ejpmr, 2021,8(9), 514-521

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

RP-HPLC METHOD DEVELOPMENT & VALIDATION FOR THE ESTIMATION OF DEFERASIROX IN BULK & DOSAGE FORM

***Mohini Khare, Jyoti Sonawane, Shaikh Siddiqua, Abhay Kulkarni, Sambhaji Shelar and Sonali Suryawanshi**

Departement of Quality Assurance S.N.D College of Pharmacy, Matoshri Institute of Pharmacy, Dhanore, Yeola 423401.

***Corresponding Author: Jyoti Sonawane**

Departement of Quality Assurance S.N.D College of Pharmacy, Matoshri Institute of Pharmacy, Dhanore, Yeola 423401.

Article Received on 06/07/2021 **Article Revised on 26/07/2021** **Article Accepted on 16/08/2021**

ABSTRACT

Objective: The present paper describes a simple, accurate, and precise reversed-phase high-performance liquid chromatography (HPLC) method for rapid and simultaneous quantification of Deferasirox in bulk and pharmaceutical dosage form.

Methods: The chromatographic separation was achieved on C18 (250mmx4.6mm, 5µ id). Mobile phase contained a mixture of methanol: water in the ratio of 50:50 v/v, flow rate 1.0ml/min and ultraviolet detection at 247 nm.

Results: The retention time of Deferasirox was 3.48min. The proposed method shows a good linearity in the concentration range of 10–60 µg/ml for Deferasirox under optimized conditions. Precision and recovery study results are in between 95.54 to102.28.

Conclusion: This method is validated for different analytical performance parameters like linearity. Precision, accuracy, limit of detection, limit of quantification, robustness, and pharmacokinetic study were determined according to the International Conference of Harmonization (ICH) Q2B guidelines. All the parameters of validation were found in the acceptance range of ICH guidelines.

KEYWORDS: Deferasirox, Methanol, Water, Method Validation.

INTRODUCTION

Deferasirox is an oral iron chelator. Its main use is to reduce chronic iron overload in patients who are receiving longterm blood transfusions for conditions such as beta-thalassemia and other chronic anaemias. Anaemia is related to less haemoglobin count in which iron is present in ferrous cation (Fe2+). It is the first oral medication approved in the USA for this purpose. The half-life of deferasirox is between 8 and 16 hours allowing once a day dosing. Two molecules of Deferasirox are capable of binding to 1 atom of iron which is subsequently eliminated by fecal excretion. The drug has two phenolic groups (-OH) and one carboxylic group (-COOH). Ferrous ion has capability to act on phenolic group, so it forms chelate (organometallic) component in-vivo. Log P of Deferasirox is 6.43 which is highly lipid soluble.

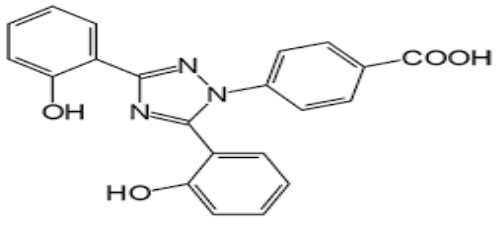

Fig. 1: chemical structure of Deferasirox.

Deferasirox is an oral iron chelator. Its main use is to reduce chronic iron overload in patients who are receiving long term blood transfusions for conditions such as beta-thalassemia and other chronic anemias. It is the first oral medication approved in the USA for this purpose.

MATERIAL AND METHODS

Apparatus

HPLC Water 486 Tunable Absorbance Detector Model No HPLC 610 Series Detector UV-visible Software Conquer Column C_{18} (250mm × 4.6mm), 5µm id. Filtered using 0.45µ membrane filter Nylon 0.45µ and ultra-sonicated Model: WUC 4L Capacity.

Chemicals and solvents

Deferasirox drug were perches from MSN Laboratory Ltd Hyderabad the marketed formulation of Deferasirox OAB-F (Alembic Pharmaceutical LTD) were perches from local market HPLC grade water, methanol were purchased from E.MerckLtd., Mumbai, India.

