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Hydrophilic interaction liquid chromatography/positive ion electrospray mass spectrometry for the quantification of deferasirox, an oral iron chelator, in human plasma

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ABSTRACT

A rapid hydrophilic interaction liquid chromatography/positive ion electrospray mass spectrometric assay (HILIC/ESI-MS) was developed, validated and applied to the determination of deferasirox, in human plasma. The sample preparation process involved liquid–liquid extraction of 50 μL plasma sample using ethyl acetate as an extraction solvent. Chromatographic separation was performed on an XBridge®-HILIC analytical column (150.0 mm × 2.1 mm i.d., particle size 3.5 μm, 135 Å) under isocratic elution. The mobile phase was composed of a 10% 8.0 mM ammonium acetate water solution pH = 5.0, adjusted with formic acid, in a binary mixture of acetonitrile/methanol (50:50, v/v) and pumped at a flow rate of 0.20 mL/min. Quantitation of deferasirox was performed with selected ion monitoring (SIM) in positive ionization mode using electrospray ionization interface. The assay was found to be linear in the concentration range of 0.20–120.0 μg/mL for deferasirox. Intermediate precision was found less than 3.9% over the tested concentration ranges. A run time of less than 6.0 min for each sample made it possible to analyze a large number of human plasma samples per day. The method can be used to support a wide range of clinical studies concerning deferasirox monitoring and it was applied to the analysis of human plasma samples obtained from patients with β-thalassemia major.

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1. Introduction

The clinical course of patients with β-thalassemia major is characterized by profound anemia, who present to medical attention in the first year of life, and who subsequently require regular blood transfusions and iron chelation therapy for survival [1]. Excess iron is deposited in the form of hemosiderin primarily in the liver, spleen, several endocrine organs, and myocardium and leads to progressive organ dysfunction and ultimately death [2]. Iron overload can also be caused in patients with sickle cell disease as a result of repeated blood transfusions and in patients with chronic anemias or hereditary hemochromatosis because of excessive dietary iron uptake [3].

Deferasirox, 4-[(3Z,5E)-3,5-bis(6-oxocyclohexa-2,4-dien-1-ylidene)-1,2,4-triazolidin-1-yl] benzoic acid, is an orally absorbed tridentate bis hydroxyl pheny-triazol chelator of Fe^{3+} [4]. The drug is currently licensed in more than 85 countries worldwide as first-line therapy for thalassemia and transfusional iron overload [5–7]. The pharmacokinetic profile of deferasirox is suitable for a convenient, once daily, oral administration schedule, as it can provide 24 h chelation coverage in patients with transfusion-dependent anemias [8,9]. Peak plasma concentration levels, C_{max}, of deferasirox for 2.5 mg/kg/day up to 80 mg/kg/day increases by a factor of 26, and the area under the curve, $AUC_{0-24\,h}$, increases by a factor of 34, that is approximately dose proportional [10]. The elimination half-life, $t_{1/2}$, for 10 and 20 mg/kg/day doses of deferasirox ranges from 7 to 16 h, however without a strict dose relationship [11]. Single oral administration of 40 mg/kg of the drug leads to peak plasma concentration levels, C_{max}, of 44.4 μg/mL, occurring approximately 1.5 h after dosing [10].

Up to now, only a few analytical methods have been published for the analysis of deferasirox in dosage forms or biological fluids. A stability indicating HPLC-UV method has been developed for the determination of deferasirox in bulk drugs and pharmaceutical dosage forms [12]. HPLC-UV and ion-pair HPLC-UV methods have been has also been used to analyze deferasirox in human plasma [13–15]. Liquid chromatography ion-trap mass spectrometry has been used to study pharmacokinetics, distribution, metabolism and excretion of deferasirox and its iron complex in rats [16]. Recently, a terbium-sensitized fluorescence method has been developed for the determination of deferasirox in biological fluid and tablets [17]. The electrocatalytic oxidation of deferasirox and deferiprone was also investigated using cyclic voltammetry, chronoamperometry and electrochemical impedance techniques [18].

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Although most of the above described methods are successful approaches, no report has been mentioned in literature for the determination of deferasirox in human plasma by hydrophilic interaction liquid chromatography (HILIC) in combination with electrospray ionization single mass spectrometric detection. There has been an increasing use of HILIC chromatography in the clinical laboratory for chromatographic separation of polar and hydrophilic analytes that presents small or even minimal retention in the more widely used reversed phase LC columns [19]. The high organic mobile phase concentration gives HILIC a number of advantages such as the enhancement of signal and overall sensitivity gained with electrospray ionization by reducing surface tension and enhancing desolvation, and faster separations due to the lower viscosity of HILIC eluents compared to standard RP eluents [20,21].

In this study a rapid, sensitive and selective hydrophilic interaction liquid chromatographic mass spectrometric method was developed and validated for the rapid simultaneous determination of deferasirox in plasma samples. The principal aim of this work was to optimize and validate an analytical procedure for the determination of the analyte in plasma samples and to investigate its chromatographic behavior on HILIC columns. Thus, as there are no reports on the chromatography of iron chelators on HILIC columns, a thorough investigation was conducted using a variety of mobile phases.

