Liquid chromatographic analysis for the determination of deferasirox in pharmaceutical formulations and spiked plasma samples using dansyl chloride reagent

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Abstract: In the current study, a new sensitive and precise method based on HPLC and derivatization with dansyl chloride was developed for quantification of deferasirox (DEF) in pharmaceutical dosage forms and spiked plasma samples. Separation procedure in the chromatographic system was performed using a mobile phase consisting of the mixture of methanol and acetic acid solution (0.5 M, pH adjusted to 7.0 with NaOH) with a ratio of 70:30 v/v at flow rate of 1.0 mL per minute under isocratic elution on a C18 column (150 mm × 4.6 mm, 5 μm I.D.). The excitation wavelength was selected as 340 nm and emission wavelength was selected as 480 nm for the measurement of the analyte signal. The retention time for DEF is approximately 3.5 min. ICH Guidelines were taken into account for method validation. For the deferasirox concentration range of 20–2000 ng/mL, the proposed analytical method exhibited a linear relationship between the drug concentration and measured fluorescence with a correlation coefficient of 0.9994.The currently developed method can be implemented efficiently for the quantification of DEF in pharmaceutical dosage forms and spiked plasma samples.

1. Introduction

Deferasirox, chemically named as 4-[3,5-bis (2-hydroxyphenyl)-1H-1,2,4triazol-1-yl]-benzoic acid (DEF), is an iron chelator agent used for prevention and treatment of iron overload in patients having thalassemia and other chronic anemic conditions resulting from long term blood transfusion [1]. DEF is considered as an effective chelation therapy agent for the management of chronic iron accumulation in patients with chronic anemias in need of blood transfusions [2, 3]. DEF achieves maximum plasma concentration (C_{max}) in 1-4 hours (T_{max}) through a fast absorption phase and wide distribution in biological fluids and tissues. Following single-dose administration and at also at steady-state conditions, the primary pharmacokinetic parameters of DEF, area under the concentration curve

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over 24 h (AUC_{0-24h}) and maximum plasma concentration (C_{max}) proportionally increase with the administered dose [4].

Only several HPLC methods are defined in the literature [5–10]. Literature search did not reveal any study using 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl chloride). Weberl was the first scientist who introduced dansyl chloride as fluorescent reagent to have fluorescent conjugates of albumin [11]. Dansyl chloride was used as a fluorescent derivatization agent in determination of several pharmaceutical active ingredients having primary amines, secondary amines, imidazoles and phenols in their chemical structure [12-16]. In the current study, Dansyl chloride was selected as an efficient fluorescent labeling reagent for quantification of DEF in pharmaceutical dosage forms and spiked plasma samples. The proposed method based on derivatization with DNS-CI, has the advantages of simple and faster sample preparation, sensitivity and selectivity resulting from fluorescence spectra, use of widely available equipment. As per the results, the currently developed analytical HPLC method is suitable for quantification of DEF in pharmaceutical dosage forms and spiked plasma samples.

2. Experimental

2.1. Chemicals and Reagents

DEF was supplied from Glenmark Pharmaceuticals Ltd. (Gujarat, India) and its pharmaceutical preparation (Ferout *Film Tablet*®) containing 90 mg of DEF per film tablet was obtained from the local drug store. All substances and reagents were analytical grade reagents. Dansyl chloride was purchased from Sigma-Aldrich (Germany). All chemicals and reagents were of analytical-reagent grade. HPLC grade ultra-pure water was prepared by an Elga Purelab Option Q (VWS Deutschland) water purification system.

2.2. Instrumentation and Chromatographic Conditions

The chromatographic separations were carried out by using a Shimadzu LC 20A (Kyoto, Japan) liquid chromatography system, its model is LC 20 AT solvent delivery system, a SIL-20AC autosampler system and the model of the oven is CTO 20A. RF 10 AXL fluorescence detector was set an excitation wavelength of 340 nm and an emission wavelength of 480 nm. The separation was performed on a Thermo Hypersil Gold C18 column (150 mm × 4.6 mm, 5 μm I.D.) with a guard column (4 mm × 3 mm, 5 μm I.D.) including with the same material. Isocratic elution was applied with methanol and acetic acid solution (0.5 M adjusted to pH 7.0 using NaOH) (70:30, v/v) by flow rate of 1.0 mL/min. The temperature of the column was stabilized at 30 °C.

