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Dried blood spot analysis of an iron chelator – Deferasirox and its potential application to therapeutic drug monitoring

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ABSTRACT

Deferasirox is an iron chelating agent for the treatment of transfusional iron over load in patients with chronic anemia. These anemic patients require close monitoring of the deferasirox exposures for ensuring its therapeutic efficacy. Dried blood spot (DBS) sampling methodology has the advantages of low volume of blood withdrawal and ease of transportation and storage over liquid blood methods. A LC–MS/MS based analytical method was developed using reversed phase column with gradient elution program and quantitated in MRM mode. Linearity range for the liquid blood was 1–1000 ng/mL and for DBS was 5–5000 ng/mL under similar mass spectrometry conditions. The method was validated with respective $(M-H)^-$ ions, m/z 372→118 for deferasirox and m/z 410→348 for fluvastatin (internal standard). The validated method was applied for the analysis of DBS samples from a rat pharmacokinetic study and results were compared against liquid blood samples from the same animal. The mean C_{max} from DBS sample (1121 ng/mL) was comparable to mean C_{max} found in blood samples (1015 ng/mL) at 2 h after oral dose of deferasirox. All the other calculated pharmacokinetic parameters were quite comparable for both liquid blood and DBS samples.

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1. Introduction

Dried blood spots (DBS) technique was well established and widely used for screening of new borns for metabolic disorders and DNA analysis. DBS as sampling technique has been receiving growing interest in both preclinical and clinical studies due to its inherent advantages over the liquid blood/plasma sampling technique which was in use for many years in quantitative bioanalysis to support drug development studies. The applicability of the DBS in drug development was initially demonstrated by Beaudette and Batemann [1]. DBS sampling technique with the recent developments in LC–MS/MS detection system has emerged as the most promising technique for quantitative analysis of samples from clinical and preclinical samples [2–4]. There are several advantages of the DBS technique in comparison to conventional liquid plasma, blood, serum sampling technique [2,4,5]. The collection of small volumes of blood (10–40 μL) ideally suited for neonatal and infant screening. DBS requires minimum resources such as finger or heel prick instead of venipuncture for blood collection cum spotting. DBS method simplifies the activities related to storage and transportation in comparison to plasma/blood/serum samples which require either refrigeration or freezing during their storage and transport. DBS possess low bio-hazard risk to carry HIV and hepatitis as disruption of their envelope on drying [6]. Serial sampling can be employed due to reduced sample volume requirements which in turn reduces the number of animals required for rodent pharmacokinetic and safety assessment studies. Given the advantages that DBS can offer, several DBS assays have been developed and validated for quantitation small molecules and peptides. Varied hematocrit value in different persons especially in diseased state is the limitation of the DBS technique. However impact of hematocrit value in bioanalysis using DBS samples was widely discussed and possible solutions were elucidated [7–14].

Excess iron due to the frequent blood transfusions in disease conditions, such as β-thalassemia or enhanced dietary iron uptake in chronic anemia patients, will get accumulated in the body organs, such as liver, heart and endocrine organs [15]. After exceeding the body's iron storage capacity the freely available iron catalyzes the formation of highly reactive hydroxyl radicals as humans got no other physiological mechanism to eliminate excess iron [16]. These hydroxyl radicals lead to membrane damage and penetration of proteins and ultimately death [16]. Patients who receive repeated blood transfusions without proper chelating therapy, cardiac disease symptoms are generally reported within 10 years of receipt

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of the first blood transfusion [17]. Cardiac damage caused by iron overload toxicity is the main cause of death in thalassemia patients [18].

Deferasirox was approved in November 2005 by USFDA, as a new iron-chelator in its class in comparison to existing Deferiprone and Deferoxamine. Two molecules of deferasirox chelates with one molecule of labile iron present in transit pool and then gets excreted through urine and/or feces [19]. Deferasirox has shown better patient compliance due to its convenient *oral* administration once a day when compared to existing iron chelating treatments including deferoxamine (*intramuscular* injection *intravenous* infusion) [20], or Deferiprone which needs to be taken thrice a day [21] and has potential adverse effects like agranulocytosis.

The half-life of unbound deferasirox varies between 8 and 19 h [22,23]. It is primarily metabolized via glucuronidation by UDP glucuronyl transferase 1A1 (UGT1A1) in hepatocytes with biliary excretion [22]. UGT1A1 has variable expression in promoter polymorphism: 40% of the white population is heterozygote for allele UGT1A1*28 which was associated with lower enzyme expression [24]. As reported, deferasirox and [Fe–(deferasirox)$_2$] complex are excreted mainly to bile: the complex is then dissociated in intestinal lumen, deferasirox might enter an entero hepatic cycle [22]. The systemic exposures of deferasirox indicates high inter individual variability leading to either insufficient chelation or increased toxicity. Deferasirox needs close monitoring by physicians during its treatment for its therapeutic efficacy.

With the advent of pharmacogenetics and development in analytical techniques, therapeutic drug monitoring has become essential tool in optimizing the usage of existing drugs by adjusting dose [25]. Measurement of deferasirox in treated patients could be useful to measure its therapeutic or toxic effect.

Quantification of drugs in biological fluids by liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become more common due to inherent selectivity and sensitivity of the technique. The present purpose of this work was to explore the possibility of the DBS technique for the therapeutic drug monitoring of the deferasirox. The pharmacokinetic parameters obtained in DBS method will be evaluated with pharmacokinetic parameters obtained in liquid blood method for its applicability and usage of a rat pharmacokinetic study. However to apply current rat DBS method for therapeutic drug monitoring of deferasirox in patients with DBS key parameters, such as selectivity, recovery and matrix factor needs to be evaluated with human DBS.