2. Experimental

2.1. Materials and reagents

All solvents were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Formic acid of analytical reagent grade was obtained from Merck (Darmstadt, Germany). Ammonium acetate was obtained from Acros Organics (New Jersey, USA). Acrodisk® GHP membrane syringe filters (13 mm, pore size 0.45 μm) were obtained from Pall life sciences (Ann Arbor, MI, USA). Deferasirox of pharmaceutical purity grade was kindly provided from Novartis Pharma AG (Basel, Switzerland) and mirtazapine (internal standard) of pharmaceutical purity was kindly provided from ILS AE (Athens, Greece). Pooled drug-free human plasma was obtained from Sotiria General Hospital, Athens, Greece.

2.2. LC/ESI-MS instrumentation

Analyses were performed on a LC–MS system consisting of a Finnigan AQA single quadrupole mass spectrometer (ThermoQuest, Manchester, UK) equipped with an electrospray ionization interface and a SpectraSeries P100 (SP ThermoSeparation, UK) LC system. A Nitrox-N2 Model UHPLC-MS12E nitrogen generator, Domnick hunter (Gateshead, England) was used to provide high purity nitrogen utilized as sheath and nebulizing gas. Data acquisition and analysis were performed using Xcalibur software (v. 1.2, ThermoQuest, Manchester, UK).

2.3. Liquid chromatographic conditions

Substances were separated using an XBridge®-HILIC analytical column 150.0 mm × 2.1 mm i.d., particle size 3.5 μm (Waters Corporation, Milford, MA, USA). An XBridge®-HILIC guard cartridge (20 mm × 2.1 mm, 3.5 μm) was used to prolong column lifetime. The mobile phase consisted of 10% 8.0 mM ammonium acetate water solution pH = 5.0, adjusted with formic acid, in a binary mixture of acetonitrile/methanol (50:50, v/v) and pumped at a flow rate of 0.20 mL/min. Chromatography was performed at 25 ± 2 °C with a chromatographic run time of 6.0 min; a 80 μL volume was injected into a 20 μL loop.

2.4. Mass spectrometric conditions

The electrospray interface was operated in the positive ionization mode. The parameters of the source were used with the following settings using nitrogen as sheath and nebulizing gas: capillary voltage 3.7 kV, temperature 230 °C, cone voltage (AQA_{max}) was set at 20.0 V. The SIM mode was chosen for the quantitation of the analytes that were monitored in different time windows. Deferasirox was evidenced from 0.00 to 2.85 min in ESI positive ion mode using a cone voltage value of 20.0 V to obtain the maximum abundances of the protonated molecule $[M+H]^+$ at m/z 374.1, while mirtazapine (IS) was quantitated by measuring the protonated molecule $[M+H]^+$ at m/z 266 using a time window of 2.86–6.00 min.

2.5. Stock and working standard solutions

Stock standard solution of deferasirox, 400 μg/mL, was prepared by dissolving the appropriate amount of the compound in acetonitrile–water (50:50, v/v). The stock standard solution was further diluted in water to prepare a series of working standard solutions containing 0.20–120.00 μg/mL of deferasirox. Stock standard solution of mirtazapine (IS) was prepared at 400.0 μg/mL in acetonitrile–water (50:50, v/v). This solution was further diluted in water to prepare a working standard solution containing 0.10 μg/mL of mirtazapine.

The stock standard solutions were stored in amber bottles at −20 °C and were found to be stable for at least 1 month. The working standard solutions were freshly prepared every week and stored in amber bottles and under refrigeration (−4 °C).

2.6. Sample preparation

Clean up of human plasma sample is carried out by liquid–liquid extraction according to the following procedure. A 25 μL aliquot of IS solution (0.10 μg/mL) is added to 50 μL plasma sample and vortex mixed for a few seconds. The plasma sample is then acidified by the addition of 25 μL 2.5 mM KH_2PO_4 (pH = 3.2), vortex-mixed for 1 min and 1.0 mL of ethyl acetate is added. The mixture is shaken for 2 min and centrifuged at 12,000 × g for 10 min. An 800 μL aliquot of the organic layer is transferred into an eppendorf tube and evaporated within 3 min under a gentle stream of nitrogen at ambient temperature (25 ± 2 °C). The residue, after reconstitution in 1 mL of acetonitrile, is filtered through a 13 mm GHP Acrodisk® membrane syringe filter (pore size 0.45 μm). Sample is further diluted in acetonitrile (1:6, v/v) prior to the injection into the LC/ESI-MS system.

2.7. Calibration spiked plasma standards and quality control sample preparation

Calibration spiked plasma samples were freshly prepared every working day for the concentration levels of 0.2, 0.6, 2, 6, 10, 20, 40, 60, 80, 100 and 120 μg/mL for deferasirox by addition of the appropriate aliquot of the above mentioned working standard solutions to 50 μL of pooled drug-free human plasma. Each calibration plasma sample contained 0.10 μg/mL of mirtazapine (IS).

Quality control (QC) samples were prepared independently, in an analogous manner as the calibration spiked plasma samples, using separate stock solutions of the analytes. QC samples were prepared at three concentration levels (0.2, 40 and 120 μg/mL) for the analyte.

