ORIGINAL ARTICLE

Development and Validation of Stereoselective Method for the Quantification of Stereoisomers of Eliglustat Using Amylose-Based Immobilized Chiral Stationary Phase

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Abstract

Aim: The main aim of the present work is to develop and validate a new simpler, selective, specific, and robust high performance liquid chromatographic method for the separation of Eliglustat and its isomers. **Materials and Methods:** For the accurate quantification method, for the all isomers was developed by performing the significant number of methods on trial and error techniques using a large number of polar and non-polar solvent mixtures as mobile phase. A distinctive resolution between Eliglustat and its stereo isomers was achieved on Immobilized Amylose *tris* (3-chloro-4-methylphenylcarbamate) stationary phase namely CHIRALPAK IF-3(4.6 mm × 250 mm, 3 μm), with flow rate of 1.0 mL/min using isocratic method containing n-Hexane/Ethanol/Methanol/Diethyl amine (90/05/05/0.1,v/v/v/v). Column temperature was maintained at 40°C and detection wavelength of 280 nm. **Results and Discussion:** The limit of detection and limit of quantification values of Eliglustat S,S-Isomer, R,S-Isomer, and S,R-Isomers were found to be 0.0027/0.0082, 0.0367/0.1112, and 0.0375/0.1137 μg/mL, respectively. The method was found to be precise, accurate, and linear (R^2 > 0.999). Recoveries of all isomers were found to be in the range of 90–110%. **Conclusion:** As per the ICH guidelines, the developed method has been shown to be linear, accurate, precise, robust, and sensitive. The method is also considered quality control friendly as it is robust, uses isocratic mobile phase and employs commonly used solvents as mobile phase.

Key words: Chiral column, Eliglustat, Gaucher disease, limit of quantitation, validation

INTRODUCTION

Many of the compounds associated with living organisms are chiral, for example, DNA, enzyme, antibody, and hormones. Thus, biology is very sensitive to chirality and the activity of drugs also depends on which enantiomer is used. Most drugs consist of chiral molecules and since a drug must match the receptor in the cell, it is often only one of the enantiomer that is of interest. Individual enantiomer of a chiral compound can potentially exhibit different pharmacology, toxicology, and metabolism activities in the living system.[1] Potentially only one of the two enantiomers may be responsible for the desired therapeutic response, whereas the other enantiomer may be inactive or may even have deleterious effect. Therefore, it is of a paramount importance to develop analytical methods that can identify and determine enantiomeric purity of a chiral active pharmaceutical ingredient (API) in bulk lots as well as in drug products. To provide pharmaceutical industry further guidance on this key topic US Food and Drug Administration (FDA) issued a guidance entitled development of new stereoisomeric drugs in 1992.[2]

ELGS, marketed by Genzyme as Cerdelga, is a glucosylceramide synthase inhibitor useful in the long-term

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treatment of type-I Gaucher disease. Patients selected for treatment with ELGS undergo an FDA approved genotype test to establish if they are CYP2D6 extensive metabolizers, intermediate metabolizers, or poor metabolizers, as the results of this test dictate the dosage of ELGS recommended. ELGS chemically represented as *N*-[(1*R*,2*R*)-1-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(1-pyrrolidinyl)-2-propanyl]octanamide with a molecular formula and weight of $C_{23}H_{36}N_2O_4$ and 404.543 g/mol, respectively. It has two chiral centers in its structure and it exists in four 4 stereoisomers. Three chemical structures of ELGS stereoisomers, namely, (1R,2S)-stereoisomer, (1S,2R)-stereoisomer, and (1S,2S)-stereoisomer, are represented in Figure 1.[3,4]

From the review of literature, there are only two high-performance liquid chromatography (HPLC) methods[5,6] that have been reported for the estimation of ELGS and no chiral HPLC method iswas reported for the quantitation of ELGS and its stereoisomers. In the present research paper, chromatographic method was developed with immobilized Amylose *tris* (3-chloro-4-methylphenylcarbamate) chiral stationary phase namely CHIRALPAK IF-3(4.6 mm × 250 mm, 3 µm), utilized for the separation and quantification of ELGS isomers. The developed method also subjected for the influence of temperature and organic modifiers to get the robust analytical method. Method validation was executed for the optimized method as pet the guidelines of ICH.[7]