Chromatographic condition

Column: Grace C18 (4.6 ID×250 mm; 5µm) column, Mobile phase: Methanol: water (50:50) at pH 3.0 adjusted with OPA Flow Rate: 1.0 ml/min, Detection

Wavelength: 247 nm, Retetion time: 3.48 min, Injection volume: 10.0 µl.

Detection Wavelength by UV Spectroscopy

The absorption maximum (λ_{max}) of Deferasirox was determined in the mobile phase of Methanol and Water in the ratio of 50:50. The 10µg/ml standard working solution of Deferasirox was selected for this experiment and the same was prepared as per procedure laid down in chapter 6. The UV spectrum obtained was as shown in **Figure 2**.

The absorbances were recorded for Deferasirox from the UV spectrum obtained and same were as demonstrated. From the results obtained Deferasirox explained absorbance values of 0.006, 0.573 and 1.394 at 374, 247 and 202nm respectively. The maximum absorbance was found at 202nm. But as the mobile phase composition consists of methanol as one of the component having solvent cutoff of UV up to 206nm. Hence, 247nm wavelength was used as detection wavelength for RP-HPLC method development of Deferasirox.

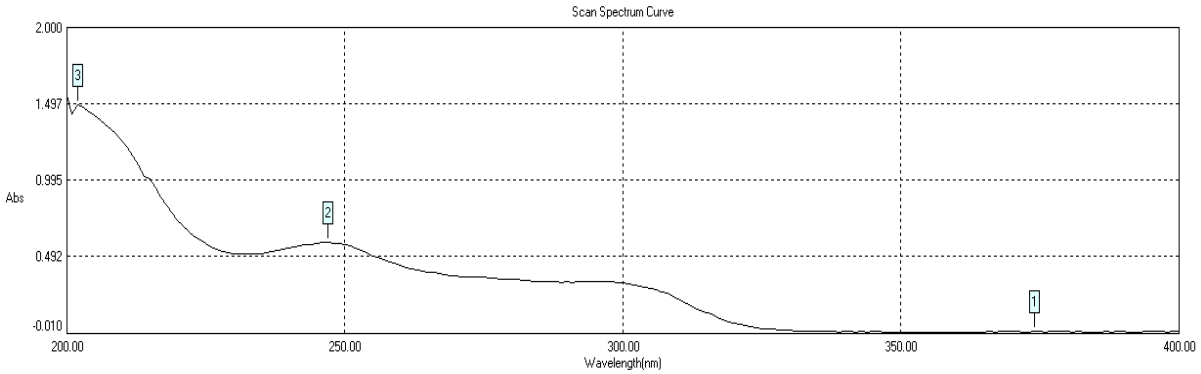

Figure 2: UV spectrum of Deferasirox.

HPLC method development

Preparation of standard stock solutions of Deferasirox

10 mg of Deferasirox was weighed accurately and transferred to 10ml volumetric flask containing a mobile phase in the ratio of 50:50v/v. The volume was made up to the mark using same mixture of solvent to obtain resulting solution of 1000µg/ml. Then 1ml was this solution was pipette out and diluted up to the 10ml which will give resultant solution of 100µg/ml.

Preparation of working solution of Deferasirox

Further, again 1ml of stock solution (100ppm) was diluted to 10ml to obtain working solution of 10ppm. This solution was filtered through 0.45µ membrane filter and sonicated for three cycles each of 10min.This solution was used to for method development using HPLC.

Selection of mobile phase

The mobile phase selection was achieved by trial and error. For this purpose few trial runs were carried out with chromatographic conditions. The first trial run was conducted with mobile phase composition of Methanol and Water in the ratio of 70:30% v/v. The flow rate was maintained at 1.0mL/min and the detection was achieved at 260 nm using UV detector. The chromatogram obtained. The chromatogram did not show any peak for Deferasirox. In addition the base line was also found to be unstable.

Trial 2 was carried out by mobile phase consisting of Methanol and water in the ratio of 70:30% v/v. Also, the pH of the aqueous phase was set at Methanol: water 70:30 (pH is adjusted 3.0 using phosphoric acid. The chromatogram observed for trial 2 was as illustrated. From the chromatogram obtained, it was seen that Deferasirox. The retention was observed as 2.12min. But, the peak shape was observed to be unacceptable. Therefore, this chromatogram was also rejected to further optimize the mobile phase composition and other parameters.