2.8. Validation procedures

To evaluate the linearity of the analytical method the calibration spiked plasma samples were prepared and analyzed in duplicate on

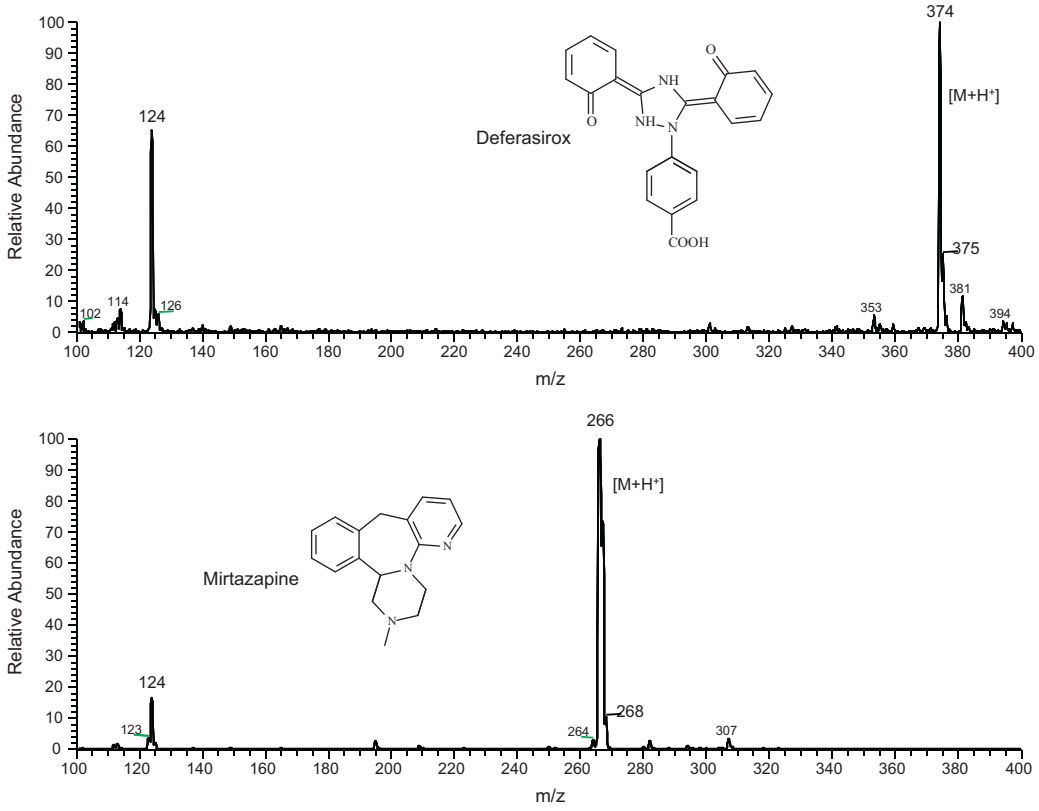

Fig. 1. ESI mass spectra of 20.0 μg/mL deferasirox standard solution and 15.0 μg/mL mirtazapine standard solution prepared in a mixture of 10% 8.0 mM ammonium acetate water solution pH = 5.0 in a binary mixture of acetonitrile/methanol (50:50, v/v) under the optimal cone voltage conditions of 20 V. MS conditions: positive ESI mode; ESI probe temperature 230 °C; capillary voltage 3.7 kV; flow rate 50 μL/min.

three different analytical runs. Quantitation was performed using the peak area ratio of deferasirox to that of the internal standard, mirtazapine. Weighted ($1/y^2$) least-squares linear regressions were used to obtain the equation of the calibration curves. QC samples were processed in five replicates at each concentration (0.2, 40 and 120 μg/mL) for five different analytical days in order to evaluate the intra- and inter-assay accuracy and precision.

Recovery of the liquid–liquid extraction (LLE) procedure was evaluated at three concentration levels (10, 40 and 120 μg/mL) for deferasirox and at 0.10 μg/mL for the internal standard and in three replicates. It was determined by comparing the analytical response obtained from the analysis of spiked plasma samples after LLE procedure with the analytical response of blank plasma samples spiked with equivalent concentrations of the analyte and the IS after the sample preparation procedure.

Matrix effect was evaluated at three concentration levels (10, 40 and 120 μg/mL) for deferasirox and at 0.10 μg/mL for the internal standard according to the method described by Matuszewski et al. [22]. In particular, six individual lots of drug-free human plasma were processed according to the sample preparation procedure and then spiked with deferasirox and the IS. Subsequently, standard solutions of deferasirox and the IS in equivalent concentrations were prepared in acetonitrile and analyzed according to the proposed method. The percentage matrix factor was used as a quantitative measure of the matrix effect and it is defined as the percentage of the ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions. Carryover and potential endogenous compound interference were also investigated.

The stability of deferasirox in spiked human plasma samples was investigated under various storage conditions. Stability samples were stored at ambient temperature (25 ± 2 °C) for 6 h and at −20 °C for 4 weeks. Freeze–thaw stability was also evaluated by successive cycles of freezing and thawing; four complete freeze–thaw cycles were performed with samples frozen at −20 °C for 7 days per cycle and thawed (without warming) at room temperature. Results obtained from the analysis of the stored samples were compared to those that were obtained from the analysis of freshly prepared spiked plasma samples and experiments were performed in three replicates.

3. Results and discussion

3.1. Optimization of MS detection

The electrospray ionization interface parameters need to be optimized for maximum abundance of the molecular ions of the compounds. Acquisition parameters were determined by direct infusion into the mass spectrometer of a 20.0 μg/mL solution (in mobile phase) of deferasirox and a 15.0 μg/mL solution of the IS at a flow rate of 50 μL/min. ESI probe temperature was set at the minimum acceptable value (230 °C) regarding the mobile phase flow rate (0.20 mL/min) to avoid thermal degradation of the compounds and capillary voltage was kept at 3.7 kV. Mass spectra were recorded in ESI positive ion mode and under these conditions it was found that the highest ion intensity for the molecular ion of the analyte was achieved when the source block voltage was set at 20.0 V. Mass spectrum of the analyte and the IS obtained in ESI positive ion mode and under the optimum mass spectrometric conditions are presented in Fig. 1.