HPLC–UV methods are available for the measurement of deferasirox [19,26–28], deferasirox and iron complex [19,26] which requires lengthy run times and these methods suffer from selectivity related issues. Few LC–MS/MS methods are reported for the quantitation of deferasirox [19,29–31], and its iron complex [19,29] using blood, urine or plasma as a matrix. However we have explored DBS sampling technique for monitoring deferasirox using LC–MS/MS.

2. Experimental

2.1. Chemicals and reagents

Deferasirox (4-[3,5-bis-(2-hydroxy-phenyl)-[1,2,4]triazol-1-yl]-benzoic acid, molecular weight 373.4, and fluvastatin were obtained from Suven R&D center, Hyderabad. HPLC-grade acetonitrile and HPLC-grade methanol were purchased from Merck (Darmstadt, Germany). Formic Acid was purchased from Fluka (Sigma Aldrich, Steinheim, Germany). Ammonium acetate was purchased from Merck (Worli, Mumbai, India). HPLC grade water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade. Whatman FTA-DMPK-C cards were obtained from GE health care (Amersham place, Little Chalfont, Buckinghamshire, UK).

2.2. Instrumentation

The HPLC SIL HTc system (Shimadzu Corporation, Kyoto, Japan) was equipped with LC-AD VP binary pump, a DGU20A5 degasser and a SIL-HTc auto sampler equipped with a CTO-10AS VP thermostated column oven. The column was Agilent® Zorbax Eclipse XDB C_8 (100 mm × 4.6 mm, 3.5 μ). AB Sciex API-4000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) with a turboionspray source with electrospray ionization was used for detection. Electronically triggered six port switching valve (Valco instruments Co., Houston, TX, USA) was used for diverting/introducing the flow into the LC–MS/MS system. Gas generator (NM20Z-A, Fountain crescent, Scotland, United Kingdom) was used to generate purified air and high purity nitrogen.

2.3. LC–MS/MS conditions

The mobile phase contains 10 mM ammonium acetate in channel "A" and acetonitrile in channel "B" with flow rate of 1 mL/min were used to achieve separation from endogenous interferences. The flow was diverted with split ratio of 10% to mass spectrometer source and 90% to waste. Program starts with equilibration at 60% buffer, 40% acetonitrile and remains same up to 0.3 min, then slowly changes up to 90% acetonitrile at 1.0 min and holds the same up to 2.0 min gets back to the initial equilibration conditions at 2.01 min till the end of the run (3.0 min).

Mass spectrometer parameters were optimized by infusing a neat solution of deferasirox and the internal standard (IS) separately by using a Harvard Apparatus syringe pump (Holliston, MA, USA). A turboionspray interface operating in negative ionization mode was used. Typical source conditions were as follows: the turbo-gas temperature was set at 350 °C and the ion spray needle voltage was adjusted to −4500 V. The mass spectrometer was operated at unit resolution for both Q1 and Q3 with a dwell time of 200 ms per MRM channel. The precursor/product ion pairs monitored were m/z 372→118 for deferasirox and m/z 410→348 for the IS. GS1 and GS2 were set at 25 (arbitrary units set in the Analyst software) and the collision gas at 6 (arbitrary units). The voltage parameters like declustering potential (DP) were set at −65, −70, and collision energy (CE) was set at −70, −22 and collision cell exit potential (CXP) was set −11, −7 for deferasirox and IS, respectively. Data acquisition was performed with *Analyst 1.6* software version.

2.4. Preparation of stock solutions, calibration standards and quality control samples

Standard stock solutions of deferasirox (1 mg/mL) and fluvastatin (1 mg/mL) were prepared in methanol by dissolving 10 mg of deferasirox and 2 mg of fluvastatin. Working solutions were prepared with water:methanol (50:50, v/v, diluent) with appropriate dilutions. Calibration standard samples were prepared by spiking 5 μL of stock solution to the 45 μL of blank blood to get their respective calibration standard at 1, 2, 5, 10, 50, 100, 500 and 1000 ng/mL. Quality control samples were prepared by the independent weighing of the analyte. LLOQ (1 ng/mL), low (3 ng/mL), mid (500 ng/mL) and high (800 ng/mL) were prepared by spiking 5 μL of the respective stock solution to the 45 μL of the blank blood. Only 20 μL of the calibration standard/quality control sample was used either for the liquid blood extraction or dried blood spots.

For DBS cards the calibration standards were prepared at 5, 10, 25, 50, 250, 500, 2500 and 5000 ng/mL. Quality control samples

were prepared at 5, 15, 800 and 4000 ng/mL for LLOQ, low, mid and high QC, respectively.

2.5. Blood spotting

20 μL of the either liquid blank blood/spiked blood (for standards and QC sample preparation) or blood collected from the animals prior to dosing or after dosing was spotted on to the FTA DMPK-C by using calibrated pipette (Brand, Germany). Precaution was taken to spot the blood in a drop form without touching the card for uniform spot size and even spreading on the surface of the card. The blood spot was allowed to dry at room temperature for 2 h; after drying the cards were placed in plastic zip lock pouches which were stored in laboratory cupboard at room temperature.

2.6. Extraction of liquid blood samples

A 20 μL of liquid blood sample was spiked into the 0.6 mL microtube (Axygen Scientific, USA) to this 5 μL of IS (concentration 2 μg/mL) was spiked. To these contents 150 μL of acetonitrile was added drop by drop for precipitation and vortexed on a multiple vortexer for 1 min. The contents were centrifuged at 10,000 rpm (5415-R, Eppendorf, Hamburg, Germany) at 10 °C for 10 min and supernatant was transferred into a vial. A 10 μL aliquot of this was injected into LC–MS/MS.