MATERIALS AND METHODS

Chemicals

API samples of ELGS and its isomers (enantiomer: SS isomer, diastereomer 1: RS isomer and diastereomer 2: SR isomer) were acquired from Daicel chiral technologies, India Private Limited. The HPLC-grade ethanol (EtOH), n-Hexane, methanol (MeOH), 2-propanol, and acetonitrile were obtained from Merck, India. Diethylamine (DEA), Isopropyl amine (IPA), ethanolamine (EA), ethylene diamine (EDA), and triethylamine (TEA) were bought from Spectrochem, Mumbai, India, and deionized water from Merck Millipore.

Liquid chromatographic (LC) system

A LC system of an Agilent instrument consisting of a G1311A QuatPump 1200 series, a G1322A degasser 1200 series, a G1329A 1200 series automatic liquid sampler, and a G1316A 1200 series thermo stated column compartment. The output chromatograms was integrated and monitored by utilizing Agilent Chemstation software.

Chiral stationary columns

Various immobilized polysaccharide chiral columns (Daicel, Japan), namely, CHIRALPAK IA (4.6 mm × 250 mm, 5 µm) (amylose tris (3,5-dimethylphenylcarbamate) immobilized on silica gel), CHIRALPAK IB (4.6 mm × 250 mm, 5 µm) (cellulose tris (3,5-dimethylphenylcarbamate) immobilized on silica gel), CHIRALPAK IC (4.6 mm × 250 mm, 5 µm) (cellulose tris (3,5-dichlorophenylcarbamate) immobilized on silica gel), CHIRALPAK ID (4.6 mm × 250 mm, 5 µm) (amylose tris (3-chlorophenylcarbamate) immobilized on silica gel), CHIRALPAK IE (4.6 mm × 250 mm, 5 µm) (amylose tris (3,5-dichlorophenylcarbamate) immobilized on silica gel), CHIRALPAK IF (4.6 mm × 250 mm, 5 µm) (amylose tris (3-chloro-4-methyl phenylcarbamate) immobilized on silica gel), and CHIRALPAK IF-3 (4.6 mm × 250 mm, 3 µm) (amylose tris (3-chloro-4-methyl phenylcarbamate) immobilized on silica gel) were employed.

Chromatographic conditions

An isocratic chromatographic elution was processed for the separation of ELGS and its stereoisomers on CHIRALPAKIF-3 (4.6 mm × 250 mm, 3 µm) chiral stationary column by utilizing n-hexane/EtOH/MeOH/DEA as a mobile phase in the proportion of 90:05:05:0.1 v/v/v/v. All the isomers were monitored at the detection wavelength of 280 nm. The analytical column was thermostated at 40°C and the mobile phase flow rate was set to 1.0 mL/min.

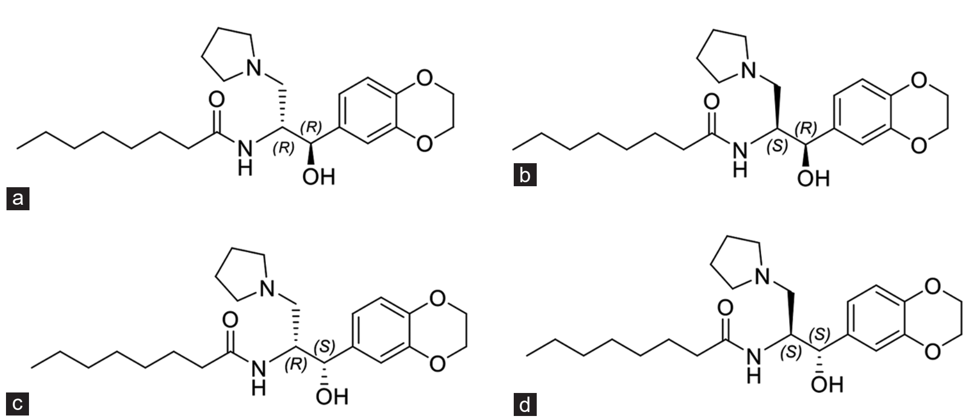

Figure 1: Chemical structures of (a) ELGS (b) (1R,2S)-isomer, (c) (1S,2R)-isomer, and (d) (1S,2S)-isomer