2.3. Preparation of Stock Solutions

The concentration of DEF stock solution in methanol was prepared as 1 mg/mL. DEF standard solutions were prepared through appropriate dilution of this stock solution. The definitive concentrations of the analyte were 20–2000 ng/mL. The stock solutions were kept at 4 °C and were stable for 30 days.

Solution of dansyl chloride was freshly prepared at 2.0 mg/mL (0.02%) in acetone. The sodium bicarbonate (0.5 M) solution was prepared in water and pH was adjusted to 10 with 0.5 M sodium hydroxide using a pH meter. This solution was kept in the refrigerator and used for about 1 week.

2.4. Preparation of Sample Solution

2.4.1. General Procedure

The aliquots were taken from DEF standard solution corresponding to the concentration interval of 20–2000 ng/mL were transferred into a series of test tubes and a 500 μL of bicarbonate solution at pH 10 and 400 μL of dansyl chloride solution were added to each test tube. The reaction

solution mixture was kept at 40°C for 10 min. They were left to cool at ambient temperature in air and then, the formed drug fluorescent derivative was extracted using 2 mL of dichloromethane during 1 min. The extract was subject to evaporation under nitrogen gas at 40°C. The remaining residue was dissolved in 0.5 mL of the mobile phase. A 20 µL aliquots of the resulting solution are used for quantification by HPLC.

2.4.2. Determination of DEF in Pharmaceutical Formulation

Tablet powder amount corresponding to the equivalent to 10 mg of DEF, was precisely weighed and transferred into a 100 mL calibrated flask. Extraction was performed with addition of approximately 100 mL of methanol followed by a mechanical movement and ultrasonic bath during 20 minutes. The volume was completed to 100 mL and the resulting solution was filtered. Aliquots of the filtrate were diluted using methanol and processed as mentioned under the preparation section of general procedure. The calibration graph or the corresponding regression equation were used for calculation of nominal contents of the tablets.

2.4.3. Determination of DEF in Spiked Plasma Samples

A volume of plasma (100 µL) in a centrifuge tube was spiked with various concentrations of the analyte (DEF) and then it was subjected to extraction with addition of 200 µL of acetonitrile [10]. The organic phases were evaporated to dryness in a water bath at 50ºC. The residual mass was reconstituted with 0.2 mL methanol. The analytical procedure was implemented as mentioned under General Assay Procedure section. For blank assay, all procedures were implemented in the same way. The corresponding calibration graphs for plasma were used for calculation of the percentage recoveries. Statistical evaluation was also carried out in terms of the standard deviation of intercept and slope. (Ethics committee approval was provided for this study by Bezmialem Vakif University (BVU) Ethics Clinical Research Committee (approval reference number 22/3, see supporting information, Figure S2).

