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A new HPLC UV validated method for therapeutic monitoring of deferasirox in thalassaemic patients

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Abstract

We describe a new high performance liquid chromatography coupled with ultraviolet detection method for the quantification of plasma concentration of oral iron chelating agent deferasirox. A simple protein precipitation extraction procedure was applied on 500 µl of plasma aliquots. Chromatographic separation was achieved on a C18 reverse phase column and eluate was monitored at 295 nm, with 8 min of analytical run. This method has been validated following Food and Drug Administration procedures: mean intra and inter day variability was 4.64 and 10.55%; mean accuracy was 6.27%; mean extraction recovery 91.66%. Calibration curves ranged from 0.078125 to 40 µg/ml. Limit of quantification was set at 0.15625 while limit of detection at 0.078125 µg/ml. We applied methodology developed on plasma samples of thalassaemic patients treated with deferasirox, finding correlation between deferasirox plasma concentrations and serum ferritin levels. This methodology allowed a specific, sensitive and reliable determination of deferasirox, that could be useful to perform its therapeutic monitoring and pharmacokinetic studies in patients plasma.

1. Introduction

In thalassaemic patients iron overload, caused by regular transfusions need and increased gastrointestinal absorption, can lead to different clinical consequences [1]. Iron accumulation in the body over the time can damage liver, myocardium, spleen, and endocrine organs, inducing heart failure, diabetes, hypothyroidism, hypogonadism, and hepatic disease as cirrhosis or liver cancer [2] and [3]. The major cause of death reported in transfusion iron overload is heart failure [4]. Achievement of safe tissue iron concentrations, by promoting a negative iron balance and iron detoxification, can be pursued with chelation treatment initiation [1]. Chelators such as deferipone and deferoxamine, standard cares for the past thirty years, have been widely used to remove excess iron in the body [5] although with evident drawbacks. Deferipone (Ferriprox©), formulated as solid tablets and administered 3 times a day, has a narrow therapeutic

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window, and its safety risks may include drug related agranulocytosis and arthropathy [6] and [7]. On the other hand, the uncomfortable way of administration of Deferoxamine (Desferal©), injected by slow subcutaneous or intravenous infusion over 8–12 h, due to the low oral drug bioavailability and to its short half life, results in a therapy compliance often poor with limited efficacy [8]. This situation has prompted to investigate for a more convenient iron chelating agent. Deferasirox (ICL670, Fig. 1), following indicated as DFX, is a tridentate orally administered iron chelator recently approved by Food and Drug Administration (FDA) and licensed by European Medicines Agency (EMA) to this purpose. Commercially known as Exjade©, DFX represents a new approach to the management of chronic iron overload in patients with chronic anemias who require blood transfusions [6], [9] and [10]. Currently approved in many countries for the treatment of patients over 2 years of age, its once daily administration leads to high patient satisfaction and compliance [11]. DFX dose between 20 and 30 mg/kg/day generally produces a net negative iron balance [6], however, a recent retrospective study demonstrated that doses of DFX greater than 30 mg/kg/day are safe and more effective in reducing the iron burden [12]. The current maximum FDA approved dose of DFX has been recently increased to 40 mg/kg/day in the Unites States [13].

DFX elimination half life is between 8 and 16 h, allowing to a convenient once daily administration. Its metabolism and that of the iron chelate (Fe [DFX]2) is primarily mediated by glucuronidation, whereas elimination of both occurs by hepatobiliary excretion into the faeces [6]. Although the mentioned half life is the most frequent reported, one study has shown that the half life of DFX may decrease to 7 h in some patients [8], and this may decrease the total effective time of drug coverage. Furthermore DFX is metabolized in hepatocytes by UDP glucuronyltransferase 1A1, with the formation of main metabolite, a glucuronide, that has no clinical use, up to now; this enzyme shows a variable expression, depending on common promoter polymorphism [14]. As previously described drug and its complex are excreted mainly in the bile: the complex is dissociated in intestinal lumen whereas DFX might enter an enterohepatic cycle [15]. In addition some patients, especially those heavily iron loaded, do not achieve adequate iron chelation and a negative iron balance, even when receiving DFX doses exceeding 30 mg/kg/day (poor responders). Others may experience DFX related adverse events (AEs) at the dose required to maintain the iron burden balance (intolerant patients). If AEs are managed by decreasing the dose of DFX or interrupting treatment, these patients will not be able to achieve adequate iron chelation and maintain a negative iron balance during their regular blood transfusions. Finally some DFX AEs may be dose dependent [6] and related to peak drug levels.