Protocol for standard solution

A stock solution of ELGS (3.0 mg/mL) and its stereoisomers (3.0 mg/mL) were processed by dissolving the required quantity of the substances in the diluent (n-hexane:EtOH [1:1]). From the resulting solutions, 100 µg/mL solutions were prepared and final dilutions were made to get the required concentrations using diluent.

Protocol for sample preparation

Twenty capsules of ELGS (Cerdelga 84 mg) were weighed and made a fine powder using a mortar and pestle. Then, 100 mg ELGS drug equivalent capsule powder was weighed and relocated into a volumetric flask of 100 mL, dissolved in 50 mL of diluent and sonicated for 20 min to ensure the dissolution. Finally, the solution was made up to 100 mL with diluent. The resulting solution was subjected for filtration through a 0.22-µm nylon membrane filter. Serial dilutions were made to obtain working sample solution of a concentration of 3.0 mg/mL.

RESULTS AND DISCUSSION

Method development and optimization

Primary screening for identification of suitable column

Different types of immobilized polysaccharide chiral columns were evaluated in normal phase (NP) and polar organic elution modes. As shown in Figure 1, ELGS is benzodioxine chiral compound having two chiral centers. It is having different alkylpyrrolidine, secondary alcohol, and carboxamide groups. These functional groups can interact with the active centers of the different chiral stationary phases (CSPs) to result in chiral separation. In immobilized polysaccharide-based CSPs with carbamate derivatives (as CHIRALPAK IA, IB, IC, ID, IE, and IF) and these carbamate groups can interact with the enantiomers through hydrogen bonding using CO and NH groups and through dipole-dipole interaction using CO moiety.[8,9] Besides these interactions, the enantiomers with aromatic groups may provide additional stabilizing effect to the enantiomer–CSPs complex by the insertion of the aromatic ring into the chiral cavity of the CSPs [R1].

Identification of the column in NP mode

To develop the stereo specific method for the quantification of enantiomer (SS isomer), diastereomer-1(RS isomer), and diastereomer-2(SR isomer), all the isomers were spiked to ELGS in screening experiments. DEA is often used as a modifier for amine groups to ensure their elution from the column and peak shape in normal-phase chromatography.[10,11] Hence, 0.1 % DEA was introduced into the mobile phase. The mobile phases having standard solvents compositions of n-Hexane/EtOH/DEA (80/20/0.1, v/v/v) [Figure 2a] and n-Hexane/2-propanol/DEA (80/20/0.1, v/v/v) [Figure 2b] were used in NP mode on Daicel's immobilized chiral columns such as CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC, CHIRALPAK ID, CHIRALPAK IE, and CHIRALPAK IF.

As shown in Figure 2a with the mobile phase composition of n-hexane/EtOH/DEA: 80/20/0.1 (v/v/v), all the isomers were not resolved in IB and IE columns. In CHIRALPAK IC column, all the four isomers were eluted with very poor asymmetry factor and theoretical plates. In the presence of IA, ID, and IF columns, out of four, three isomers were separated with poor chromatographic performance. In Figure 2b with the mobile phase composition of n-hexane/IPA/DEA:80/20/0.1 v/v/v), all the isomers were not separated with IB column and only three isomers were separated through IA, IC, ID, IE, and IF columns with poor chromatographic performance.

