - 0.0003$	0.999
Brain	0.0075–75	$y = 0.0397x + 0.00007$	1.000
Stomach	0.0075–75	$y = 0.0762x + 0.0003$	0.999
Intestine	0.0075–75	$y = 0.0447x + 0.0005$	0.999
Particulate homogenate	0.0075–75	$y = 0.0313x + 0.0009$	0.999

r: correlation coefficient.

lipid nanoparticle formulation is the emulsion formation/solvent evaporation method. Briefly, lipid and edelfosine were dissolved in chloroform and homogenized by ultrasonication with an aqueous solution of 2% Tween® 80. The obtained emulsion was then subjected to mechanical stirring for the organic solvent evaporation and consequent lipid nanoparticle formation. Particles were then centrifuged at 4500 × *g* for 10 min using an Amicon Ultra-15 filter device and washed twice with distilled water. The obtained particular suspension was fast frozen at −80 °C for at least 3 h and freeze-dried in order to store it at 4 °C [24].

3. Results and discussion

A sensitive method for edelfosine detection in plasma and tissue samples was needed for their concentration-time course measurements during dose escalation and other pharmacokinetic preclinical studies. To achieve this aim it is critical to optimize the chromatographic conditions to obtain symmetrical peak shapes and a short chromatographic analysis time with high sensitivity and selectivity. Previously, we developed a HPLC–MS method for the quality control of edelfosine drug delivery systems and the edelfosine quantitation in cell internalization studies. Under these chromatographic conditions edelfosine is eluted as tailing and band-broadening chromatographic peaks (values of USP asymmetry factor between 1.1 and 1.3), with insufficient chromatographic efficiency to measure this drug at low concentrations [14]. Chemically, edelfosine is an anionic amphiphilic compound that is positively charged at acidic pH, which is the pH of the mobile phase. The observed peak tailing may be a result of an ionic interaction of residual package silanols and positively charged nitrogen of edelfosine.

One of the first efforts was the attempt to reduce the value of the limit of quantitation while maintaining the employed extraction procedure. In our previous work, edelfosine determination was performed with polymerically bounded C_{18} reversed-phase narrow-bore column packed with double encapped spherical silica particles (Alltima®). To overcome the technical difficulties observed, a substantial improvement of the chromatographic performance of the method is the only valid solution. For the HPLC method, we have used a Gemini® column instead of the Alltima® package. Gemini® column has been developed with a technology that grafts additional silica–organic layers onto the surface of the internal base silica. These additional layers protect the particle from ionic interactions with ionized compounds, such as edelfosine at acidic pH, and as a result, sharp, symmetrically chromatographic peaks are obtained.

On the other hand, and according to the van Deemter equation, one helpful way to improve the efficiency and analysis time of the HPLC column is to decrease the particle size. However, the improved column efficiency gained from using small particles comes along with a tremendous increase in the column pressure, which is prohibitive for traditional HPLC hardware, but not for UHPLC hardware, which can easily resist pressure values up to 15,000 psi [15,19]. For an ideal comparison, we would prefer to use columns with identical chemistry. However, at the time of the study, columns packed with Gemini® C18 particles < 2 μm were unavailable. Thus, Acquity BEH UPLC™ C_{18} columns packed with bridged ethylsiloxane-silica particles were employed.

Fig. 2 shows the typical chromatograms of (A) a blank and (B) a spiked plasma sample with edelfosine and (C) the internal standard analyzed by the HPLC technique and (D) a blank and (E) a spiked plasma sample with edelfosine and (F) the internal standard analyzed by the UHPLC technique. The typical retention time for edelfosine and internal standard was 3.65 and 3.50 min for the HPLC–MS method, and 1.23 and 1.19 min for edelfosine and internal standard, respectively, for the UHPLC–MS/MS method. Compared with HPLC, UHPLC reduced the retention times threefold on average.

The resolution between the chromatographic peaks of the edelfosine and internal standard was 0.08 and 0.09 for the HPLC–MS and the UPLC–MS/MS methods, respectively. Therefore, we can affirm that there is little difference in column selectivity between the two types of package employed. The HPLC asymmetry factor for edelfosine was 2, whereas it was 1.2 for the UHPLC method. The main improvement in the chromatographic behavior has been reflected in method sensibility. In fact, a sixfold increase in the LOQ value was observed for the UHPLC–MS/MS method compared to that of the HPLC–MS method, which made edelfosine quantitation possible in small samples, such as that obtained in pharmacokinetic studies in mice, after intravenous administration of sub-milligram doses.