Therefore it is clear that, due to all these mentioned parameters, an high inter individual variability of DFX exposure may occur, leading to inadequate chelation treatment or to a toxicity increase.

Therapeutic drug monitoring (TDM) has become recently an essential tool for the management of patients with different pathologies and may be useful also for thalassaemic patients. Measurement of DFX plasma concentrations in treated patients, in fact, could be useful to evaluate patient adherence to daily oral therapy, potential drug–drug interactions, and pharmacokinetic (PK)/pharmacodynamic (PD) relationship studies [16] and [17]. Furthermore recent data [18] show an inverse correlation between preadministration labile plasma iron, target of chelators, and DFX trough concentration (i.e. 24 h after last intake), following indicated as Ctrough, sustaining the hypothesis that DFX Ctrough could be related to treatment response.

In recent years, numerous papers have reported the use of high throughput bioanalytical procedures for the quantification of iron chelating drugs [8], [10], [13], [19], [20], [21], [22] and [23]. Those reporting the use of high performance liquid chromatography coupled with ultraviolet determination (HPLC UV) methods [8], [10], [13], [20] and [23], all applied methodology developed by Rouan in 2001 [19]. More recently liquid chromatographic methods based on mass spectrometry (LC MS MS) detection have been developed to this purpose [21] and [22], although MS facilities are not always available in standard hospital laboratories. Chauzit et al. [22] reported also the analysis of DFX metabolite, with the evidence that glucuronide not interferes with DFX at its retention time and that it do not convert usually into the parent drug, increasing the DFX concentration in vitro. Method developed by Rouan [19] permitted separation and simultaneous plasma determination of DFX and its iron complex in a range of concentrations from 0.25 to 20 µg/ml. In order to preserve the ratio between complex and total form, method required plasma samples storage at 4 °C immediately after collection, and all samples processing maintained at low temperature, procedures often difficult both for hospital setting and laboratory analytical routine.

Therefore aim of the present study was to develop and validate an easier HPLC UV method for DFX plasma quantification, broadly applicable and defined by a wider range of concentrations (0.078125–40 µg/ml).

2. Materials and methods

2.1. Chemicals

DFX (ICL 670) and Imatinib (STI 571), used as internal standard (IS), were kindly provided by Novartis Pharma AG (Basel, Switzerland). Acetonitrile HPLC grade, methanol HPLC grade and triethylamine were purchased from VWR International (Milan, Italy). HPLC grade water was produced with Milli DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Blank plasma from healthy donors was kindly supplied by the Blood Bank of San Luigi Hospital (Orbassano, Italy).

2.2. Stock solutions, calibration standards (STDs) and quality controls (QCs)

Stock solution of DFX was prepared by dissolving an accurately weighed amount of drug in ethanol to obtain a final concentration of 1 mg/ml, then stored at −20 °C till analysis [22]. Stock solution of IS was prepared by dissolving an accurately weighed amount of drug in methanol to obtain a final concentration of 1 mg/ml, then stored at −20 °C till analysis, stable up to 3 months [24]. The highest calibration standard (STD10: 40 µg/ml) and 3 QCs, QChigh (20 µg/ml), QCmedium (5 µg/ml) and QClow (0.3125 µg/ml) were prepared adding a determined volume of stock solution to blank plasma. Others STDs were prepared by serial dilution from STD10 to the lowest calibration standard (STD1: 0.078125 µg/ml) with blank plasma, to obtain 10 different spiked concentrations. A blank sample plus IS (STD0) was also included. Calibration range, from STD10 to STD1, and QCs concentrations are listed in Table 1. STDs and QCs were stored at −20 °C until analyses.

2.3. STDs and QCs extraction

The extraction procedure was based on protein precipitation: 100 µl of IS working solution, made at the final concentration of 100 µg/ml in methanol and used immediately, was added to 500 µl of plasma sample. Then 750 µl of protein precipitation solution (methanol:acetonitrile 50:50, v/v) was added to each sample. After brief mixing (30 s), samples were centrifuged at 12,000 rpm for 15 min and 800 µl of the obtained supernatant were transferred to vials, for injection in column (20 µl).