2.7. Extraction of DBS samples

A 6 mm disc was punched out from the center of the DBS with Harris UNICORE[TM] device by keeping the DBS card on the top of the Harris cutting mat. The punched spot was transferred to pre labeled tube and fortified with 100 μL of internal standard (2 μg/mL) mixed in water. These tubes were sonicated initially for 3 min. To these tubes 100 μL of acidic acetonitrile (0.1% formic acid in acetonitrile) was added and vortex mixed for 3 min and then sonicated for 5 min and finally centrifuged for 5 min at 10,000 rpm at 10 °C. Supernatant was transferred to labeled vials and 10 μL was injected into LC–MS/MS.

2.8. Validation of the deferasirox assay

2.8.1. Selectivity

Blood samples from six different sources of rats/humans were tested for the presence of endogenous components in blood which might interfere with detection of deferasirox or IS. Blank blood from six different rats/humans was spiked on to DBS cards and dried to get blank DBS cards. LLOQ samples were prepared in six lots of blood and they were spiked onto DBS cards and dried to get LLOQ samples.

2.8.2. Matrix effect and recovery

Matrix effect and recovery of rat/human blood were tested at three concentrations using low, mid and high QC ($n = 6$) samples. The experiment for matrix effect and recovery consists of three types of sample preparations and analysis: the analysis includes (1) the analyte area response obtained by injecting the neat solutions, (2) the equal amount of analyte added to extracted blank blood samples, (3) the equal amount of analyte added to blood before extraction. Similarly, DBS samples were prepared and analyzed.

Matrix effect was determined by comparing the analyte area response in the extracted blank plus post spiked samples to the response of analyte in neat solutions. Recovery of deferasirox was determined by comparing the analyte area response in extracted samples to the extracted blank plus post spiked samples. Matrix effect and recovery were calculated using the following formulae:

$$\text{Matrix effect} = \frac{\text{analyte response of blank plus post spiked samples}}{\text{analyte response of neat samples}}$$

$$\text{Recovery} = \frac{\text{analyte response of extracted samples} \times 100}{\text{analyte response of blank plus post spiked samples}}$$

2.8.3. Linearity

The linearity was constructed by using the eight point calibration curve consisting of 1, 2, 5, 10, 50, 100, 500 and 1000 ng/mL by spiking respective working solutions for blood. Calibration standards of 5, 10, 25, 50, 250, 500, 2500 and 5000 ng/mL for DBS were prepared and analyzed.

2.8.4. Accuracy and precision

Accuracy and precision were evaluated by analyzing the QC samples of LLOQ, low, mid and high (each $n = 6$) at the concentration of 1, 3, 500 and 800 ng/mL, respectively. Quality control samples ($n = 6$) of DBS method consist of 5, 15, 800 and 4000 ng/mL. Five such batches were analyzed for calculating the between and within batch accuracy and precision.

2.8.5. Dilution integrity

Dilution integrity was performed to quantify the amount of deferasirox in blood samples which contain concentrations more than the ULOQ in liquid blood method. Six aliquots of 10 folds dilution samples were analyzed and calculated the results for % nominal concentrations. However for DBS method dilution integrity was assessed by diluting an extracted dilution DBS QC sample with extracted zero samples (containing IS only), followed by analysis of regular DBS calibration and QC samples.

2.8.6. Stability

Liquid blood stability studies were performed with low and high QC samples (3 and 800 ng/mL, $n = 6$ each). The stability samples were analyzed and compared against the freshly prepared calibration standards. All the samples were prepared as described in sample preparation procedure. The Freeze-thaw stability (2–8 °C) samples were thawed unassisted at room temperature and frozen again for a minimum of 12 h. Such five freeze-thaw cycles were completed and analyzed.

Long term stability of deferasirox in liquid blood at 2–8 °C was evaluated after 4 weeks of storage time by using low and high QCs.

Room temperature stability was assessed by analyte determination at room temperature for various time periods (8 and 24 h) and analyzed after the specified time periods.

For DBS samples in injector stability and long term stability was performed. For in injector stability of DBS method samples were prepared as per the method mentioned and stored at auto sampler temperature (5 ± 1 °C) for 36 h and injected in to LC–MS/MS. For long term stability DBS cards spiked with low and high QC ($n = 6$) were stored at room temperature in a zip lock pouch. After completion of 4 weeks storage time the samples were processed and analyzed as per the mentioned method.

2.9. Pharmacokinetic study

Male wistar rats were housed in a room with 15 air changes per hour. The temperature was maintained at 21 ± 3 °C and relative humidity between 30 and 70%, respectively, under a 12 h dark/light cycles. The rats had access to food and water ad libitum. The rats were cannulated into the jugular vein and left for recovery for 48 h. On the day of experiment, deferasirox (10 mL/kg) was dissolved in reagent grade water for *oral* (gavage) administration in fasted