Deferasirox was evidenced in ESI positive ion mode using a cone voltage value of 20.0 V to obtain the maximum abundances of the

Table 1
Analytical concentration parameters of the calibration equations for the determination of deferasirox by LC/ESI-MS.

Concentration range (μg/mL)	Regression equations[a]	r[b]	Standard deviation		S_r[c]	α/S_α[d]
			Slope	Intercept		
Representative calibration data of one of the runs						
0.20–120.00	$R_{Dfx} = 0.02991 \times C_{Dfx} + 0.00036$	0.997	5.8×10^{-4}	3.5×10^{-4}	0.054	1.03
Mean of three calibration curves over a period of 1 month						
0.20–120.00	$R_{Dfx} = 0.0291 \times C_{Dfx} + 0.00036$	≥0.991	0.0015	3.0×10^{-4}	≤0.11	≤1.03

[a] Ratios of the peak areas signals of deferasirox to that of the internal standard (mirtazapine), R_{Dfx}, vs the corresponding concentration of deferasirox, C_{Dfx}.
[b] Correlation coefficient.
[c] Standard error of the estimate.
[d] Theoretical value of t at $P = 0.05$ and $f = n - 2 = 8$; df, 2.31.

protonated molecules $[M+H]^+$ at m/z 374, while mirtazapine (IS) was quantitated by measuring the protonated molecule at m/z 266.

3.2. Hydrophilic interaction liquid chromatography

The BEH XBridge®-HILIC column that was used in this work consists of bis-triethoxysilyl ethane (BEH) particles. In BEH particle synthesis the bis (triethoxysilyl) ethane monomer contains a preformed ethylene bridge, which is responsible for the high pH dissolution resistance of polymers into the silica backbone. Hydrophilic interaction chromatography utilizes a high organic mobile phase and a polar stationary phase to facilitate chromatographic separation. The separation mechanism in HILIC is complex and under varying experimental conditions has been shown to involve hydrophilic partitioning into an absorbed water layer, adsorption onto the water layer or stationary phase, ion exchange onto a charged stationary phase and hydrophobic interactions with the stationary phase [23,24]. When developing robust methods in HILIC it is important to understand the dominant mechanism that governs retention.

To optimize the chromatographic conditions for deferasirox and mirtazapine (IS) on the BEH XBridge®-HILIC column, the percentages of water and the concentration of ammonium acetate in the mobile phase were selected for examination. The method development was conducted in detail using a univariate procedure (change one-variable-at-a-time approach). In an effort to reduce the acetonitrile content in the mobile phase we found that a mixture of acetonitrile–methanol (50:50, v/v) gave good chromatographic peak shapes for deferasirox and the IS and this mixture was used in the following studies as the organic modifier.

The dependencies of $\log k'$ values of the analyte and the IS on the percentage of water, φ_{water}, which is the strongest eluent on HILIC, were thoroughly investigated. The percentage of water, φ_{water}, was varied from 5 to 25%, while ammonium acetate concentration was maintained constant at 0.8 mM in whole mobile phase with the pH in the aqueous component of the mobile phase held at pH 5.0. In all cases mirtazapine (IS) is eluted after the elution of deferasirox. Deferasirox is a chelating agent with pK_a values of 4.57, 8.71 and 10.56 for the carboxylic acid and the two hydroxyl groups, respectively, and it is 50% positively charged at pH = 5.0 of the mobile phase. It was found that the $\log k$ values of the analyte decrease linearly ($r = 0.992$) as the percentage of water (φ_{water}) in the mobile phase increases pointing to a partition mechanism for the separation. Mirtazapine is a basic drug with a pK_a value of 8.10 ± 0.20 and it is 100% positively charged at pH 5.0 of the mobile phase. The retention factor ($\log k'$) for mirtazapine increased using higher percentages of water in the mobile phase pointing to an ion exchange mechanism that dominates retention. As the aqueous content of the mobile phase is decreased the MS signal is increased probably due the improvement of the desolvation process. The analyte and the IS are adequately retained and exhibit the best MS signal using a 10% of water in the mobile phase that was chosen as the optimum.

The concentration of ammonium acetate in the aqueous content of the mobile phase was varied from 4.0 to 20.0 mM, while the water content of the mobile phase was kept constant at the previously determined optimum value of 10%. The retention of deferasirox was slightly increased upon increasing the concentration of ammonium acetate and this can be ascribed to the fact that the salt is enriched in the aqueous layer, which in turns increases the hydrophilicity of this liquid layer around the stationary phase [25,26]. On the contrary the decrease of retention factor of mirtazapine (IS) with increased ammonium acetate concentration is consistent with ionic retention associated with ionized silanols in the BEH HILIC column. A decrease in MS signal is observed upon increasing the concentration of the buffering salt probably due to ion suppression. An ammonium acetate concentration of 8.0 mM in the aqueous content of the mobile phase gave was chosen as the optimum to perform the LC/ESI-MS analysis.

