Quantitative determination of deferiprone in human plasma by reverse phase high performance liquid chromatography and its application to pharmacokinetic study

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Abstract: Deferiprone (1, 2 dimethyl-3-hydroxypyrid-4-one) is considered to be the standard iron chelator. Pharmacokinetic studies of generic formulations are required in local condition before placed on the market. High performance liquid chromatographic (HPLC) method was used for quantification of deferiprone in human plasma using UV/VIS detector. Chromatographic separation was carried out on C_{18} column, with a mobile phase of methanol-buffer (18:82, v/v), pH 3.5, and caffeine was used as an internal standard. The calibration curve was linear over the range 0.25-10 µg/mL in human plasma (R^2 = 0.9994). After oral administration of deferiprone (500 mg) to human, the plasma concentration-time curve of deferiprone was conformed to two-compartment open model. The deferiprone plasma concentration showed a rapid absorption and average area under the plasma concentration-time curve (AUC) of deferiprone was 17.0 ± 1.23 h.µg/mL. Average absorption and elimination half-life values of deferiprone of 24 volunteers were 0.62 ± 0.12 and 2.65 ± 0.43 hours. This study confirms the rapid absorption of deferiprone in humans. AUC was similar to that previously reported but C_{max} was slightly lower than that stated in the literature.

Keywords: Deferiprone; HPLC; human; validation; pharmacokinetics.

INTRODUCTION

β-thalassemia is one of the most severe health problems in the world, where iron overload plays a imperative role (Nurchi *et al*., 2008). Iron is thought to play a role in carcinogenesis in human through the generation of oxygen free radicals (Cragg *et al*., 1998). Iron, mainly in its non-protein bound form, causes cellular damage by participating in the generation of the hydroxyl radical (Kontoghiorghes, 1995), thought to be the principal effector of oxidative DNA damage and mutagenesis (Cerutti, 1985). Treatment of thalassemia major includes regular RBC transfusions, iron chelation therapy and management of secondary complications of iron over load (Galanello and Origa, 2010).

Iron chelators are currently used to avert the long-term consequences of iron overload. Deferiprone (1, 2 dimethyl-3-hydroxypyrid-4-one) has been widely used for the treatment of iron overload diseases and has also ability for treatment of non-iron loading conditions like malaria and anemic rheumatoid arthritis. Deferiprone remains the only life-saving drug for β-thalassaemia patients in developing countries (Limenta *et al*., 2008), and is more effective in removing heart iron (Anderson *et al.,* 2002; Pepe *et al*., 2011).

Several HPLC methods for quantification of deferiprone have been published (Goddard and Kontoghiorghes, 1990; Klein *et al*., 1991; El-Jammal and Douglas, 1994; Guo *et al*., 2001). But the quantification limit of these methods was not enough to determine the low plasma concentration. Other limitations of these methods included the less sensitivity, use of costly reagents/ chemicals, long retention time and use of complicated internal standard that was not commercially accessible. Therefore it is incongruous to use these methods for the routine quantitative assay of deferiprone.

Also the yearly cost of public health care is very high in Pakistan. The high cost of prescription medicine is one of the primary reasons leading to a gap in accessibility. The availability of high quality generic drugs with good safety profiles might reduce such costs and gaps. To comply with the regulatory requirements of Pakistan, it is required to study the disposition kinetics of deferiprone in local population. Thus, the intention of this study was to develop a simple, sensitive and precise analytical method for quantification of deferiprone in biological samples, and further to assess the pharmacokinetic characteristics in human after oral administration. The purpose of the present study was to evaluate the pharmacokinetic of deferiprone in healthy Pakistani adult male volunteers.

MATERIALS AND METHODS

HPLC analysis

The plasma samples were analyzed for deferiprone quantification by high-performance liquid chromato-

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graphy (HPLC) as described by Goddard and Kontoghiorghes (1990) with some modifications. The modification included the change of internal standard (because it was easily available, cost-effective and no need to synthesize it), change in pH of buffer (for good peak resolution) and different mobile phase ratio (due to solvent front and to increase the retention time).