3. Results and Discussion

3.1. Conditions for Chromatography

Different stationary phases were trialed. C18 column with sizes 150 x 4.6 mm, 5µm I.D. and a mobile phase including methanol and acetic acid solution (0.5 M, pH 7.0) with a ratio of 70:30 v/v at flow rate of 1 mL provided the most efficient separation. In order to use fluorimetric detection DEF was derivatized with dansyl chloride, which is a widely used fluorogenic agent. The fluorescent intensity of DEF derivative was measured using a fluorimetric detector at 480 nm by excitation at 340 nm. Retention time of DEF derivative was about 3.45 min under these chromatographic process (Figure 1).
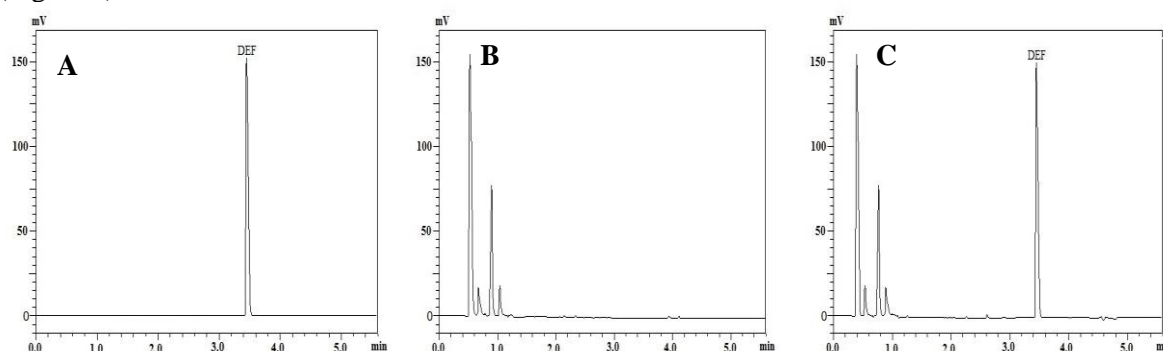

Figure 1. Representative chromatograms of LC measurements
(**A**) standard sample of DEF (900 ng/mL) , (**B**) blank plasma and (**C**) plasma spiked with DEF (1000 ng/mL)

3.2. Optimization of Derivatization Reaction Parameters

DEF was derivatized with dansyl chloride in order to gain fluorescence ability. This study is the first derivatization study for DEF for fluorescent activity. According to reach the optimum conditions for the most effective derivative 400 µL of dansyl chloride in acetonitrile solution was found to be sufficient. The effect of pH on fluorescence intensity was trialed using various pH values range from 9 to 11 using bicarbonate solution and borate buffer. Due to the fact that dansyl chloride gives reactions under alkaline conditions. The maximum fluorescence intensity was reached with bicarbonate solution of pH 10 with an optimum volume of 200 µL. The effect of temperature and different time intervals on fluorescence intensity was also trialed between 40-60°C. The optimum fluorescence values were obtained and remained stable in 40°C water bath for 10 min. Various solvents such as ethyl acetate, toluene, chloroform, dichloromethane, diethyl ether, benzene were used to achieve the highest fluorescence intensity. It was found out that dichloromethane gave suitable fluorescence intensity to use for the quantitation process. Higher of lower intensities in different solutions couldn't provide precise and efficient quantitation. The derivatives prepared under these conditions remained stable for at least 2 h.

3.2 Method Validation

The validation of the optimized method was performed in agreement with the ICH guidelines [17-19]. The following parameters were considered: specificity, linearity, accuracy, precision, LOD and LOQ, and robustness.

3.2.1 Linearity

The solutions for the linearity test were prepared from the solution of DEF at six different levels of concentration ranging from 20–2000 ng/mL. The calibration curve was created by plotting the substance area versus the concentration. The correlation coefficients, slopes and *y*-intercepts of the calibration plots were obtained and reported. The mean linear regression equation of the calibration curves was calculated as $y = 396,22x - 4753,3$ $r^2 = 0.9994$. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated according to the following equation: LOD or LOQ = κSDa/b, where 10 for LOQ and κ= 3 for LOD, SDa represents the standard deviation of the intercept, and b represents the slope. The LOD was 0.034 ng/mL and the LOQ was determined as 0.11 ng/mL.

3.2.2 Precision

The precision studies were carried out for five consecutive days by analyzing the quantity of DEF (each n=5). The RSD values were found to be in the range of 0.23-0.55% for intraday and inter-day precision studies. All these values were below 2%, confirming that the method was precise. The results of the tests have been shown in Table 1.