Validation data for edelfosine quantitation by the HPLC and UHPLC methods are compared in Tables 1–3. Assay performance of the present methods was assessed by all the following criteria: selectivity, linearity, accuracy, precision, LOD, LOQ, applicability to quantitation of edelfosine in different matrices and quantitation of edelfosine in lipid nanoparticles. Selectivity was assessed by the comparison of the chromatograms of six different batches of blank mouse plasma with the corresponding spiked plasma. There was no relevant interference from endogenous substances observed at the retention times of the analytes, as can be seen in Fig. 2.

The HPLC–MS assay exhibited linearity divided into two intervals between the response (y) and the corresponding concentration of edelfosine (x) from 0.1 to 1 μg/mL in the small concentration interval and from 1 to 75 μg/mL in the high concentration

Table 3
Accuracy, precision and between- and within-day measured concentrations for analysis of edelfosine by the HPLC and UHPLC methods.

	Conc. (μg/mL)	Accuracy (bias %)	Precision (%RSD)		Measured conc. (μg/mL, mean ± SD)	
			Between-day	Within-day	Between-day	Within-day
HPLC method						
QC1	0.1	4.88	7.77	5.42	0.10 ± 0.01	0.09 ± 0.01
QC2	5	7.13	7.11	4.23	5.22 ± 0.37	5.49 ± 0.23
QC3	10	−5.83	5.57	1.66	9.50 ± 0.53	9.28 ± 0.15
QC4	50	−0.62	2.04	3.22	50.16 ± 1.02	48.90 ± 1.58
UHPLC method						
QC1	0.075	−6.84	10.91	12.23	0.069 ± 0.007	0.070 ± 0.008
QC2	0.75	−4.06	7.20	8.33	0.70 ± 0.05	0.73 ± 0.06
QC3	15	6.49	7.70	6.60	15.80 ± 1.19	15.97 ± 1.03
QC4	60	2.73	4.59	3.72	62.06 ± 2.84	61.29 ± 2.28

interval for plasma samples. Tissue and nanoparticulate system samples presented linearity from 0.2 to 31.75 μg/mL and from 1 to 75 μg/mL, respectively. The UHPLC–MS/MS method showed a much higher sensitivity and calibration range than the obtained with the HPLC method. A linear range was achieved for all types of samples from 0.0075 to 75 μg/mL and the limit of quantitation was 0.0075 μg/mL. Results are shown in Tables 1 and 2. For each point of calibration standards, the concentrations were back-calculated from the equation of the regression curves and relative standard deviations (%RSD) were measured. %RSD did not exceed 15% in any case. For all calibration curves, linear regression provided r values greater than 0.999. As can be seen in Tables 1 and 2, the slope of the calibration curves from each tissue sample was similar to that obtained after the chromatographic analysis of plasma calibrator samples in a similar concentration range. It is clear that the developed method is adequate to quantify edelfosine in different biological samples, where an adequate extraction procedure has been applied.

The lower limit of quantitation for edelfosine with the HPLC method was 0.1 μg/mL (S/N ≥ 5) with 5 μL injected into the chromatographic column with accuracy within ±20% and %RSD lower than 20%. The UHPLC method showed a limit of quantitation of 0.0075 μg/mL with 2 μL injected into the chromatographic column, presenting similar accuracy values. Compared with the HPLC method, the present UHPLC method gave sixfold higher sensitivity. The high sensitivity of the UHPLC method could be attributed to the peak sharpness produced by the column package and the lower analyte dilution in the column.

To determine recovery, concentrations of edelfosine and PAF in extracted plasma and tissue QC samples were compared to standards prepared in blank matrix extract. The recovery was evaluated in triplicate and presented acceptable values ranging from 85.11% and 100.93%, and from 83.64% to 107.48% for edelfosine and internal standard, respectively, by the HPLC method, and from 95.25% to 99.35% and from 93.43% to 93.79% for edelfosine and PAF, respectively, by the UHPLC method. Matrix ionization suppression is considered to be a problem when using the protein precipitation method for sample preparation. Nevertheless, this method has been chosen as the sample preparation procedure in our work due to its simplicity and the lack of impact on the accuracy of the assay. The matrix effect values of edelfosine and the I.S. in both plasma and tissue homogenate samples were 15.55 ± 1.79% and 9.03 ± 3.96% for the HPLC method, and 6.17 ± 2.77% and 3.71% for the UHPLC method. It is interesting to note that the matrix effect is slightly reduced when the UHPLC analysis is performed. This may be due to the fact that the signal suppression is diminished drastically in the UHPLC–MS/MS method compared with the HPLC–MS method.

Similar data were obtained by the HPLC and UHPLC methods after accuracy and precision evaluation (Table 3). Accuracy values were within acceptable limits ranging between −6.84% and 7.13%. The results for intra-batch and inter-batch precision for the samples ranged between 1.66% and 12.23%. The precision and accuracy of the present method is in accordance with the criteria for the analysis of biological samples according to the guidance of the FDA, where the precision (expressed as %RSD) determined at each concentration level is required not to exceed 15%.