A mobile phase consisting of 10% 8.0 mM ammonium acetate water solution pH = 5.0, adjusted with formic acid, in a binary mixture of acetonitrile/methanol (50:50, v/v) was finally used and pumped at a flow rate of 0.20 mL/min. Each chromatographic run was completed within 6.0 min. The chromatographic run time was 5–6 min, thereby allowing high sample throughput. A representative MS chromatogram obtained from the analysis of blank plasma sample along with a chromatogram obtained from the analysis of a sample spiked with 0.20 μg/mL deferasirox (LOQ level) and 0.10 μg/mL mirtazapine (IS) is presented in Fig. 2 and illustrate the selectivity of the proposed chromatographic procedure. Under the current chromatographic conditions deferasirox and mirtazapine were eluted at 1.69 and 3.51 min, respectively. Chromatographic peak at 1.91 is due to plasma matrix and does not affect the quantification of deferasirox.

3.3. Statistical analysis of data

Linear relationships between the ratios of the peak area signals of deferasirox to that of the internal standard and the corresponding concentrations were observed as shown by the results presented in Table 1. Back-calculated concentrations in the calibration curves were less than 11.9% of the nominal values for the analyte, which are in agreement with international guidelines [27]. The insignificance of intercepts that was proved by Student's t-tests indicates that there is no matrix effect. The limits of detection, LOD, and the limits of quantitation, LOQ, for deferasirox were calculated taking under consideration data obtained from the calibration equations and were found to be 0.061 μg/mL and 0.18 μg/mL, respectively.

One-way analysis of variance (ANOVA) was used to evaluate the intra- and inter-assay precision. Results presented in Table 2 indicate that intra-assay coefficients of variations, % CV, were between 3.4 and 5.3% for deferasirox, while the inter-assay % CVs were lower than 3.9%. The overall accuracy was assessed by the relative percentage error, absolute % E_r that was ranged from 1.4 to 3.8%.

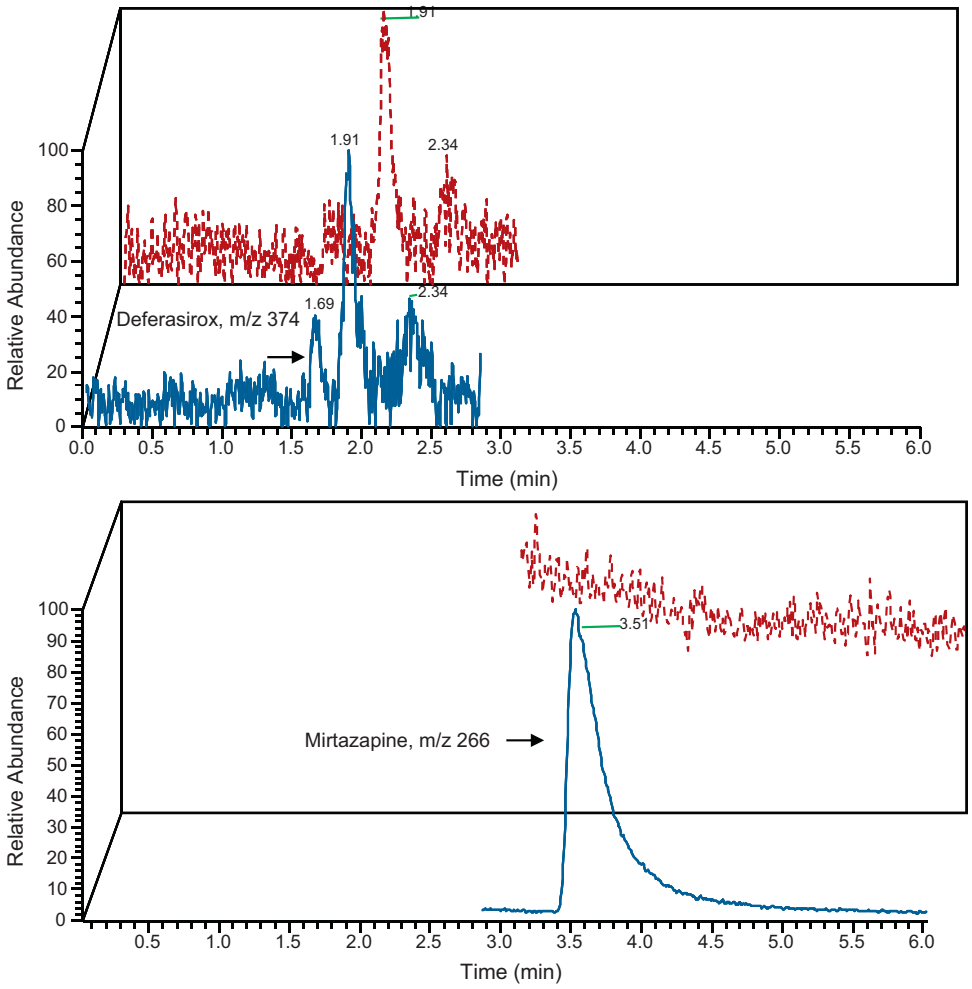

Fig. 2. Smoothed and normalized mass chromatogram of a blank plasma sample (dashed line), along with a smoothed and normalized LC/ESI-MS chromatogram of a calibration plasma sample spiked with 0.20 μg/mL deferasirox (LOQ level) and 0.10 μg/mL of the IS; the retention times of deferasirox and mirtazapine are 1.69 and 3.51 min, respectively (solid line). Chromatographic conditions: hydrophilic interaction liquid chromatography on a BEH XBridge®-HILIC analytical column; mobile phase: 10% 8.0 mM ammonium acetate water solution pH = 5.0 in a binary mixture of acetonitrile/methanol (50:50, v/v); flow rate 0.20 mL/min.