Table 1: Chromatographic conditions for trial runs.

Sr. no.	Mobile Phase	Flow rate	Wavelength
1	Methanol: Water (70:30)	1ml/min	260nm
2	Methanol: Water (80:20) at pH 3.0 adjusted with OPA	1ml/min	260nm

System suitability testing

The test was performed by using 10µg/ml standard solution of Deferasirox. The chromatograms resulting from six repeated measurements were recorded and integrated to determine chromatographic parameters such as peak area of Deferasirox, theoretical plates and tailing factor were calculated. The chromatographic conditions as shown in **Table 2** obtained the preponderance wonderful symmetry factor, peak shape and theoretical plates. Consequently, the setting cited as in **Table 2** were

selected for supplementary research for evaluation of Deferasirox.

The best resolution and peak shape, without needless tailing, were obtained by use of chromatographic conditions. The most excellent resolution with sensible retention time of 3.48min was obtained with mobile phase containing Methanol: Water (50:50). A representative chromatogram was shown in **Figure 2.**

Table 2: Table for chromatographic conditions set for the experiment.

Chromatographic Conditions	
HPLC System	Water 486 Tunable Absorbance detecter
Software	Conquer
Detector	UV Visible
Column	Phenomenex C18 (250mm×4.6mm), 5µm id
Mobile phase	Methanol 50: Water 50 at pH 3 adjusted with ortho phosphoric acid, Isocratic
Detection Wavelength	247 nm
Flow rate	1.0 ml/min
Temperature	Ambient
Sample size	10µl
Run Time	7 min

HPLC method validation

Linearity and Range

Volume of standard stock solution (100µg/ml) of Deferasirox equivalent to 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0ml was pipette out using micro-pipette and transferred to six different 10ml volumetric flasks. The aliquot of each volumetric flask was diluted up to 10ml using the mobile phase, Methanol: Water (50:50) to attain the consequent solutions of 10, 20, 30, 40, 50, and 60µg/mlin that order. Each of this solution was injected to the given chromatographic conditions and chromatograms were recorded. The same function was persistent three times for all standard solutions prepared to study linearity.

Precision: Precision of the method was studied at three points across the range for Deferasirox. This was performed by setting three quality control standards for Deferasirox in the given series of 15,35 50µg/ml. Precision experiment was performed to study two dissimilar parameters viz. repeatability and intermediate precision.

Accuracy: % Accuracy was determined from the particulars obtained for precision study. At this point it was determined from the observations of mean peak area obtained in the case of three QC standards of Deferasirox defined for precision study.

Robustness: In Robustness, the system parameters like organic concentration of the mobile phase (Methanol) and flow rate were varied as per **Table 10 & 11** The 10µg/ml concentration standard solution of Deferasirox was selected for this study and it was kept constant throughout all system parameters varied. The preferred concentration (10µg/ml) was injected to given chromatographic conditions three times at each level of change and chromatograms recorded. From the chromatograms acquired mean peak area was determined in all measurements made.

% Recovery

% Recovery is determination % purity of Deferasirox in finished product (tablet dosage form). This study was proposed to explore the applicability of the method for determination of Deferasirox in finished product formulation (herein tablet dosage form). This would emphasize that the method can be used for routine analysis of drug substance in drug product during its life cycle. The percent recovery was determined by calculating recoveries of Deferasirox at three different levels. Deferasirox % recovery was determined using spike method. This method consists of addition of known amount of sample to the standard solution. Therefore, during this experiment known amount of standard solutions (three) of Deferasirox (10µg/ml) were prepared in three 10ml volumetric flasks. Sample solutions (viz. 8, 12 and 12µg/ml) of Deferasirox were spiked to above three standard solutions to finally obtain concentrations of test solutions 18, 20, 22µg/ml for 80%, 100%, 120% levels respectively. Each of this solution was injected in triplicate and the mean peak area in each case was determined. The mean area of standard solution (10µg/ml) was subtracted from later to get the actual peak area corresponding to the sample solution at each level.