All procedures (stock solutions, STDs and QCs preparation and extraction steps) were carried out at room temperature.

2.4. Chromatographic system and conditions

HPLC was performed with a VWR Hitachi system (LaChrom Elite) equipped with autosampler, spectrophotometer, and heated column compartment. System management and data acquisition were performed with the EzChrom Elite software. Separation was achieved with GraceSmart© RP18 column, 5 µ, 250 mm × 4.6 mm (Grace, Milan, Italy), preceded by a Security Guard Cartridge C18 4 mm × 3 mm (Phenomenex, Milan, Italy). Mobile phase consisted of 40% solvent A, 20% methanol, 40% acetonitrile. Solvent A consisted of water (72.5%) methanol (25%) and triethylamine (2.5%), adjusted for pH 9.3 by orthophosphoric acid. Analysis was carried out at the constant flow rate of 1 ml/min at 25 °C in isocratic condition. The eluate was monitored at 295 nm. Total runtime was 8 min.

2.5. Method validation

Method was validated following recommended FDA procedures over 3 days [25]. Every day, 2 calibration curves and 6 replicates of QCs (specifically, for each QC concentration 2 samples were extracted 3 times for a total analysis of 18 QCs) were extracted using the protocol described above and then analyzed to assess linearity, variability, and accuracy.

2.5.1. Linearity

Range within that testing method linearity was from 0.078125 to 40 µg/ml of DFX, according phase II DFX studies data [8], [15] and [26]. Calibration curves, over the concentrations range chosen, were built with the spike height ratios of each STD and IS, and fitted using linear regression. Totally 6 calibration curves were analyzed, 2 curves for each validation day.

2.5.2. Variability

Variability was assessed as intra and inter day parameter. Intra day was defined as relative standard deviation (RSD) calculated from the values measured from 6 QCs replicates performed each day at concentration of 0.3125, 5, 20 µg/ml, respectively. Inter day variability was defined as RSD calculated using the values measured from 18 samples (6 samples/day) at concentration of 0.3125, 5, 20 µg/ml, respectively. The variability was considered acceptable for each QC if it did not exceed 15%.

2.5.3. Accuracy

Accuracy was calculated as the medium percent deviation from the nominal concentration from 18 samples (6 samples/day) at concentration of 0.3125, 5, 20 µg/ml, respectively. The variability was considered acceptable for each QC if it did not exceed 15%.

2.5.4. Recovery, limit of detection (LOD) and LOQ

Percent recovery was obtained from the spike height ratio between extracted sample and drug in mobile phase solution at equal concentration (0.3125, 5, 20 µg/ml, respectively for DFX, 20 µg/ml for IS). Final value was obtained as mean from 9 ratios.

As requested by international guidelines [25] LOD in plasma was defined as the concentration that yields signal to noise ratio of 3/1, while LOQ was considered the lowest concentration level that could be determined with a percent deviation from the nominal concentration and RSD <20%. LOQ was tested for intra and inter day variability and accuracy as previously described for QCs.

2.5.5. Selectivity and stability

Interference from endogenous compounds was investigated by analysis of eight different blank plasma samples.

Stability of DFX was assessed by variation of heights. If the measured concentration remained within 15% of nominal concentration, analyte was considered stable.

Stability was investigated for DFX stock solution at room temperature for 8 h, and within 3 months of storage at −20 °C.

Stability was also investigated for DFX STDs and QCs within 3 months of storage at −20 °C, in order to evaluate long-term stability.

For freeze and thaw stability evaluation, analyte was quantified after three freeze and thaw cycles (STDs and QCs stored at −20 °C for 24 h and then thawed at room temperature).

For short-term stability evaluation, STDs and QCs were thawed at room temperature and kept at this temperature for 8 h and then analyzed.

2.5.6. Carry-over

Carry-over was assessed by injection of blank samples after STD10. Signal reported in the blank sample following STD10 was considered acceptable if it did not exceed 20% of LOQ for DFX and 5% for IS.

2.6. Application to thalassaemic patients

During first 6 months of 2011 HPLC UV methodology developed has been applied to plasma of patients affected by thalassaemic syndromes (transfusion dependent) treated with DFX, followed by our institution. Patients receiving regularly standard dosing of DFX, ranging from 10 to 40 mg/kg/day without difference between the genders (mean dose 27 ± 7 mg/kg/day), underwent blood sampling, after obtaining their informed consent for the measurement of DFX concentrations (study has been furthermore submitted to the local ethic committee).