The selectivity towards endogenous plasma compounds was also tested in six different lots of drug-free human plasma. Mass chromatograms of six batches of drug-free plasma contained no co-eluting peaks greater than 20% of the area of the analyte at the LOQ level, and no co-eluting peaks greater than 5% of the area of the internal standard mirtazapine. The concentrations of deferasirox obtained after the analysis of the six different lots of human plasma were found to be 0.197 ± 0.011 μg/mL and indicate that the matrix effect does not appreciably affect the assay.

Recoveries data presented in Table 3 indicate average recovery of more than 86.3% for deferasirox while the recovery for mirtazapine (IS) was found to be 81.7% at 0.10 μg/mL. To evaluate potential matrix effects, the percentage matrix factors were calculated. Results presented in Table 3 indicate low ion suppression and are in agreement with international guidelines and [26].

The carry-over test met the pre-defined criteria as no interfering peaks with peak areas greater than 10% of the peak areas at the LOQ level of each analyte were detected in blank plasma samples injected after a high concentration calibration plasma sample.

The stability results presented in Table 4 indicate that the analyte can be considered stable under the various conditions

Table 2
Accuracy and precision evaluation of quality control samples for deferasirox ($n = 5$ runs, five replicates per run).

Compound	Concentration (μg/mL)		
Deferasirox			
Added concentration	0.20	40.00	120.00
Run 1 (mean + s.d.)	0.2141 ± 0.0064	41.66 ± 0.87	122.6 ± 6.3
Run 2 (mean + s.d.)	0.2112 ± 0.0052	41.8 ± 2.4	125.1 ± 1.6
Run 3 (mean + s.d.)	0.194 ± 0.012	40.3 ± 2.4	123.1 ± 4.2
Run 4 (mean + s.d.)	0.2008 ± 0.0101	38.2 ± 2.1	124.4 ± 5.6
Run 5 (mean + s.d.)	0.1949 ± 0.0042	40.8 ± 2.4	127.61 ± 0.81
Overall mean	0.2066	40.6	124.6
Intra-assay CV (%)[a]	4.6	5.3	3.4
Inter-assay CV (%)[a]	3.9	2.7	0.4
Overall accuracy % E_r[b]	1.6	1.4	3.8

[a] Coefficient of variation; intra- and inter-assay CVs were calculated by ANOVA.
[b] Relative percentage error = [(overall mean assayed concentration − added concentration)/(added concentration) × 100].

Table 3
Recovery and ion suppression data for the determination of deferasirox and mirtazapine (IS) by LC/ESI-MS.

Compound	Concentration levels (μg/mL)		
Deferasirox	10	40	120
% Recovery (mean ± s.d.)$_{n=3}$	86.3 ± 1.8	89.5 ± 1.4	87.4 ± 1.2
% Matrix factor (mean ± s.d.)$_{n=3}$	95.8 ± 1.4	96.1 ± 1.8	96.4 ± 0.1
Mirtazapine (IS)	0.10		
% Recovery (mean ± s.d.)$_{n=3}$	81.7 ± 0.5		
% Matrix factor (mean ± s.d.)$_{n=3}$	98.8 ± 1.5		

Table 4
Stability data for deferasirox in human plasma under various storage conditions.

Deferasirox	Storage conditions/time					
	Ambient temperature (25 ± 2 °C)/6 h		−20 °C/28 days		−20 °C/4 Freeze–thaw cycles	
Concentration levels (μg/mL)	Mean ± s.d. (n = 3)	$\%E_r$	Mean ± s.d. (n = 3)	$\%E_r$	Mean ± s.d. (n = 3)	$\%E_r$[a]
10	9.27 ± 0.85	−7.3	10.04 ± 0.64	0.4	9.95 ± 0.74	−0.5
40	38.43 ± 1.02	−3.9	38.1 ± 1.5	−4.8	40.4 ± 1.5	0.9
120	116.53 ± 0.94	−2.9	116.3 ± 4.6	−3.1	118.1 ± 2.8	−1.6

[a] Relative percentage error = (overall mean assayed concentration − added concentration)/(added concentration) × 100.

investigated since its concentration deviate by no more than −7.3% relative to the reference for any of the tests and no degradation products were detected at the selected mass spectrometric conditions. Plasma samples containing deferasirox may therefore be kept for up to 6 h at ambient temperature, 28 days at −20 °C, and after 4 freeze–thaw cycles (7 days per cycle) at −20 °C without any significant degradation.

3.4. Application of the method to the analysis of real samples

In order to evaluate the applicability of the proposed analytical method, deferasirox was determined in plasma samples collected from three β-thalassemia major patients treated orally with deferasirox under different protocols so as to cover all possible cases that might appear in a clinical study. The Committee on the Medical Ethics of the University Hospital of Patras, Hematology Division, in Patras, Greece authorized this study on human patients. The first sample was collected from a 20-year-old male patient that received a single oral administration of a 40 mg/kg dose of deferasirox along with 0.1 mg levothyroxine sodium, 10 mg omeprazole, 2 mg acenocoumarol and 100 mg acetylsalicylic acid orally once daily. One day before sample collection the male patient was also receiving 500 mg clarithromycin orally twice daily (morning and night) along with 1 g of aztreonam intravenously three times. The second sample was collected from a 27-year-old female patient that was in continuous oral administration of 40 mg/kg dose of deferasirox along with 0.1 mg levothyroxine sodium orally once daily. The third sample was collected from a 31-year-old female patient that was in continuous oral administration of 40 mg/kg dose of deferasirox and stopped receiving the drug 1 day before sampling. During the period of sample collection the patient was also receiving 2 mg estradiol valerate and 0.5 mg norgestrel orally once daily. Blood samples of 500 μL were collected a Venoject® tube 2 h before and 2 h after the oral administration of deferasirox (Exjade®, Novartis Pharma AG). Immediately after drawing, the samples were shaken gently and centrifuged at 4000 rpm for 10 min at 4 °C. All