The equivalent mean measured concentration for each level was determined from equation of regression line. % recovery was estimated by the formulas as mentioned. The recovered amount (in terms of % assay) was observed in the range of 99.74 to 102.28 the results obtained were as illustrated in **Table 8**. From the results obtained for recovered amount of Deferasirox in terms of percent assay was within limit as per standard prescribed for Deferasirox. The representative chromatogram recorded for this study was as revealed in **Figure 7**.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of thepeak areas using the formulae LOD

= 3.3 σ/s and LOQ = 10σ/s. The results were given in Table.

RESULT AND DISCUSSION

The test was performed by using 10µg/ml standard solution of Deferasirox. The chromatograms resulting from six repeated measurements were recorded and integrated to determine chromatographic parameters such as peak area of Deferasirox, theoretical plates and tailing factor were calculated.

Table 3: Observation table for system suitability testing of Deferosirox and their comparison with the standards.

Sr. No.	Parameter	Mean observations	SD	%RSD	Acceptance criteria	Inference
1	Peak Area	136775	920.52	0.67	< 2	Pass
2	Retention time	3.48	0.01	0.15	< 0.5	Pass
3	Number of Theoretical plates*	3986	--	--	> 2000	Pass
4	Tailing factor*	1.06	--	--	< 2	Pass

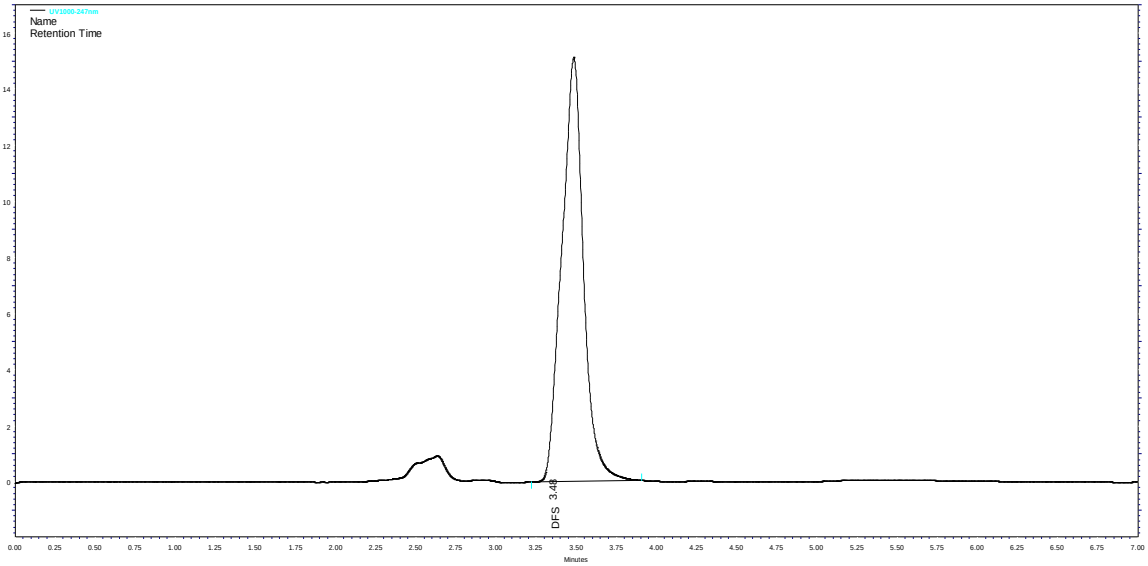

Figure 3: Chromatogram of Deferasirox obtained in system suitability testing at 10ppm conc.

Linearity and range

Table 4: Observation Table for linearity experiment.