Identification of the column in polar mode

To develop the stereo specific method for the quantification of enantiomer (SS isomer), diastereomer-1(RS isomer), and diastereomer-2(SR isomer), all the isomers were spiked to ELGS in screening experiments. The mobile phases having standard solvents compositions of MeOH/DEA (100/0.1, v/v), EtOH/DEA (100/0.1, v/v), and CAN/DEA (100/0.1,v/v) were used in polar mode on six Daicel's immobilized chiral columns such as CHIRALPAK IA, IB, IC, ID, IE, and IF. All these six columns were not resolved ELGS isomers in polar mode.

Further in the development process, we have chosen CHIRALPAK IF column for high resolution of ELGS isomers and favorable elution order in NP. At same time, we have introduced methanol into the mobile phase composition and utilized n-hexane/EtOH/MeOH/DEA 90/05/05/0.1 (v/v/v/v) without changing the other chromatographic conditions. This mobile phase has produced better resolution between the isomers and API.

Selection and optimization of column temperature

Column temperature plays an important role in the isomeric separation of analytes in the chiral HPLC. Hence, we have planned different trials on column temperatures from 15°C to 45°C at 5°C increment. The resulting chromatograms were evaluated for resolution at each temperature and the temperature of 40°C was shown better resolution. The results are shown in Table 1 and Figure 3a.

Selection and optimization of basic additives

The use of basic mobile phase additives allows the chiral separation of underivatized phenylalanine analogs on a

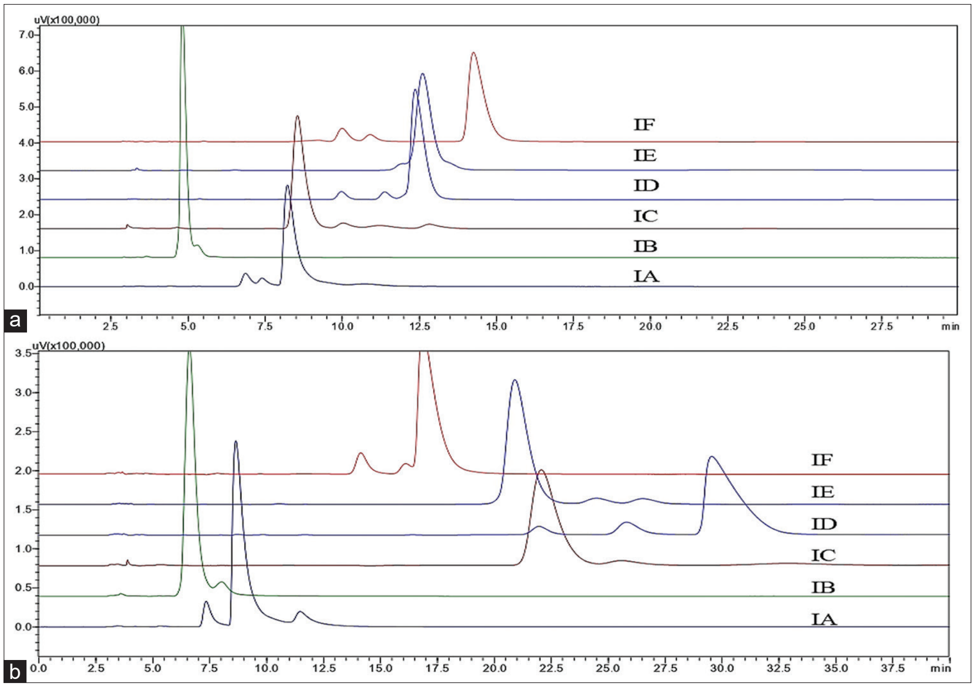

Figure 2: Separation of ELGS and its isomers on different CHIRALPAK columns (IA, IB, IC, ID, IE, and IF) with mobile phase composition of (a) n-Hexane: EtOH: Diethylamine (DEA) (80/20/0.1, v/v/v) and (b) n-Hexane: 2-propanol: DEA (80/20/0.1, v/v/v)