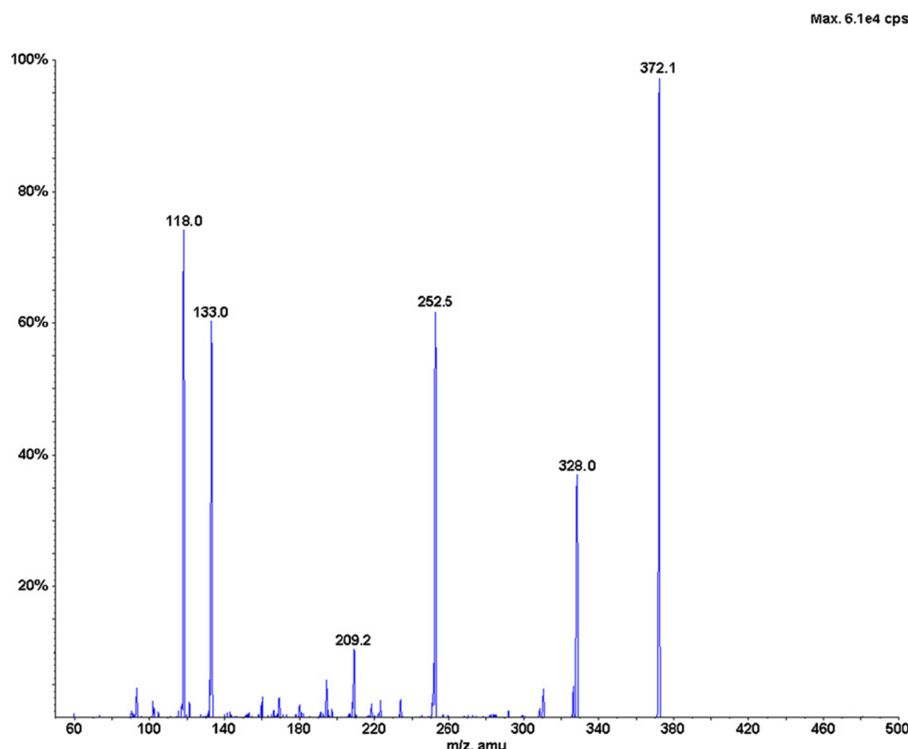

Fig. 1. Full scan negative turboionspray product ion mass spectra of the deferasirox.

rats; for *intravenous* administration water for injection was used at 2 mL/kg. In a parallel study two rats were dosed at 10 mg/kg *oral* and two rats were dosed *intravenous* through tail vein at 3 mg/kg. Blood samples of 0.25 mL were collected at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h after *oral*, at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h after *intravenous* post dose and transferred to pre labeled heparin coated sampling tubes for blood method, whereas 20 μL blood was spotted on to the DBS cards for DBS method.

Pharmacokinetic calculations were performed using Phoenix WinNonlin® version 6.2 (Pharsight Corporation, Mountain View, CA). Linear trapezoidal method with linear interpolation was used to calculate pharmacokinetic parameters.

3. Results and discussion

3.1. Method development

The quantitation of deferasirox was performed by LC–MS/MS system described earlier using turbo-ion spray source in negative ionization mode. The use of stable isotope labeled internal standard is desirable in mass spectrometry as it nullifies the variations of the assay due to endogenous matrix effects. The stable labeled internal standard was commercially unavailable, hence we have explored the method with fluvastatin as internal standard and it has readily served the desired purpose.

When the neat solution of deferasirox was infused into the mass spectrometer, product ion spectra have produced fragments of *m/z* 328, 252, 133 and 118 ions predominantly. Deferasirox undergoes ionization at one of the phenolic oxygen atoms as the ionization energy of aromatic alcohol (≈8.5 eV) was less than the carboxylic acid (≈9.7 eV) [32]. Deferasirox [M−H]$^-$ upon loosing the CO_2 produces a fragment of *m/z* 328. Fragment of *m/z* 252 appeared in parallel both from [M−H]$^-$ by loss of C_6H_5–CO_2H and by loosing benzene from *m/z* 328, this was confirmed by the precursor ion scan of *m/z* 252. As described by Potts et al. [33] 1,2,4 triazole ring gets fragmented to produce an ion of *m/z* 133 by loosing OH–C_6H_5–CN from parent. An intense fragment of *m/z* 118 was observed for O^-–C_6H_5–CN. The full scan product ion spectrum of deferasirox was shown in Fig. 1 and the probable structure of the fragments formed was depicted in Fig. 2. The sensitive precursor/product ion pairs monitored were *m/z* 372→118 for deferasirox and *m/z* 410→348 for the IS.

Eclipse XDB C_8 (100 mm × 4.6 mm, 3.5 μ) with 0.01 M ammonium acetate with acetonitrile has produced the better

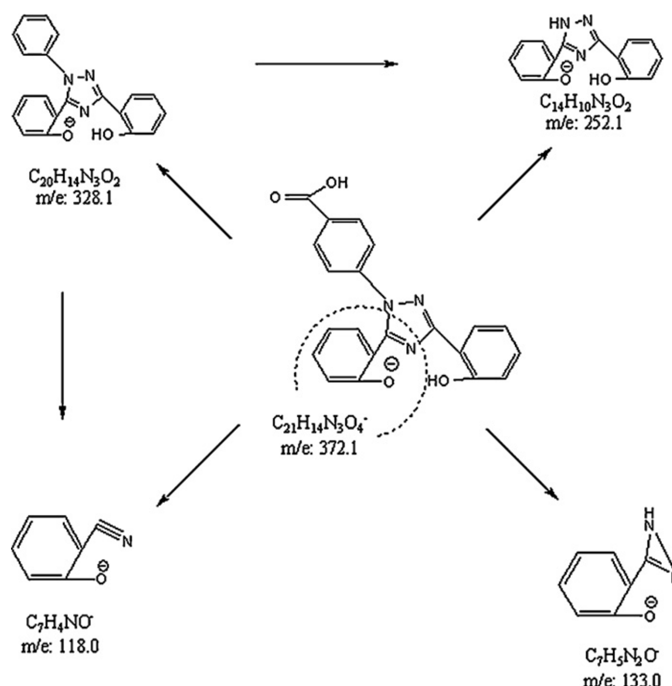

Fig. 2. Chemical structures of deferasirox and probable fragments formed in Q3 region.

chromatography. Under the gradient chromatography method mentioned above the retention time was 1.8 min for deferasirox and 1.9 min for IS. The total flow was diverted to waste by using valco valve for initial 1.5 min and after 3 min to avoid endogenous components deposition into mass spectrometer source there by to prevent response variation with time. This has improved the overall performance of the LC–MS/MS and the method being used.