Sr. No.	Conc. of Deferasirox (µg/ml)	Mean Peak Area*
1	10	140435
2	20	285637
3	30	420732
4	40	579922
5	50	708113
6	60	848361

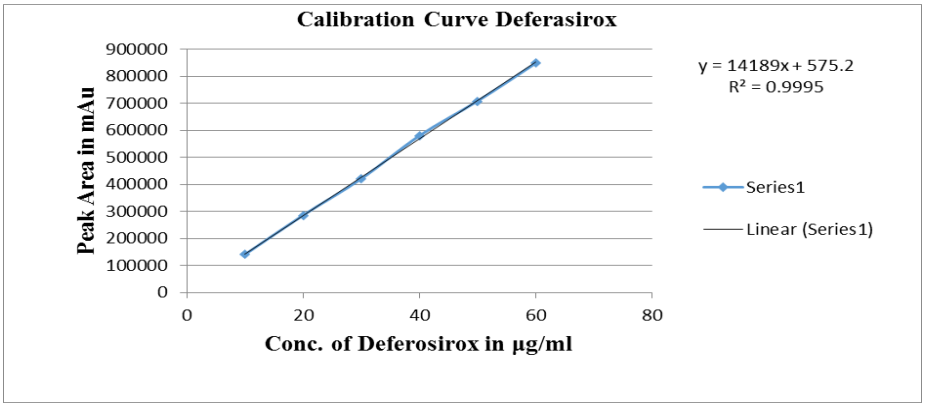

Figure 4: Calibration curve of Deferasirox.

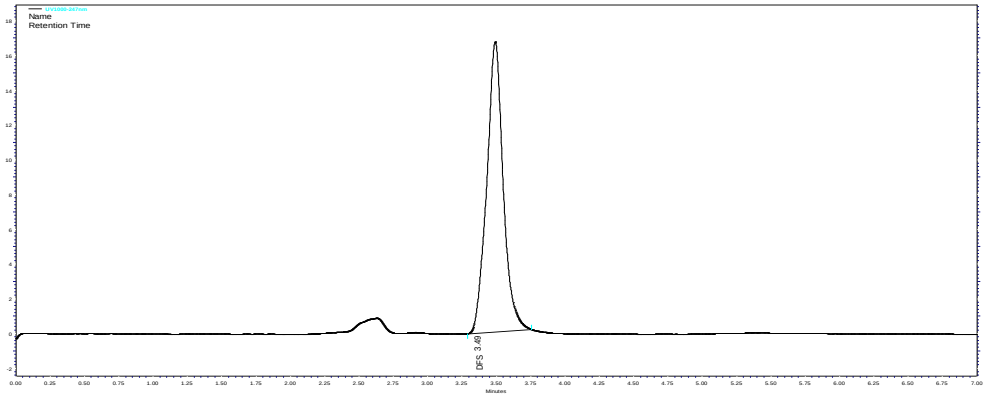

Figure 5: Representative chromatogram in linearity study observed at 10ppm

Table 5: Peak Table for Linearity experiment at 10ppm.

Inj. No.	Name	Retention Time(min)	Area	No. of Theoretical Plates	Tailing Factor
1.	DIFIRASEROX	3.49	139434	4085	1.11

Precision

Table 6: Results obtained in precision experiment of Deferasirox

Conc. (µg/ml)	Intra-day precision			Inter-day precision		
	Mean area ± SD	% RSD	Inference	Mean area ± SD	% RSD	Inference
15	204728.00 ± 924.07	0.45	Pass	203918.33 ± 1812.69	0.89	Pass
35	505061.33 ± 1444.86	0.29	Pass	508622.67 ± 4706.44	0.93	Pass
55	776088.00 ± 1813.48	0.23	Pass	777182.67 ± 5151.01	0.66	Pass