Reagents and chemicals

Deferiprone was gifted by Hilton Pharma (Pvt.) Ltd., Karachi, Pakistan. Caffeine was obtained from Merck (Germany). HPLC-grade acetonitrile, methanol and perchloric acid ($HClO_4$) were purchased from Panreac (Barcelona, Spain). Potassium dihydrogen phosphate (KH_2PO_4) and heptane sulfonic acid ($CH_3(CH_2)_6SO_3Na$) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Phosphoric acid was procured from Riedel-de Haen AG, (Hanover, Germany). Blank Plasma was collected from Cheniot Dialysis Center, Faisalabad, Pakistan.

Drug

The drug deferiprone commercially known Ferriprox® in the dosage form of oral tablets 500 mg each, manufacturer by Apotex Europe Ltd., UK (Batch No. GY 4120) were used in this study.

Analytical procedure

Standard stock solutions

Deferiprone and Caffeine stock standard solutions (1 mg/mL) were prepared in double distilled water. Working standards were prepared by diluting stock solutions in double distilled water. All standard solutions were stored at - 4°C and protected from light.

Instrumentation and chromatographic conditions

Shimadzu high performance liquid chromatography (HPLC) system (LC-10A) equipped with a fixed wavelength UV-Vis detector (Model SPD 10A, Shimadzu Corporation, Kyoto, Japan), column oven CTO 10A, liquid pump (LC-10AS) and acquisition software (Class LC-10) were used for the qualitative and quantitative determination of deferiprone. Chromatographic separation was achieved using C_{18} column, Discovery Supelco (25cm x 4.6mm, 5µm, cat No. 568523-U, Bellefonte, USA). Column oven temperature was maintained at 30°C. The compound were separated isocratically with a mobile phase consisting of (18:82) methanol:potassium phosphate buffer (50 m*M*) containing heptane sulfonic acid (5 m*M*) adjusted the pH = 3.5 ± 0.05 with phosphoric acid. Mobile phase was filtered by 0.45 µm membrane filters (Millipore, USA). The flow rate was 1.2 mL/min. The effluent was examined spectrophotometrically at a wavelength of 280 nm.

Sample preparation

In a Eppendorf tube (2.0 mL), 500 µL plasma, 100 µL of the internal standard (50 µg/mL), and 100 µL of acetonitrile:perchloric acid (50:50) were added. The mixture was vortexed (Scientific Industries, USA) for 3 min and centrifuged at 10,000 x *g* rpm for 10 min (Jouan, GR 412, Saint Mazaire, France). After centrifugation, the supernatant was filtered through 0.2 µm membrane filters (Micropore, Sorrento Valley Blvd., San Diego, USA) and the filtered sample 20 µL was directly injected onto the HPLC system.

Method validation

The method was validated as described in guidelines (ICH Guideline, 2005; FDA Guidelines, 2001) to get consistent results that could be accurately interpreted. The method was validated for specificity, LOD and LOQ, linearity, precision and accuracy, stability and recovery.

Specificity

Specificity was assessed by analyzing blank plasma samples (n=6) containing potentially co-prescribed drugs and other components in the same way as the standards. Selectivity of the analyte was estimated by spiking the drug in blank plasma in graded concentration and ascertaining from the retention time.

Limit of detection (LOD) and Lower limit of quantification (LLOQ)

The limit of detection (LOD) was defined as the concentration yielding a signal to noise ratio of 2:1. The lower limit of quantification (LLOQ) was the lowest concentration of standard curve having CV % < 20 % and accuracy > 80 %. The LLOQ response was greater than 5 times the response compared to blank.

Linearity

Linearity was investigated by preparing the deferiprone concentrations of 0, 0.25 0.5, 1, 2, 4, 6, 8 and 10 µg/mL in plasma. Each standard concentration was run in duplicate for consecutive three days. Calibration curves were obtained daily for three days by plotting concentration of deferiprone verses peak area ratio of deferiprone to internal standard (caffeine). Simple regression analysis was used to define calibration curves.

Precision and accuracy

The precision (Intra-day and inter-day) and accuracy of the assay was established by quality control (QCs) samples prepared in plasma. Quality control sample concentrations of deferiprone were 1.5, 5, 7.5 µg/mL and six replicate QCs at each concentration were investigated on three successive days. The intra and inter day means, standard deviations and coefficient of variation (CV %) and accuracy were calculated (FDA Guidelines, 2001)

Recovery and stability

Recovery was determined by comparing concentration from extracted QCs with those obtained from standard QCs solution prepared in mobile phase. Stability was

assessed by comparing the deferiprone in fresh extracts and in extracts stored in mobile phase for 24 h and 3 days at room temperature.