All patients with a compliance at least of 70% in last six months, verified by TDM, were treated continuously for minimum 7 days before blood sampling, so that collection was done at DFX steady state; furthermore 87% of patients received drug for more than 1 year. During time lapse indicated, 109 blood samples, corresponding to 67 patients, 34 females and 33 males (median age 34.00 years, 1° quartile 22.50; 3° quartile 37.00) were collected in lithium heparin tube at the Ctrough (24 ± 2 h after last drug intake). Plasma was separated from red cells after 10 min centrifugation (1500 rpm) at 4 °C, then frozen at −20 °C till analysis. For HPLC determination DFX and IS were extracted from patients plasma as described above (see Section 2.3), then injected in HPLC for quantification.

The serum ferritin concentrations, as marker of iron load, were obtained at the same time of Ctrough samples collection.

3. Results

Time of analytical run was chosen as 8 min, according to the retention times of substances and their good separation. DFX retention time was 3.51 ± 0.15 while for IS it was 5.98 ± 0.35. Representative chromatograms of a blank plasma (plus IS) extracted and DFX STD2 and STD9 are shown in Fig. 2 and Fig. 3.

3.1. Method validation

3.1.1. Linearity

Calibration curves were linear over the concentrations range selected for validation (0.078125–40 µg/ml), with a mean regression coefficient (r2) of 0.99.

3.1.2. Variability and accuracy

Variability results, assessed as intra and inter day parameters, and accuracy are listed in Table 2. All observed data were below 15%.

3.1.3. Recovery, LOD and LOQ

Final extraction recovery value for DFX was obtained as mean from 9 ratios: results are listed in Table 2.

According international guidelines [25], the lowest calibration point (0.078125 µg/ml) was defined as LOD, while LOQ was set at STD2 (0.15625 µg/ml).

Accuracy for LOQ was 11.55%, intra and inter day variability was 3.98 and 19.85%.

3.1.4. Selectivity and stability

No signal increase due to endogenous plasma substances was observed at the retention time of DFX and IS.

Analyses of freeze and thaw, short-term and long-term stability for DFX STDs, QCs and stock solution were all within 15% of nominal concentration.

3.1.5. Carry-over

No signal increase due to carry over of substances was observed at the retention time of DFX and IS.

3.1.6. Application to thalassaemic patients

Patients data (DFX Ctrough, age, drug dose and serum ferritin concentrations) are reported in Table 3, divided by sex. DFX Ctrough ranged from 0.16 to 107.85 µg/ml with a mean value of 14.87 ± 15.64 µg/ml and a difference between genders: females had mean DFX Ctrough of 16.79 ± 17.46 µg/ml, higher than value reported for males, 12.90 ± 13.49 µg/ml. No patients had DFX plasma concentration below method LOQ (0.15625 µg/ml) and no linear correlation was observed between DFX plasma Ctrough and drug dose (r = −0.08, Fig. 4).

A representative chromatogram of patient treated plasma extracted for DFX and IS is shown in Fig. 5.

Serum ferritin concentrations ranged from 50 to 7344 ng/ml, with a mean value of 1853 ± 1424 ng/ml. A linear negative correlation was observed between DFX plasma Ctrough and patients ferritin concentrations (r = −0.43, Fig. 6), that reached statistical significance (P = 0.000003, Wilcoxon non parametric test). In fact, patients grouped for ferritin over 1000 ng/ml (n = 65) had a mean DFX concentration of 10.03 ± 10.55 µg/ml, while patients characterized by ferritin concentrations under 1000 ng/ml (n = 41) had a mean value of 27.57 ± 25.80 µg/ml. This analysis was conducted on 106 plasma samples, because for 3 samples, ferritin concentration data were missing.

Furthermore, a statistically significant difference, was observed between ferritin concentration gender related (P = 0.000008, Wilcoxon non parametric test).

4. Discussion

Chelation treatment is necessary to control iron overload occurring in thalassaemic patients. In order to monitor drug related AEs appearance, at the dose required to maintain the iron burden balance, and/or dose dependent AEs, methodology able to quantify iron chelating agents circulating levels, such as DFX, should be developed. Furthermore, inter individual variability of drug exposure, leading to potential inadequate chelation treatment, also should be investigated by TDM related techniques.