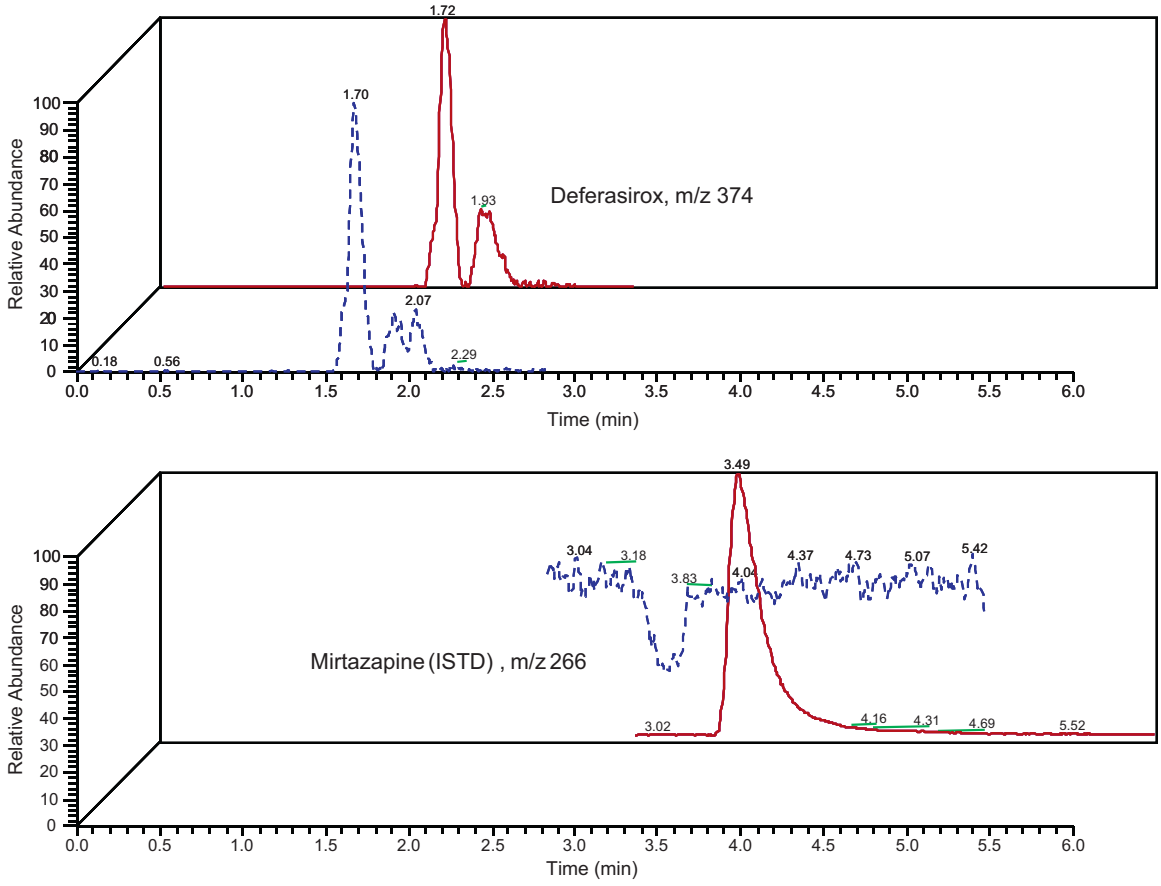

Fig. 3. Smoothed and normalized mass chromatogram of a calibration plasma sample containing 40 μg/mL deferasirox and 0.10 μg/mL mirtazapime (solid line) along with a smoothed and normalized LC/ESI-MS chromatogram obtained from the analysis of a β-thalassemia patient's plasma sample 2 h after the administration of exjade® at a dose of 40 mg/kg (dashed line). Chromatographic conditions: hydrophilic interaction liquid chromatography on a BEH XBridge®-HILIC analytical column; mobile phase: 10% 8.0 mM ammonium acetate water solution pH = 5.0 in a binary mixture of acetonitrile/methanol (50:50, v/v); flow rate 0.20 mL/min.

Table 5
Clinical data in patients treated with deferasirox at a dose of 40 mg/kg.

Patient/age (years)	Time of sampling (h)	Dose peros	Concentration (μg/mL) mean ± s.d.$_{(n=3)}$
Male/20	−2	Single oral administration of a 40 mg/kg dose	–
	2		23.3 ± 1.4
Female/27	−2	Continuous oral administration of a 40 mg/kg dose	26.9 ± 2.9
	2		44.1 ± 1.1
Female/31	−2	Continuous oral administration of a 40 mg/kg dose[a]	–
	2		30.2 ± 0.3

[a] The patient stopped receiving deferasirox 1 day before sampling.

plasma samples were stored at −20 °C and analyzed within 3 weeks after storage by the proposed LC/ESI-MS method. Three aliquots (50 μL) of each plasma sample were analyzed according to the sample preparation procedure (IS was added) in order to calculate the concentration of the analyte. In Fig. 2 is presented a smoothed and normalized LC/ESI-MS chromatogram of a calibration plasma sample containing 40 μg/mL deferasirox and 0.10 μg/mL mirtazapime (solid line) along with a smoothed and normalized LC/ESI-MS chromatogram obtained from the analysis of a β-thalassemia patient's plasma sample 2 h after the administration of exjade® at a dose of 40 mg/kg (dashed line) (Fig. 3).