Table 1. Intra-day and Inter-day precision results of DEF

Added concentration (µg/mL)	Found Concentration (µg/mL)± SD	RSD% *
Intra-day		
20	20.02±0.09	0.45
1000	1012.3±2.36	0.23
2000	2009.4±5.14	0.26
Inter-day		
20	20.18±0.11	0.55
1000	1013.4±3.54	0.35
2000	2012.8±6.07	0.30

*RSD: Relative Standart Deviation

3.2.3 Accuracy

The standard addition technique was carried out to prove the accuracy of the proposed method. After adding a certain amount (200 ng/mL) of pure sample solution to the 20, 1000, 1800 ng/mL concentrations of standard DEF. The percentage recoveries for the drug ranged from 99.94–100.31%. The results of the recovery study have been presented in Table 2.

Table 2. Accuracy results of DEF

Compound	Nominal value (µg/mL)	Amount Added (µg/mL)	Total amount found[b](µg/mL) (Mean± S.D.[c])	Recovery	RSD
DEF	200	20	219.87±0.28	99.94	0.13
		1000	1203.72±4.01	100.31	0.33
		1800	2001.6±5.09	100.08	0.25

[a] Ferout *Film Tablet*®(90 mg)
[b]Five independent analyses.
[c]Standard deviation

3.2.4 Robustness

The method was found to be robust based on the observed changes in the mobile phase flow rate (±0.1 mL/min), the determined organic phase composition (±2%). During the analyses, the mobile phase pH (7.0 ± 0.5) and column oven temperature (30 ± 5°C) were measured and noted. The study demonstrated that minor variations in the method variables did not significantly affect the results, proving the robustness of the currently proposed method.

The chemical stability of the stock solutions, which were composed of the study compounds in the mobile phase mixture, was assessed after storing the solutions at room temperature for 48 hours. All of the studied compounds were found out to be stable in the mobile phase for 48 hours at room temperature and in the refrigerator (at 4°C). The stability studies yielded no further peaks in the chromatograms.

3.3. Assays in Matrices

3.3.1. Determination of DEF in Pharmaceutic Formulation

The proposed method was applied for the quantitative determination of DEF in the pharmaceutical forms. The obtained results were in agreement with the results specified in the approved labeled content of DEF. (Table 3).

Table 3. Determination of DEF in tablets by the proposed methods (*n*=5)

	Label claim [a] (mg/per tablet)	Mean[b]± S.D	Recovery (%)	RSD[c] (%)
Proposed method	90	90.14± 0.26	100.16	0.29

[a] Ferout *Film Tablet*® (90 mg)
[b]Five independent analyses.
[c]Relative Standard deviation

3.3.2. Determination of DEF in Spiked Plasma Samples

The C_{max} of DEF after administration of 1500 mg/day is reported as 308.7 µg/mL [20]. Regarding the study, the proposed methods could be successfully applied for the determination of DEF in spiked plasma.

The extraction procedures and applying of the proposed methods to plasma samples were described at section 2.4.3. The obtained results shown in Table 4 are satisfactorily accurate and precise.

Table 4. The recoveries of DEF from spiked plasma (n=5)

Proposed method	Added (µg mL^{-1})	Determined ± S.D (µg mL^{-1})	Recovery	RSD (%)^a
	20	18±0.17	90	0.94
	1000	890±5.05	89	0.57
	2000	1760±8.36	88	8.36

^{a}Five independent analyses.

In conclusion, a new HPLC method based on dansyl chloride is proposed and successfully applied for quantification of a novel compound DEF in commercially available pharmaceutical dosage form and spiked plasma samples.

DEF is an important chelation agent in order to prevent iron accumulation thought out body. It reaches C max very quickly at about 1-4 hours and its therapeutic range is narrow. Because of these pharmacokinetic properties, its plasma concentration is a critical point. New sensitive analytical methods are required to determine DEF in plasma. This proposed study provides a simple, fast and sensitive quantitation of DEF, and with spiked samples, it is proved to use this method for routine or toxicologic plasma analysis. In addition, the novel method is applied to pharmaceutical preparations of to indicate that it is possible to use the method in quality control or bioequivalence studies. In the literature survey it is noticed that there is a lack of analytical methods for these purposes. It is foreseen that this HPLC method will meet these needs.

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