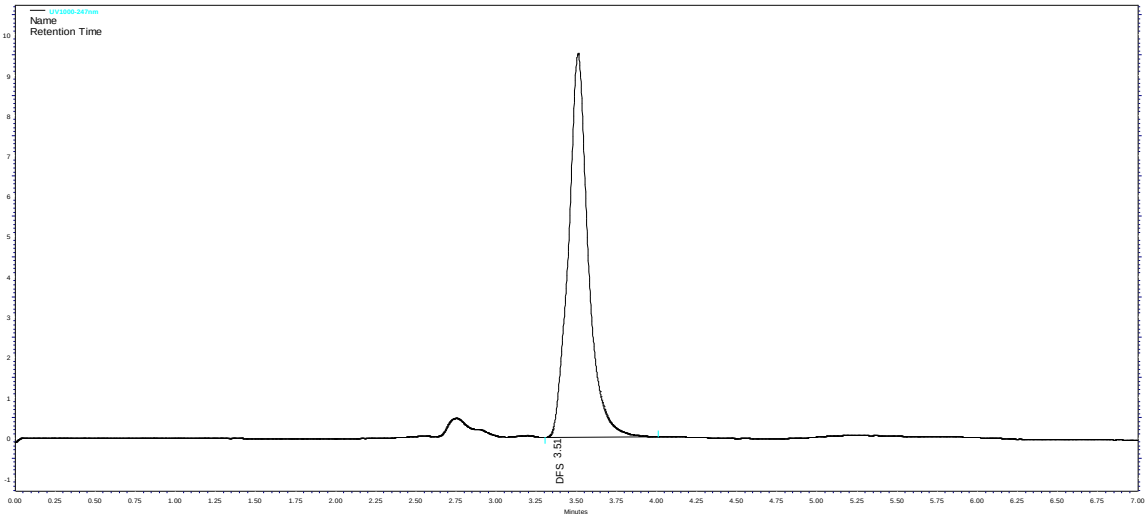

Figure 6: Chromatogram obtained for DGR at 15ppm Intra-day precision.

Table 7: Peak Table for Intra-day precision experiment at 15ppm.

Inj. No.	Name	Retention Time (min)	Area	No. of Theoretical Plates	Tailing Factor
1.	DIFIRASEROX	3.51	203484	4750	1.10

% Accuracy

Table 8: Observation table for percent accuracy results obtained from the precision experiment data.

Sr. No.	Conc. (µg/ml)	Mean Peak Area*	Mean Measured Conc. (µg/ml)	% Accuracy (w/w)	Inference
1	15	203918.33	14.33	95.54	Passed
2	35	508622.67	35.80	102.28	Passed
3	55	777182.67	54.73	99.51	Passed

Robustness

Table 9: Observation table for percent accuracy results obtained from the precision experiment data.

Sr. No.	Conc. (µg/ml)	Mean Peak Area*	Mean Measured Conc. (µg/ml)	% Accuracy (w/w)	Inference
1	15	203918.33	14.33	95.54	Passed
2	35	508622.67	35.80	102.28	Passed
3	55	777182.67	54.73	99.51	Passed

Table 10: Observation Table for robustness experiment with deliberate variation in methanol concentration.

Methanol Concentration (%)	Standard Conc. (µg/ml)	Mean peak area*	Mean measured conc. (µg/ml)	% Assay (%w/w)	Inference
50	10	140435	9.86	98.57	Passed
52	10	142643	10.01	100.13	Passed
48	10	138643	9.73	97.31	Passed

% Recovery

Table 11: Observations table for percent recovery experiment of Deferasirox in tablet dosage form.

% Recovery Level	Conc. of standard spiked (µg/ml)	Conc. of sample (µg/ml)	Mean peak Area of sample conc.*	Amount recovered# (µg/ml)	% Recovery (%w/w)	Inference
80	10	8	117396	8.23	103.07	Passed
100	10	10	145493	10.21	102.19	Passed
120	10	12	170396	11.97	99.74	Passed