Our methodology, developed for DFX plasma determination, reveals a good performance to this aim. Characterized by a wider range of concentrations and by an easier procedure than methodology presented by Rouan [19], our technique allows DFX quantification without MS facilities, not always present in standard laboratory [22]. Based on recent findings, analysis of glucuronide, has been avoided, considering that metabolite has no actual clinical use in patients and do not interferes with DFX in the chromatographic run [22]. Mean regression coefficient of calibration curves obtained during validation method assay indicates an excellent linearity of the methodology developed; accuracy, intra and inter day variability data, listed in Table 2, are all acceptable because within allowed limits. High extraction efficiency shows a good final recovery and absence of interference peaks at the analyte retention times, lets an accurate measurement of DFX plasma levels. The LOQ (0.15625 µg/ml) of the developed assay makes methodology suitable to perform DFX therapeutic monitoring in thalassaemic treated patients. DFX values obtained from

plasma of patients resulted in the expected range of concentrations according to available literature data [8], [15] and [26]. As shown in Fig. 5, in fact, reporting a representative chromatogram of patient treated plasma extracted for DFX and IS, DFX peak is sharp and free from close analytes. The absence of correlation between DFX plasma Ctrough and dose, as shown in Fig. 4, substantiates the prediction of an high inter individual variability in pK, as shown by recent phase II studies observations [8], [15] and [26]. The negative correlation, instead, observed between DFX plasma Ctrough and patients ferritin concentrations (Fig. 6), indicates a potential role of DFX plasma level on treatment efficacy. This link is confirmed by the comparison between the group of patients with ferritin over 1000 ng/ml, which had DFX concentration around 10 µg/ml, and patients with ferritin concentrations under 1000 ng/ml, that had greater DFX plasma level. This correlation reached statistical significance, suggesting that an higher plasma DFX concentration could be associated with a major treatment efficacy, as shown already by Chauzit et al. [22], even if, may be due to the low samples number involved, in this case difference did not reach statistical significance. Also Chirnomas et al. [13], reported that non responding patients had a lower DFX exposure than responding patients, leading to the hypothesis of a relationship between chelation treatment efficacy and DFX plasma level. Furthermore, the statistically significant difference observed between ferritin concentrations gender related may suggests that sex could be a variable to be considered in choosing drug dosage schedule. In fact, same DFX dosage administered seems to be more efficient in females than in males, in terms of ferritin concentrations reduction, confirmed by the higher DFX plasma level reached by females.

However, these preliminary results are not sufficient up to now to perform a reliable analysis of DFX levels impact on chelation treatment efficacy. Other 6 months data collection could be maybe enough to conduct a stratified analysis of patients by DFX levels related to efficacy treatment (paper in progress). In fact, because no therapeutic window has been clearly defined for DFX, it could be interesting quantify plasma level reached in treated patients, assessing concentration response relationship, in order to define at least a threshold of treatment efficacy.

5. Conclusion

Aim of the present study was to develop and validate an easy and reproducible HPLC-UV method, broadly applicable to clinical routine and defined by a wide range of concentrations, in order to measure DFX plasma level in thalassaemic treated patients. Resulting methodology is rapid, selective, sensitive and suitable to perform TDM in patients, as demonstrated by initial analyses done in plasma patients treated with DFX.

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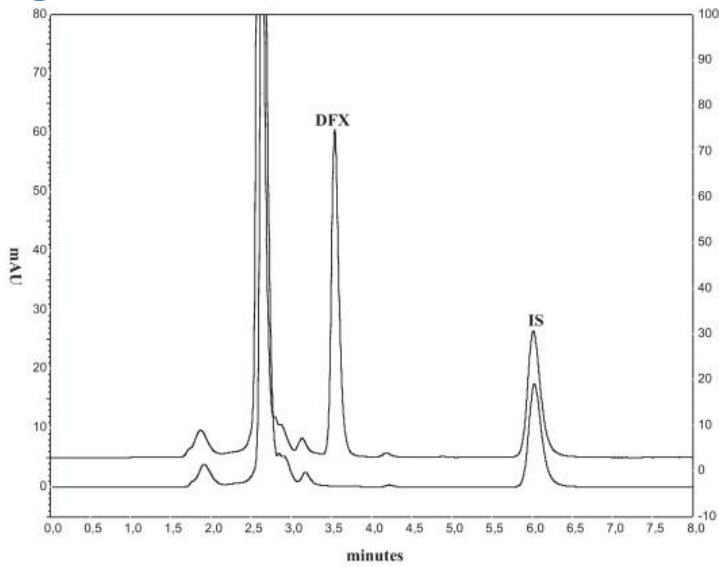
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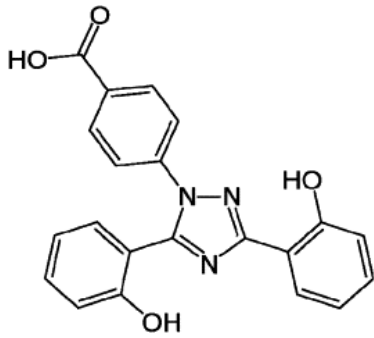