Extraction of deferasirox from DBS was challenging unlike in plasma due to endogenous substances present in blood and impurities of DBS paper. During the initial trials 3 mm and 6 mm punches were taken from DBS cards and were extracted with various combinations of solvents like water:methanol; water:acetonitrile; water:acidic acetonitrile (0.1% formic acid in acetonitrile) in the ratio of 1:1; 4:1; 1:4 (v/v). Due to larger area of 6 mm punch higher amount of analyte was desorbed from the DBS. Nearly equal amount of analyte was recovered in water:0.1% formic acid in methanol and water:0.1% formic acid in acetonitrile in combination of 1:1 (v/v). But water:0.1% formic acid in methanol

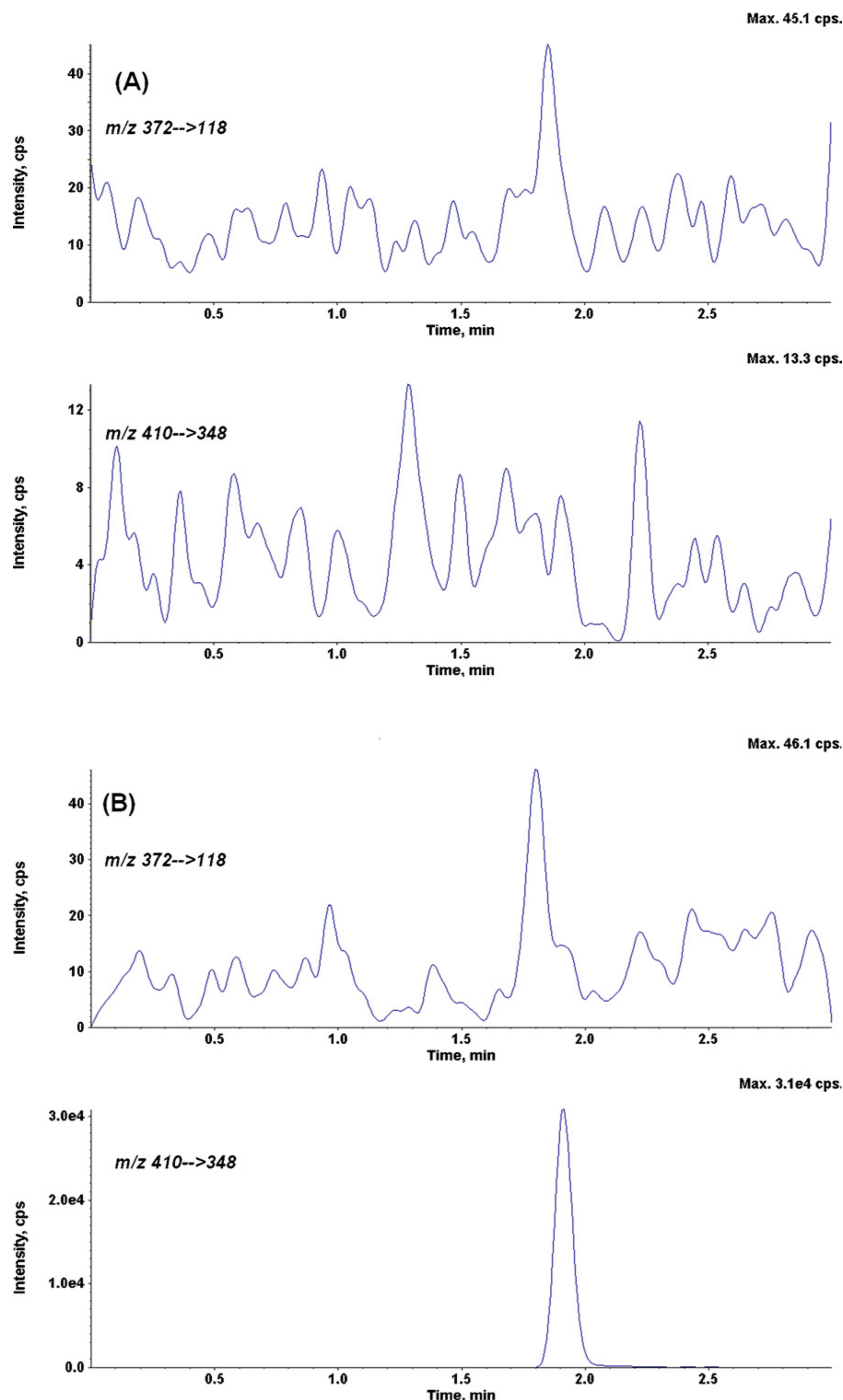

Fig. 3. MRM chromatograms for deferasirox and IS resulting from analysis of (A) blank (drug and IS free) rat blood and (B) zero sample (drug free spiked with IS) rat blood in DBS.