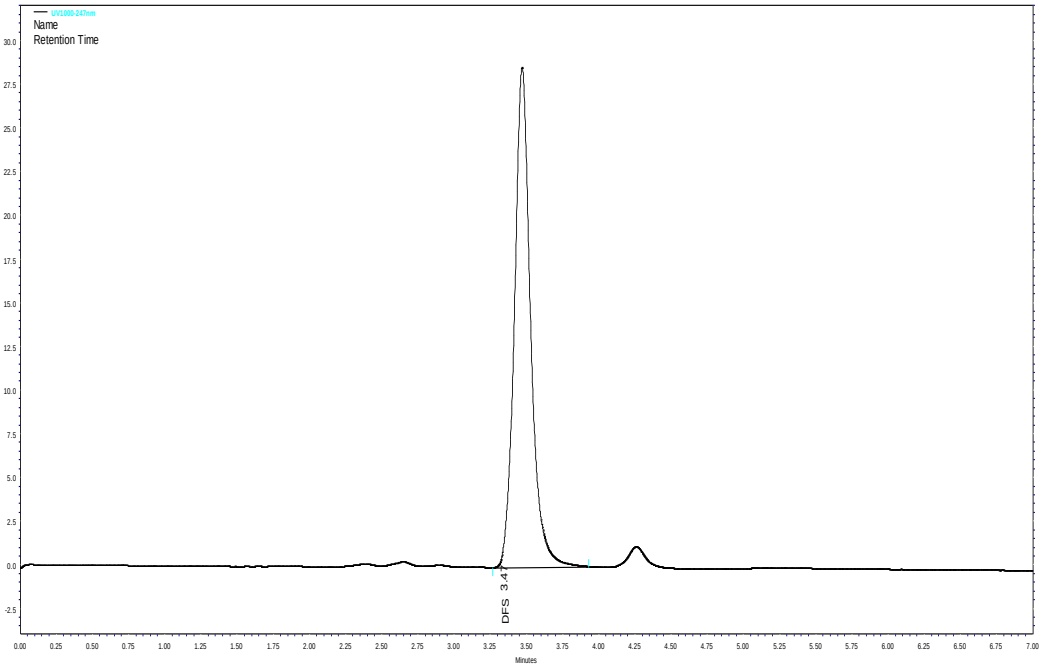

Figure 7: Chromatogram obtained for Deferasirox at 100% recovery level.

LOD and LOQ

Table 12: Results obtained for LOD and LOQ.

Standard Drug Solution	LOD (µg/ml)	LOQ (µg/ml)
Deferasirox	1.61	4.87

SUMMARY AND CONCLUSION

The predictable investigate job was projected to expand an uncomplicated, susceptible, precise and true RP-HPLC method for the examination of Deferasirox as API. Also, method was planned to explore its applicability for analysis of Deferasirox in marketed formulation. Amalgamation of Methanol and water in the ratio of 50:50 v/v, at pH 3 of aqueous phase on a C18 stationary phase was found to be the practically an appropriate. Considering the chromatographic peaks were better defined and almost flattering from tailing. The retention time obtained for Deferasirox was 3.48min

with C18 stationary phase (Column 250mm x 4.6mm, 5µm particle size).

System suitability experiment was proposed to study an efficiency of the system and to make it appropriate for supplementary study with six repeated measurements of standard solution of the Deferasirox. The calculated statistical parameters were within the acceptance criteria as per ICH Q2R1 guidelines for Deferasirox. The equivalent peak areas of Deferasirox were reproducible as indicated by % RSD within limit (<2).

Linearity of the method was proved by observed regression coefficient near to unity (r^2=0.9995) between the standard concentration of Deferasirox and the respective peak areas. The regression curve was constructed by linear regression fitting and its regression equation was y = 14189x + 575.2 (Where, Y gives peak area and X is the concentration of the Deferasirox).

Precision of the RP-HPLC method was established by performing experiment of repeatability and intermediate precision. When Deferasirox standard solutions containing 15, 35 and 55µg/ml of Deferasirox were measured by this developed RP-HPLC method for finding out intra-day and inter-day precision, % RSD values obtained by experiment were less than 2 for all above standards tested.

Robustness of the method was assessed for the parameters like variation in methanol concentration of the mobile phase and flow rate variation. The effect of purposeful disparity in method parameter up to 2% in methanol concentration and 0.1ml/min flow rate was not affected the quantification of Deferasirox. Therefore, it was proved that the developed RP-HPLC could attain the reproducible results as per compendial standards even after above variations.

Applicability of the method was discovered in tablet dosage form. Recovery values observed in terms of percent assay were in the range of 99.74 to 103.07 %w/w. The results obtained were in agreement with the compendial limits prescribed for Deferasirox tablets. In addition, nonappearance of supplementary peaks indicated non-interference of usual excipients used in the tablet dosage form. The drug content in tablet dosage form was successfully enumerated by the presented RP-HPLC method of Deferasirox.

Consequently, it was concluded that this RP-HPLC method was proved to be sensitive, simple, precise and reproducible for the quantification of Deferasirox as API. Also, it was sensitive for determination of Deferasirox in tablet dosage form even in the presence of excipients.

Eventually, we accomplished that the each single one exact rationale of this study was accomplished and acknowledged.

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