Pharmacokinetics

The plasma concentration of deferiprone versus time plot was used to calculate pharmacokinetic parameters using the PC-Computer Program, APO (MWPHARM version 3.02, a MEDIWARE product, Holland). Concentration of deferiprone was measured by HPLC method in the plasma samples from the blood collected at time intervals 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5. 3.0, 4.0, 5.0, 6.0, 8.0, 10, 12 and 24 hours after oral administration of 500 mg tablet in 24 healthy male subjects. In all 24 experiments none of the samples collected at 24 hours revealed any drug concentration. Therefore, the data has been calculated on the basis of last detectable concentration time.

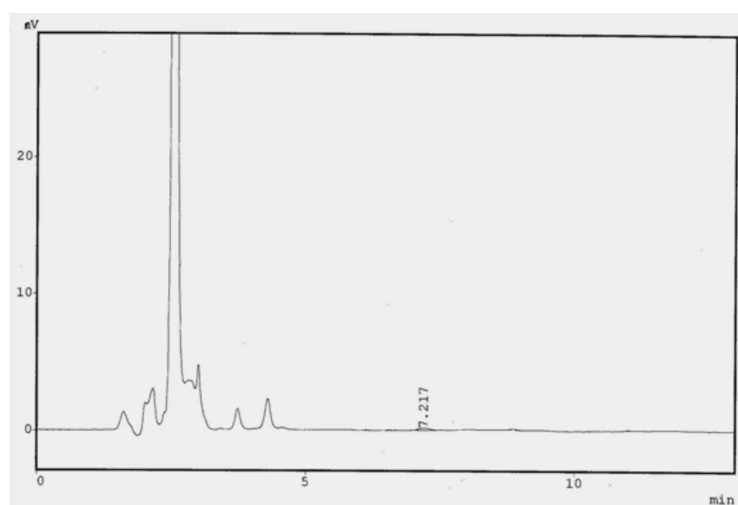
STATISTICAL ANALYSIS

For computation and analysis of the drugs plasma concentration versus time data and the graphics, Microsoft Excel 3.0 was used. All data are reported as the mean ± SE (Steel *et al*., 1997).

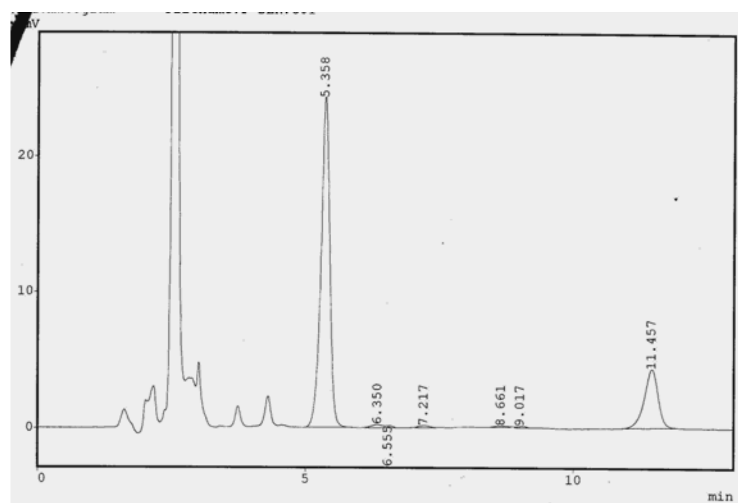
RESULTS

Specificity

Specificity of the deferiprone and internal standard was estimated by spiking the drug and internal standard in blank plasma and ascertaining from the retention time. There was no interference around the retention time of deferiprone and caffeine. Representative chromatograms of blank plasma (a) plasma sample (b) containing deferiprone and caffeine (IS) are shown in fig. 1. The blank chromatogram confirmed no interference from the



(a) HPLC chromatogram of blank human plasma.



(b) HPLC chromatogram of human plasma containing deferiprone and caffeine.

Fig. 1: HPLC chromatograms of (a) human blank plasma (b) plasma sample containing deferiprone (RT= 5.3) and caffeine (RT=11.4).

endogenous substances. The retention times of deferiprone and the internal standard were 5.3 min and 11.4 min, respectively.

Limits of detection and quantification

Lower limits of detection (LLOD) and quantification (LLOQ) were projected from the signal-to-noise ratio. The LOD obtained from plasma samples was 0.1 and 0.25 µg/mL, respectively.