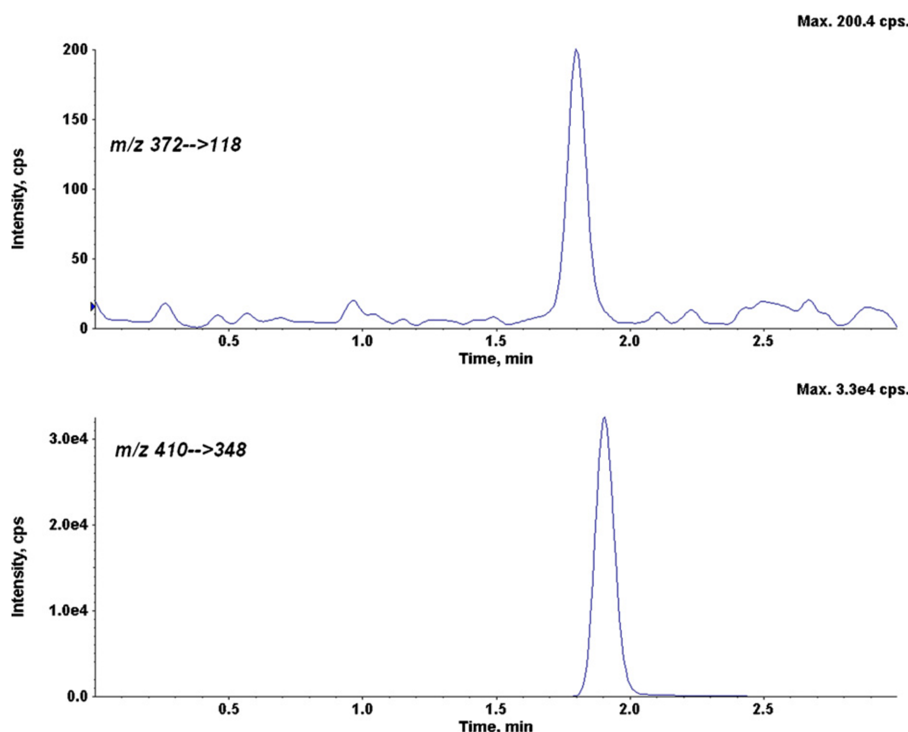

Fig. 4. LLOQ chromatogram of deferasirox and IS in rat blood in DBS.

produced hazy supernatants due to which columns were clogged on repeated injections along with gradual decrease in response. Hence water:0.1% formic acid in acetonitrile was pursued in various combinations [1:1, 1:4 and 4:1, v/v] and 1:1 (v/v) ratio of water: 0.1% formic acid acetonitrile was finalized as that has produced the neat solution along with the stable response. Few initial trials were done with chemically treated DMPK-A cards and untreated DMPK-C cards (plain cellulose) gave similar results. To keep the LC–MS/MS assay simple and economical we used untreated DBS (DMPK-C) cards as the constituents of treated DBS cards sometimes poses a significant interferences and matrix effects [34] on analytes of interest.

3.2. Blood spotting

The weights of the discs ($n = 6$) punched from the DMPK-C cards after drying time of 2 h has exhibited excellent drying process and precision for the disc cutting procedure. The weights of the blank paper discs were found to be 2.93 ± 0.12 mg (RSD 4.17%); for non-spiked liquid blood 4.47 ± 0.13 mg (RSD 2.94%) confirming suitability of the procedure involved in DBS.

3.3. Method validation

3.3.1. Selectivity

Deferasirox area response at its retention time in blank rat/human blood was compared to the LLOQ area response both in liquid blood method and DBS method. The area response at the analyte RT was less than 20% of LLOQ area response both in liquid blood method and rat/human DBS method. Interference in blank blood at the retention time of internal standard was less than 5% of the internal standard response in LLOQ sample. Chromatogram of the extracted blank DBS (Drug and IS free) was shown in Fig. 3A and zero sample (Drug free IS added) was shown in Fig. 3B. Chromatogram of the extracted blood spiked with LLOQ along with IS (2 μg/mL) was shown in Fig. 4.

3.3.2. Matrix effect and recovery

The mean recovery of the deferasirox at three levels ($n = 6$ each) in liquid blood method with acetonitrile precipitation was 95.6%. Experiments were conducted for recovery of deferasirox from rat/human DBS cards and the resultant recoveries were explained in the Table 1. The relative recovery of deferasirox from 6 mm punch of nearly 8 mm dried blood spot formed was 36.4%. For consistency of the assay reproducible recoveries are sufficient and for this method deferasirox has shown an absolute recovery of 48.5% for 8 mm spot. Absolute recovery of the deferasirox from human DBS was found to be 44.3.

The mean matrix factor for the deferasirox at all the tested three levels was 1.02 in liquid blood method. The mean matrix factor for the deferasirox at all the tested three levels was 0.92 in rat DBS method and 0.94 in human DBS (Table 2). The matrix factor for internal standard was comparable to analyte in both the methods.

3.3.3. Linearity

Linearity was obtained by weighted linear regression ($1/X^2$) showed good linearity ($\geq r = 0.998$) over the whole concentration range 1–1000 ng/mL in liquid blood method, 5–5000 ng/mL in DBS method. The back calculated concentrations for all the standards were within the limits (±15% for LLOQ ± 20%). The accuracy at LLOQ was 103.7% with precision of ≤6.7% in DBS method. The accuracy at ULOQ was 100.4% with a precision of ≤1.1%. The back calculated concentrations of calibration standards in both the methods were within the acceptable limits (100 ± 15%). Linearity range can be further extended (data not shown here) upon requirement of the assay.

Table 1
Recovery experiments of deferasirox from DBS cards using different solvents in various combinations.

Solvent	Composition (%, v/v)	Area response of deferasirox (counts)	Relative recovery	Absolute recovery
Neat sample	–	135,723	–	–
Water:Methanol	50:50	18,214	13.4	17.9
Water:ACN	50:50	19,302	14.2	19.0
0.03% formic acid:ACN	50:50	46,912	34.6	46.1
Water:0.1% FA in ACN	50:50	49,347	36.4	48.5
Water:0.1% FA in ACN	80:20	12,119	8.9	11.9
Water:0.1% FA in methanol	50:50	45,075	33.2	44.3
Water:0.1% FA in ACN	20:80	21,546	15.9	21.2
Water:0.1% FA in ACN (human DBS)	50:50	45,060	33.2	44.3

Table 2
Matrix factor table of deferasirox in rat and human dried blood spots 3.

Samples	QC level	Rat DBS		Human DBS	
		Mean ($n = 6$)	%CV	Mean ($n = 6$)	%CV
Neat	Low	4927	3.4	5030	4.4
	Mid	742,136	3.4	737,448	1.9
	High	1,193,655	5.0	1,149,517	4.5
In presence of matrix	Low	4586	4.4	4681	3.9
	Mid	684,970	1.4	704,778	2.6
	High	1,096,593	5.4	1,090,318	4.4
Matrix factor (mean)		0.92		0.94	

3.3.4. Accuracy and precision

The within batch accuracy was ranging from 98.9% to 102.2% and precision was found to be 4.2% to 8.6%, whereas between batch accuracy for deferasirox was ranging from 95.5% to 105.8%, where as precision was ranging from 3.2% to 9.8% in DBS methodology (Table 3). The within and between batch accuracy and precision were within the acceptable limits in liquid blood method.