Deferasirox structure.

Figure 2
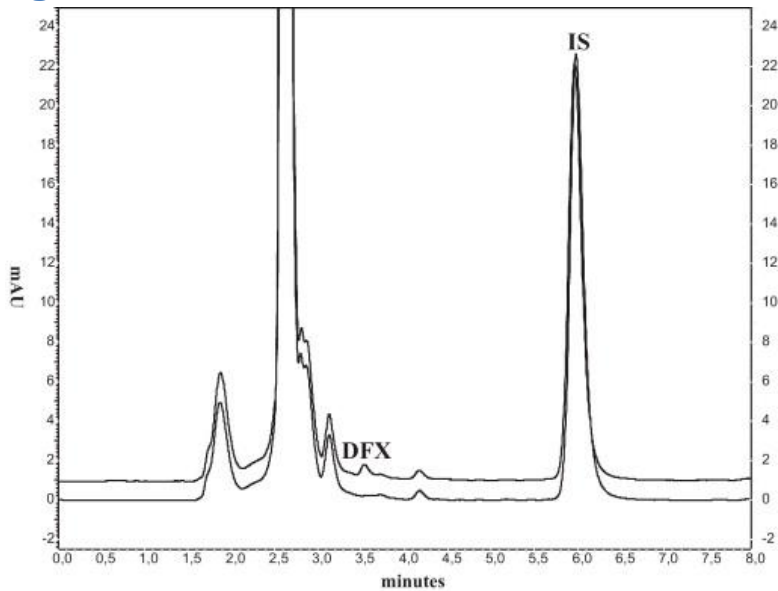
Representative overlapped chromatograms of extracted plasma: blank plus IS and DFX STD2 (0.15625 µg/ml) plus IS. Blank line starts from 0 mAU, while DFX STD2 line starts from 1 mAU. Retention time for DFX and IS is 3.51 ± 0.15 and 5.98 ± 0.35 min, respectively (DFX: deferasirox; IS: internal standard).
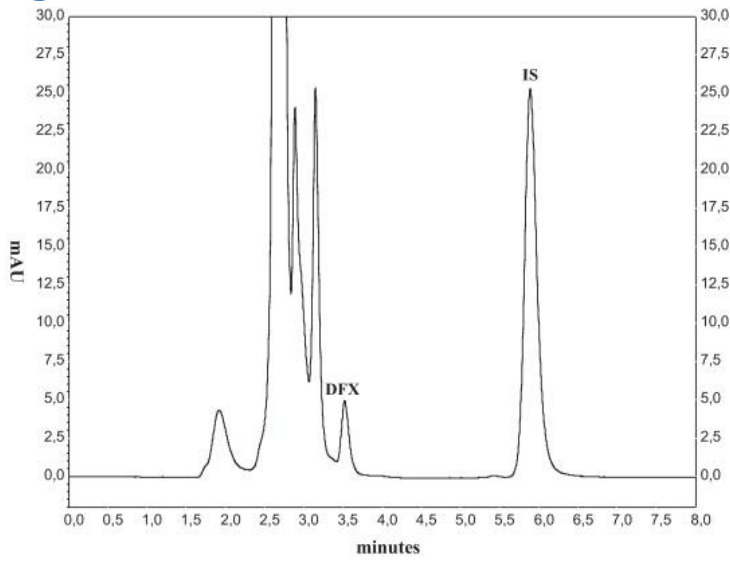
Figure 3