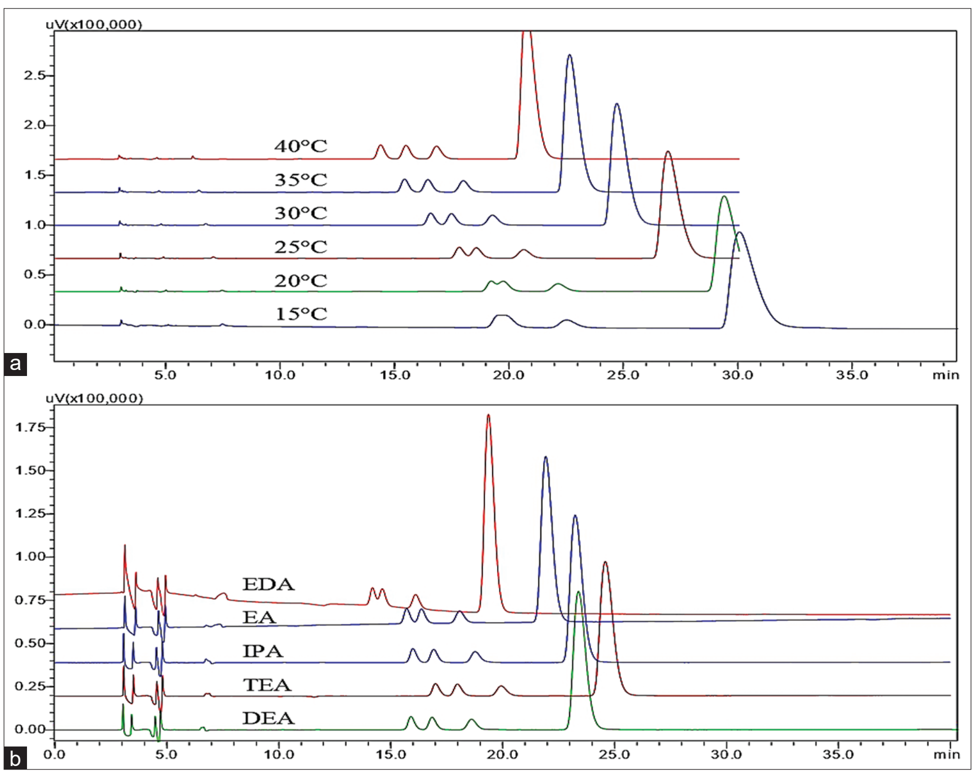

Figure 3: (a) Influence of temperature on separation of ELGS and its isomers with mobile phase composition of n-Hexane: EtOH: MeOH: diethylamine (DEA) (90/05/05/0.1, v/v/v/v) and (b) Influence of basic additives DEA, Isopropyl amine, ethanolamine, ethylene diamine, and triethylamine on mobile phase composition of n-Hexane: EtOH: MeOH (90/05/05, v/v/v) for the separation of ELGS and its isomers

common amylosic column. In addition to decrease retention time and band-broadening arising from non-ideal interactions, basic additives also increases the selectivity. This appears to be due to the minimization of non-selective binding in the recognition site. Effects of the additives are related to additive pKa and size.[12] Initially, we have optimized the mobile phase composition of n-hexane/Ethanol/Methanol (90/05/05, v/v/v) and further we have tried with different basic additives such as DEA, IPA, EA, EDA, and TEA. The resulting chromatograms were evaluated for resolution of different additives and DEA has shown excellent resolution when compared with other additives. The results are shown in Table 2 and Figure 3b.

Finally, the method was optimized with a well-packed CHIRALPAK IF column with 3 µm particle size in place of 5 µm for the outstanding separation efficiency [Figure 4b]. Optimized mobile phase was n-hexane/EtOH/MeOH/DEA in the proportion of 90:05:05:0.1 v/v/v/v. All the isomers were monitored at the detection wavelength of 280 nm. The analytical column was thermostated at 40°C and the mobile phase flow rate was set to 1.0 mL/min.