3.3.5. Dilution integrity

Mean percent nominal concentration of the dilution QC in liquid blood was 97.6% with the precision of 1.2% (data was not shown) for dilution integrity of 10 folds dilution, extending the range from 1000 ng/mL to 10,000 ng/mL. Dilution integrity data of DBS method for five fold dilution was within the acceptable limits (100 ± 15%).

Table 3
Within batch and between batch accuracy and precision of deferasirox by using DBS method.

Within batch				
Nominal concentration (ng/mL)	5	15	800	4000
Determined concentration (ng/mL)	4.9	14.8	779	3564
	5.4	16.5	745	4154
	4.9	14.5	902	4486
	5.2	16.6	741	4037
	5.2	14.9	834	3810
	5.0	13.7	749	3683
Mean	5.1	15.2	792	3956
SD	0.2	1.1	64	339
% CV	4.2	7.6	8.1	8.6
% Accuracy	102.2	101.0	98.9	98.9
Between batch				
Nominal concentration (ng/mL)	5	15	800	4000
Determined concentration (ng/mL)	5.1	17.0	746	4122
	5.1	16.5	794	4052
	5.1	15.2	792	3956
	4.2	16.3	765	3894
	4.3	14.4	835	4221
Mean	4.8	15.9	786	4049
SD	0.5	1.0	33.7	130.0
% CV	9.8	6.6	4.3	3.2
% Accuracy	95.5	105.8	98.3	101.2

3.3.6. Impact of hematocrit value on deferasirox concentrations

Hematocrit is directly proportional to blood viscosity thus it affects blood diffusion on the surface of DBS card. We tested four hematocrit levels to evaluate whether the accuracy of this DBS method would be affected by hematocrit. Mid QC concentrations were spotted on to DBS card with hematocrit levels of 24%, 35%, 50% and 60% individually. Table 4 shows summary of data obtained from QC samples with hematocrit levels spotted on to DBS card. The determined concentrations were increased with increasing hematocrit value. Preparing calibration and QC samples in blank blood as similar (in terms of hematocrit value) as possible to study samples (i.e. pre-dose sample of that subject) would overcome the issue.

3.3.7. Stability

All the stability parameters were well within the acceptable limits for their accuracy (100 ± 15%) and precision (≤15%) in liquid blood method. In DBS method long term stability (4 weeks) and autosampler stability (36 h) were performed and the results (Table 5) were well within the commonly accepted criteria [35].

3.4. Pharmacokinetic study

The linearity range of the DBS method was designed to capture the concentrations of samples obtained in rat pharmacokinetic study as the calculated concentrations of deferasirox were within the limits of 5–5000 ng/mL. The liquid blood samples with

Table 4
Impact of hematocrit value on precision and accuracy of deferasirox assay.

Nominal concentration (ng/mL)	800			
Hematocrit (%)	24	35	50	60
Determined concentration (ng/mL)	673	737	820	840
	682	728	771	880
	676	720	790	859
	701	736	764	876
	679	716	821	869
Mean	682.1	727.3	793.3	864.8
S.D	11.1	9.4	26.7	15.8
% CV	1.6	1.3	3.4	1.8
% Accuracy	85.3	90.9	99.2	108.1
% Difference from 35% Hct	−6.2	0.0	9.1	18.9

Table 5
Stability evaluation of deferasirox in DBS and liquid blood method.

Liquid blood method				
(ng/mL)	Room temperature (24 h) ($n = 6$)		Long term (4 weeks) ($n = 6$)	
Nominal concentration	3.0	800	3.0	800
Determined concentration	3.0	778	3.0	745
% CV	4.2	3.3	4.7	2.6
% Accuracy	101.5	97.3	98.9	93.1
DBS method				
(ng/mL)	Autosampler (36 h) ($n = 6$)		Room temperature (4 weeks) ($n = 6$)	
Nominal concentration	15	4000	15	4000
Determined concentration	15.3	4015	15.5	3627
% CV	5.0	4.5	6.1	6.4
% Accuracy	102.1	100.4	103.5	90.7