Representative overlapped chromatograms of extracted plasma: blank plus IS and DFX STD9 (20 µg/ml) plus IS. Blank line starts from 0 mAU, while DFX STD9 line starts from 5 mAU. Retention time for DFX and IS is 3.51 ± 0.15 and 5.98 ± 0.35 min, respectively (DFX: deferasirox; IS: internal standard).
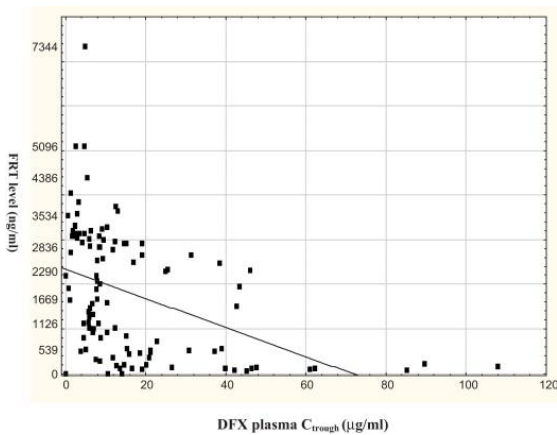
Figure 4
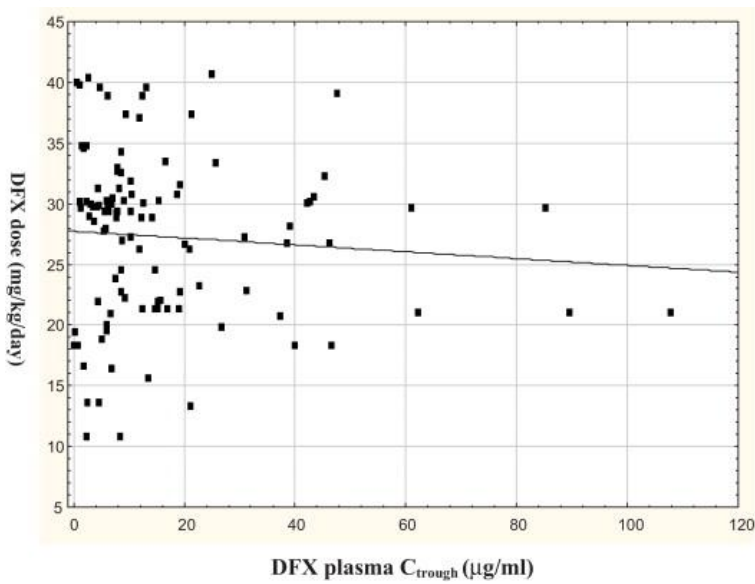
Correlation between DFX plasma Ctrough (µg/ml) and drug dose administered to patients (mg/kg/day) (DFX: deferasirox; Ctrough: DFX trough concentration measured 24 ± 2 h after last intake).

Figure 5

Representative chromatogram of patient treated plasma extracted for DFX and IS. Retention time for DFX and IS is 3.51 ± 0.15 and 5.98 ± 0.35 min, respectively (DFX: deferasirox; IS: internal standard).

Figure 6

Correlation between DFX plasma Ctrough (µg/ml) and patients FRT levels (ng/ml) (DFX: deferasirox; Ctrough: DFX trough concentration measured 24 ± 2 h after last intake; FRT: ferritin).

Table 1.
Calibrations standards from STD_1 to STD_{10} and quality controls (low, medium, high) concentrations.

Concentrations (µg/ml)

STD_1 [a]	STD_2 [b]	STD_3	STD_4	STD_5	STD_6	STD_7	STD_8	STD_9	STD_{10}	QC_l	QC_m
0.078125	0.15625	0.3125	0.625	1.25	2.5	5	10	20	40	0.3125	5

a Corresponds to LOD: Limit of detection.

b Corresponds to LOQ: Limit of quantification.

Table 2.
Validation data.

QC_{low} 0.3125 µg/ml				QC_{medium} 5 µg/ml				QC_{high} 20 µg/ml		
Accuracy (%)	Variability (RSD%) [a]		Recovery (%)	Accuracy (%)	Variability (RSD%)		Recovery (%)	Accuracy (%)	Variability (RSD%)	
	Intra-day	Inter-day			Intra-day	Inter-day			Intra-day	Inter-day
5.77	7.77	12.42	87.12	2.29	2.95	3.75	90.31	5.55	3.96	6.23

a Relative standard deviation.