The concentrations of deferasirox (μg/mL) in plasma samples as determined by the proposed LC/ESI-MS method are presented in Table 5. The results demonstrate the applicability of the method to support several clinical studies related to deferasirox therapy.

4. Conclusions

The proposed LC/ESI-MS method enables a rapid, accurate and selective assay for the determination of deferasirox in human plasma. The method is a part of a larger study that aims to investigate the chromatographic behavior of several drugs on HILIC columns. Hydrophilic interaction liquid chromatography was performed on a BEH XBridge®-HILIC column, 3.5 μm particle size, with a run time lower than 6.0 min. Mass spectrometry was conducted in ESI positive ion mode and enabled the sensitive and selective quantitation of the analyte. The method was successfully applied to the analysis of plasma samples obtained from β-thalassemia patients treated with deferasirox. The proposed method with a LOQ of 0.2 μg/mL for deferasirox is suitable to support a wide range of clinical studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.044.

References

[1] A.T. Taher, K.M. Musallam, M.D. Cappellini, D.J. Weatherall, Br. J. Haematol. 152 (2011) 512.
[2] N.F. Olivieri, N. Engl. J. Med. 341 (1999) 99.
[3] J.B. Porter, Br. J. Haematol. 115 (2001) 39.
[4] J.B. Porter, Drugs Today 42 (2006) 623.
[5] M. Otto-Duessel, M. Aguilar, N. Hanspeter, R. Moats, J.C. Wood, Exp. Hematol. 35 (2007) 1069.
[6] E. Wimazal, T. Nosslinger, C. Baumgartner, W.R. Sperr, M. Pfeilstocker, P. Valent, Eur. J. Clin. Invest. 39 (5) (2009) 406.
[7] G. Metzgeroth, D. Dinter, B. Schultheis, A. Dorn-Beineke, K. Lutz, O. Leismann, R. Hehlmann, J. Hastka, Ann. Haematol. 88 (2009) 301.
[8] S. Steinhauser, U. Heinz, M. Bartholoma, T. Weyhermuller, H. Nick, K. Hegetschweiler, Eur. J. Inorg. Chem. 21 (2004) 4177.
[9] K. Miyazawa, K. Ohyashiki, A. Urabe, T. Hata, S. Nakao, K. Ozawa, T. Ishikawa, J. Kato, Y. Tatsumi, H. Mori, M. Kondo, J. Taniguchi, H. Tanii, L. Rojkjaer, M. Omine, Int. J. Hematol. 88 (2008) 73.
[10] R. Galanello, A. Piga, D. Alberti, M.C. Rouan, H. Bigler, R. Sechaud, J. Clin. Pharmacol. 43 (2003) 565.
[11] A. Piga, R. Galanello, G.L. Forni, M.D. Cappellini, R. Origa, G. Donato, E. Bordone, A. Lavagetto, L. Zanaboni, R. Sachaud, N. Hewson, J.M. Ford, H. Opitz, D. Alberti, Haematologica/Hematol. J. 91 (2006) 873.
[12] R.K. Kaja, K.V. Surendranath, P. Radhakrishnanand, J. Satish, P.V.V. Satyanarayana, Chromatographia 72 (2010) 441.
[13] M.C. Rouan, C. Buffet, L. Masson, F. Marfil, H. Humbert, G. Maurer, J. Chromatogr. B 754 (2001) 45.
[14] M.C. Rouan, C. Buffet, L. Masson, F. Marfil, H. Humbert, G. Maurer, J. Pharm. Biomed. Anal. 25 (2001) 995.
[15] M.C. Rouan, F. Marfil, P. Mangoni, R. Sechaud, H. Humbert, G. Maurer, J. Chromatogr. B 755 (2001) 203.
[16] G.J.M. Bruin, T. Faller, H. Wiegand, A. Schweitzer, H. Nick, J. Schneider, K.O. Boernsen, F. Waldmeier, Drug Metab. Dispos. 36 (2008) 2523.
[17] J.L. Manzoori, A. Jouyban, M. Amjadi, V. Panahi-Azar, E. Tamizi, J. Vaez-Gharamaleki, Luminescence 26 (2011) 244.
[18] M. Hajjizadeh, A. Jabbari, H. Heli, A.A. Moosavi-Movahedi, A. Shafiee, K. Karimian, Anal. Biochem. 373 (2008) 337.
[19] A. Alpert, J. Chromatogr. 499 (1990) 177.
[20] C.J. Van Platerink, H.G.M. Janssen, J. Haverkamp, Anal. Bioanal. Chem. 391 (2008) 299.
[21] S. Georgakakou, M. Kazanis, I. Panderi, Anal. Bioanal. Chem. 397 (2010) 2161.
[22] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[23] I.S. Lurie, L. Li, S.G. Toske, J. Chromatogr. A 1218 (2011) 9336.
[24] D.V. McGalley, J. Chromatogr. A 1217 (2010) 3408.
[25] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[26] P.J. Boersema, S. Mohammed, A.J.R. Heck, Anal. Bioanal. Chem. 391 (2008) 151.
[27] Guidance for Industry. Bioanalytical Method Validation, FDA, May 2001, http://www.fda.gov/cder/guidance/4252fnl.pdf.