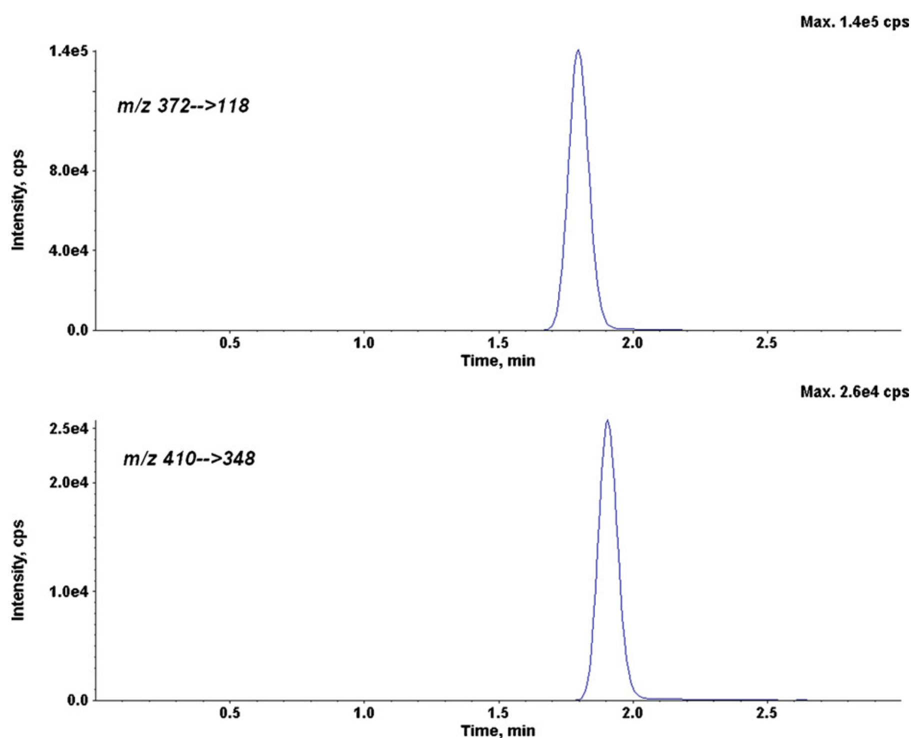

Fig. 5. MRM chromatogram showing the deferasirox concentration of 1271 ng/mL in rat blood in DBS after 2-h post dose of deferasirox 10 mg/kg.

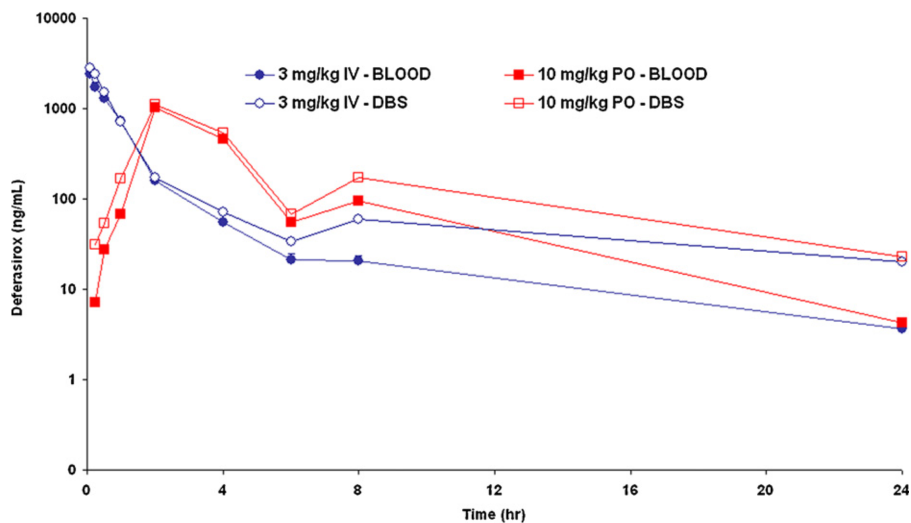

Fig. 6. Mean blood time concentration profile (ng/mL) of deferasirox in male wistar rats after *intravenous* dose of 3 mg/kg and *oral* dose of 10 mg/kg.

Table 6
Pharmacokinetic parameters of deferasirox after *oral* (10 mg/kg) intravenous (3 mg/kg) administration.

Parameter	*Oral*, liquid blood	*Oral*, DBS
C_{max}	1015 ± 2	1121 ± 213
T_{max}	2.0 ± 0.0	2.00 ± 0.0
AUC_{0-24h}	3497 ± 37	4778 ± 731
MRT last	4.3 ± 0.1	5.3 ± 0.2
F	43% ± 0	44% ± 7
Parameter	***Intravenous*, liquid blood**	***Intravenous*, DBS**
AUC_{0-24h}	2415 ± 46	3254 ± 317
$t_{1/2}$	6.8 ± 0.1	6.9 ± 4.0
CL	20.4 ± 0.3	14.6 ± 1.5
V_{dss}	2.8 ± 0.4	4.3 ± 0.5

concentrations above 1000 ng/mL were diluted and analyzed. Deferasirox concentrations were calculated from the equation $y = mx + c$, by weighted ($1/x^2$) linear regression of the calibration curve constructed from area ratios of deferasirox to IS versus nominal concentration of deferasirox. Chromatogram of the DBS sample measuring the concentration of 1271 ng/mL spiked with internal standard was shown in Fig. 5. Mean plasma concentrations versus time profiles of *intravenous* and *oral* doses in both DBS and liquid blood method were depicted in Fig. 6. The pharmacokinetic parameters calculated for *oral* and *intravenous* doses in both liquid blood and DBS method were given in Table 6.

The mean C_{max} was found to be 1015, 1121 ng/mL at 2 h after oral dose in liquid blood and DBS methods, respectively. AUC_{0-24}(ng h/mL) was found to be 3497 ± 37, 4778 ± 731 ng h/mL in liquid blood and DBS methods, respectively, after oral administration. Deferasirox mean bioavailability was found to be 43 ± 0%, 44 ± 7% in liquid blood and DBS methods. This states the applicability of the DBS method in evaluation of the deferasirox therapeutic drug monitoring and pharmacokinetic studies.

4. Conclusion

A rapid LC–MS/MS method was developed and validated in negative polarity for deferasirox by DBS technique. This method was applied to a rat pharmacokinetic study to compare the pharmacokinetic parameters obtained in liquid blood and DBS sampling. This is the first method in the literature to the best of author's knowledge for illustrating the analytical quantitation of deferasirox by dried blood spot (DBS) approach. This method was selective and sensitive enough to calculate the concentrations of deferasirox by taking 6 mm punch from the spot formed after spiking 20 μL of liquid blood. The simple sample clean up procedure with gradient elution has produced clean chromatograms with acceptable analytical results. The stability of the deferasirox was evaluated up to 4 weeks. To translate rat DBS method to patients for therapeutic drug monitoring critical parameters were evaluated with human DBS and the results were comparable to that of rat DBS. This method can be potentially employed by heel prick for new borns or finger prick to thalassemia patients where withdrawal of large volume of the blood would be harmful

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