Linearity

The linearity was determined by linear regression analysis. The calibration curves were found linear in the range between 0.25 to 10 µg/mL in plasma and was described by the equation (y = 0.1616x + 0.0178) and regression coefficient of $R^2 = 0.9994$.

Precision/Reproducibility and accuracy

Reproducibility (Intra-day precision and inter-day precision) and accuracy was determined by repeat assay (n = 6) of three quality controls. Precision was expressed as the percentage coefficient of variation (CV %). The accuracy of this method was found to be between 97 and 103%. The intra-day and inter-day precision in plasma are depicted in table 1.

Recovery

Recovery was determined for low, medium and high quality control concentrations in plasma. The average recovery of the assay was more than 90%.

Stability

All three quality controls (Low, medium and high quality controls) in plasma were tested for stability. There was no

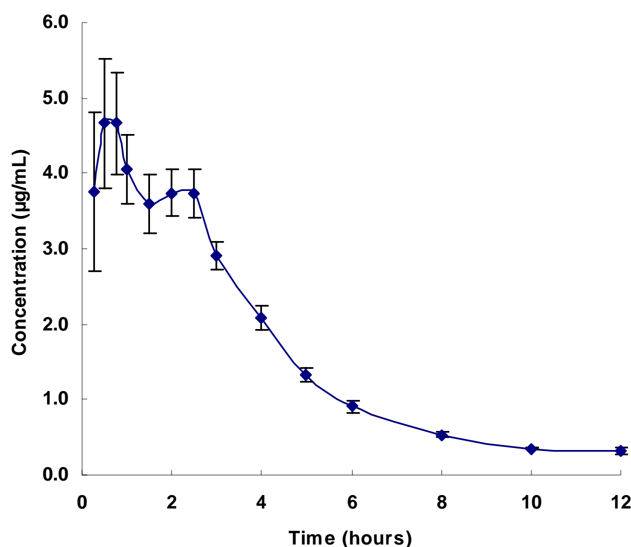


Fig. 2: Mean ± SE plasma concentration (µg/mL) of deferiprone on ordinary scale after oral dose of 500 mg tablet to healthy male subjects (n=24).

Table 1: Intra- and inter-assay precision and accuracy for deferiprone in plasma

Reproducibility	Concentration (µg/mL)		CV (%)	Recovery (%)
	Added	Found (mean ± SD)		
Intra-assay Reproducibility[a]				
Quality controls	1.5	1.42 ± 0.04	2.31	97.52
	5	5.15 ± 0.09	1.65	102.9
	7.5	7.42 ± 0.02	0.71	98.42
Inter-assay Reproducibility[b]				
Quality controls	1.5	1.40 ± 0.03	2.18	97.61
	5	4.97 ± 0.05	0.81	99.42
	7.5	7.27 ± 0.09	1.52	98.57

[a] QC samples (n= 6) per concentration for three days. [b] QC samples (n = 18) per day for three days.

variability in stability of stock standard solution after 2 months at 4°C. The processed QCs samples were stable at + 4°C for at least 72 h. No significant degradation of deferiprone was observed.

Pharmacokinetics

The validated assay was used to determine the blood concentration profile of deferiprone in human after a single oral dose of 500 mg. Absorption of drug is rapid and maximum concentration of drug 4.67 ± 0.86 µg/mL in blood was achieved at 0.5 hour which rapidly declined and thereafter gradually decreased to 0.32 ± 0.04 µg/mL at 12 hour as shown in fig. 2. The pharmacokinetic parameters of deferiprone after oral dose of 500 mg tablet were determined in 24 healthy male subjects. Average pharmacokinetic parameters of each volunteer are presented in table 2.

Table 2: Pharmacokinetic parameters of deferiprone after oral dose of 500 mg tablet to human male subject.