The applicability of this method has been demonstrated *in vivo* by the determination of edelfosine in plasma samples from BALB/c mice treated with 200 μg of edelfosine. Fig. 3 depicts the concentration of edelfosine in mouse plasma plotted against time after a single-dose intravenous administration of 200 μg of edelfosine (10 mg/kg) to BALB/c mice determined by the HPLC method. Edelfosine in blood plasma showed a C_{max} of 50.7 ± 28.1 μg/mL and a C_{min} of 2.5 ± 1.3 μg/mL, 24 h after intravenous administration. Tissue levels of edelfosine were also measured and compared to those

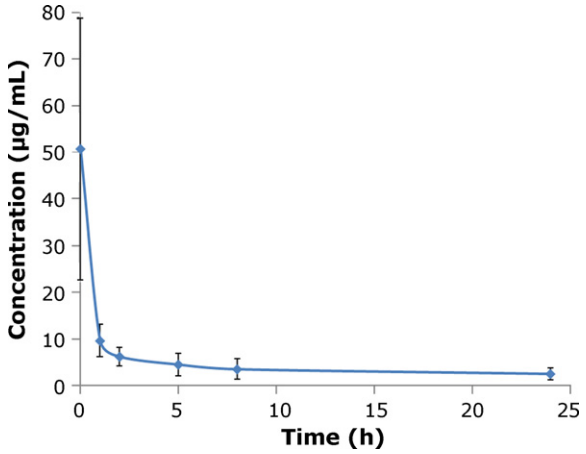

Fig. 3. Time-concentration curve data of edelfosine over 24 h after single dose intravenous administration of 200 μg (10 mg/kg) to BALB/c mice (n = 6, Mean ± S.D.) calculated by the HPLC method.

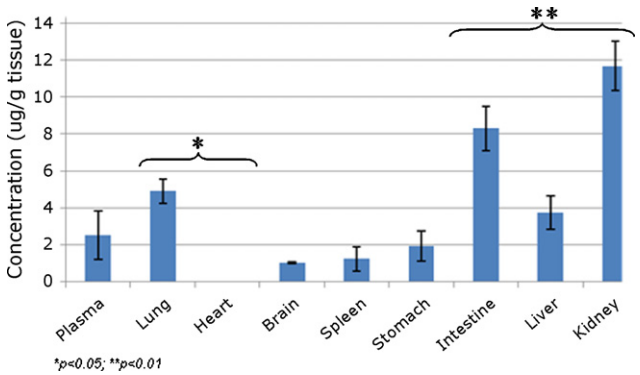

Fig. 4. Tissue distribution of edelfosine 24 h after a single intravenous administration of 200 μg to mice (10 mg/kg) calculated by the UHPLC method.

of plasma. Fig. 4 depicts the distribution of edelfosine to different organs in mice after intravenous administration of 200 μg of edelfosine determined by the UHPLC method. No drug was detectable in the heart and very little was found in the brain. The edelfosine levels present in the kidney and intestine were statistically very significant compared to the plasma levels of edelfosine ($p < 0.01$). Edelfosine levels in the lung and heart were statistically different from plasma levels ($p < 0.05$). No significant differences were found between plasma and liver, spleen, brain and stomach levels ($p > 0.05$). The drug was also extracted from previously formulated lipid nanoparticles and quantified. Nanoparticulate systems made of lipid material Compritol® 888 ATO showed an encapsulation efficiency of about 95%.

The same samples were analyzed with both the HPLC–MS and UHPLC–MS/MS method to ensure the interchangeability of both methods. The edelfosine results obtained from liver samples analyzed by the UHPLC–MS/MS method were highly correlated ($r^2 = 0.91$; $y = 0.9706x + 0.2123$) with those from the HPLC–MS method. A good relationship between both techniques was found over the concentration range of 0.2–31.75 μg/mL for the HPLC–MS and UHPLC–MS/MS.

4. Conclusion

Two liquid chromatographic methods, an HPLC–MS method and a UHPLC–MS/MS method for the bio-analysis of edelfosine were developed and evaluated. The UHPLC–MS/MS method developed in this work was more sensitive for the quantitation of edelfosine in plasma, tissue and lipid nanoparticles than the HPLC–MS method. Under the UPLC conditions we are able to achieve a shorter chromatographic run time while still avoiding a matrix ion suppression problem. UHPLC and HPLC are valuable methods for the determination of the pharmacokinetic behavior of edelfosine and bio-distribution in mice after intravenous administration of a dose of 10 mg/kg of edelfosine and the quality control of lipid nanoparticulate systems.

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