Parameters	Average	± SE
Area Under the Curve (AUC) (h.µg/mL)	17.0	1.23
AUC polyexponential (t= 12)	16.0	1.26
AUC trapezoidal rule (t= 12)	16.2	1.30
Clearance (CL) (L/h)	32.7	2.78
Volume of distribution comp.1 (L)	50.7	6.34
Volume of distr. steady state (L)	90.3	19.2
Volume of distribution (L)	129	32.0
Half-life phase 1 (h)	0.62	0.12
Half-life phase 2 (h)	2.65	0.43
Rate constant k10 (L/h)	1.95	1.34
Rate constant k12 (L/h)	1.66	1.00
Rate constant k21 (L/h)	1.21	0.47
Mean Residence Time (MRT) (h)	4.20	0.40
Absorption rate constant (ka) (L/h)	2.84	0.71
Absorption half-life (h)	0.57	0.10
Lag-time (h)	0.64	0.14
Time to peak t_{max} (h)	1.50	0.19
Peak concentration C_{max} (µg/L)	5.03	0.52

Following oral administration of 500 mg deferiprone tablet, the maximum concentration (C_{max}) 5.03 ± 0.52 µg/mL was achieved at the peak concentration time (t_{max}) of 1.50 ± 0.19 hour. Average area under the plasma concentration-time curve (AUC) of deferiprone was 17.0 ± 1.23 h.µg/mL. Total body clearance, volume of distribution and elimination rate constant of deferiprone after administration of deferiprone were 32.7 ± 2.78 L/h, 129 ± 32.0 L and 1.21 ± 0.47 L/h, respectively. Average absorption and elimination half-life values of deferiprone of 24 volunteers were 0.62 ± 0.12 hours and 2.65 ± 0.43 hours.

DISCUSSION

The statistical assessment of the anticipated method exposed its good linearity, precision and its other validation parameters which led us to the conclusion that it could be used for the rapid and reliable determination of deferiprone in human fluids.

The deferiprone plasma concentration showed a rapid absorption until 0.5 hours and then declined gradually and could not be detected in the plasma samples collected at 24 hours. The maximum concentration (C_{max}) of deferiprone in twenty volunteers following oral administration of 500 mg tablet (8.68 mg/kg) observed in this study was 5.03 ± 0.52 µg/mL comparable to the C_{max} (15.15 ± 3.47 µg/mL) reported by Thuma *et al*. (1998) by giving nearly three times higher dose (25 mg/kg). The values of C_{max} studied by Limenta *et al*. (2008) by giving nearly three times higher dose 25 mg/kg in male and female were higher than the present study. Other studies showed that the C_{max} of deferiprone was also higher than the present study (Al-Refaie *et al*., 1995; Kontoghiorghes *et al*., 1990). Average time (t_{max}) at which the maximum concentration of deferiprone achieved was 1.50 ± 0.19 hours being comparable to the literature (Thuma *et al*., 1998; Kontoghiorghes *et al*., 1990; Limenta *et al*., 2007; Al-Refaie *et al*., 1995; Matsui *et al*., 1991).

Average AUC of deferiprone in this study was 17.0 ± 1.23 h.µg/mL. By giving nearly three times higher dose 25 mg/Kg orally AUC 53.63 h.µg/mL in male volunteers reported by Limenta *et al*. (2007) was almost comparable. However, the value of AUC in female volunteers 74.98 h.µg/mL reported by Limenta *et al*. (2007) was higher than the present study. According to Thuma *et al*. (1998) a mean 8 h AUC of 43.04 ± 8.34 h.µg/mL with 25 mg/kg dose was also nearly comparable to the present study. Volume of distribution 129 ± 32.0 L (2.28 ± 0.61 L/kg) of deferiprone was higher than the literature (Thuma *et al*., 1998; Limenta *et al*., 2007). Elimination half-life of deferiprone was (2.65 ± 0.43 hours) greater than the Thuma *et al*. (1998). Elimination half-life of deferiprone in thalassemia patients was significantly longer than that in the healthy volunteers (Stobie *et al*., 1993). Total body clearance of deferiprone in the present study was 32.7 ± 2.78 L/h comparable to the clearance studied by Thuma *et al*. (1998) and higher than the clearance studied by Limenta *et al*. (2007) indicating the role of elimination processes in the body.

CONCLUSION

A specific, sensitive and simple HPLC method for the quantification of deferiprone in human plasma was developed, which was applied to pharmacokinetic exploration in human. Application of the method to pharmacokinetic studies has been successful. The sensitivity of the assay is ample for application in the

study of bioequivalence and clinical pharmacokinetic of deferiprone. It would also be useful for the determination of deferiprone in β-thalassemia patients. So, the assay is suitable for regular use in clinical pharmacology and therapeutic drug monitoring.

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