Method validation

To ensure that method is suitable for the intended purpose, key method characteristics, that is, specificity, accuracy, linearity, precision, limit of detection (LOD), and limit of quantitation (LOQ) were evaluated.[7,13-15]

System suitability

System suitability test (SST) is a test to determine the suitability and effectiveness of chromatographic system before use. The performance of any chromatographic system may continuously change during their regular use, which can affect the reliability of the analytical results. The operation parameters of the whole chromatographic system was performed with replicate injections (n = 6) of the SST mixtures and the parameters such as N_{eff}, resolution, asymmetry, retention time, detection limit, and selectivity were evaluated. The parameters were within acceptance limits and the results are summarized in the Table 3.

Table 1: Results for influence of temperature on n-Hexane: EtOH: MeOH: DEA (90/05/05/0.1, v/v/v/v) mobile phase

Temperature in °C	Rs1	Rs2	Rs3	Tf1	Tf2	Tf3	Tf4
15	0.3	2.5	4.7	0.6	5.6	1.3	1.8
20	0.6	2.3	5.9	0.8	1.7	1.3	1.2
25	1	2.3	5.2	1.0	1.3	1.3	1.6
30	1.3	2.3	5	1.2	1.2	1.3	1.6
35	1.6	2.1	4.8	1.2	1.2	1.3	1.6
40	1.8	1.9	4.4	1.2	1.2	1.3	1.6

Rs: Resolution; Tf: Tailing factor. DEA: Diethylamine

Table 2: Results for influence of basic additives on n-Hexane: EtOH: MeOH (90/05/05, v/v/v) mobile phase

Additive name	Rs1	Rs2	Rs3	Tf1	Tf2	Tf3	Tf4
DEA	1.6	3.0	5.3	1.2	1.2	1.2	1.4
TEA	1.6	2.6	4.8	1.2	1.2	1.3	1.4
IPA	1.6	2.7	5.0	1.2	1.2	1.4	1.4
EA	1.2	2.7	4.7	1.1	1.2	1.3	0.7
EDA	0.8	2.6	4.7	1.8	1.3	0.9	1.4

Rs: Resolution; Tf: Tailing factor. IPA: Isopropyl amine, DEA: Diethylamine, EA: Ethanolamine, EDA: Ethylene diamine, TEA: Triethylamine

Specificity

Specificity of a chiral assay method for enantiomeric and diastereomeric impurities should unequivocally assess the presence of analyte in the presence of enantiomer and diastereomers. The specificity of the developed method was determined by spiking pure substance and enantiomeric impurities with appropriate level. Specificity of this method was evaluated by injecting blank, 0.15% of test concentration of EGLS and other diastereomers together and individually. The chromatogram results are shown in Figure 4a and 4b and the chromatographic parameters are represented in Table 4. It was observed that there is no interference drug peak with blank and impurities peaks indicating that the method is specific for ELGS.[16,17]

Sensitivity

Sensitivity is often interpreted as related to the detection/determination ability. In the recent FDA's, bioanalytical method validation guidance document sensitivity is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision.[16] LOD and LOQ were determined by signal-to-noise ratio method, wherein the concentration that gave S/N ratio of 3:1 (LOD) and the concentration that gave S/N ratio of 10:1 (LOQ). LOD and LOQ values of ELGS S,S-Isomer, R,S-Isomer, and S,R-Isomers were found to be 0.0027/0.0082, 0.0367/0.1112, and 0.0375/0.1137 µg/mL, respectively, and findings are shown in Table 5 and Figure 5a.

Precision

Precision is to determine the variability of results obtained from the same sample or sub samples of the same sample. Precision of

Table 3: SST for ELGS and its stereoisomers

Sample name	S, S-Isomer	R, S-Isomer	S, R-Isomer	ELGS
Resolution	--	1.94	2.3	5.3
Asymmetry factor	1.2	1.2	1.3	1.6
Theoretical plates	9264	9020	8468	6123
Retention time	13.245	14.447	15.571	18.58

SST: System suitability test

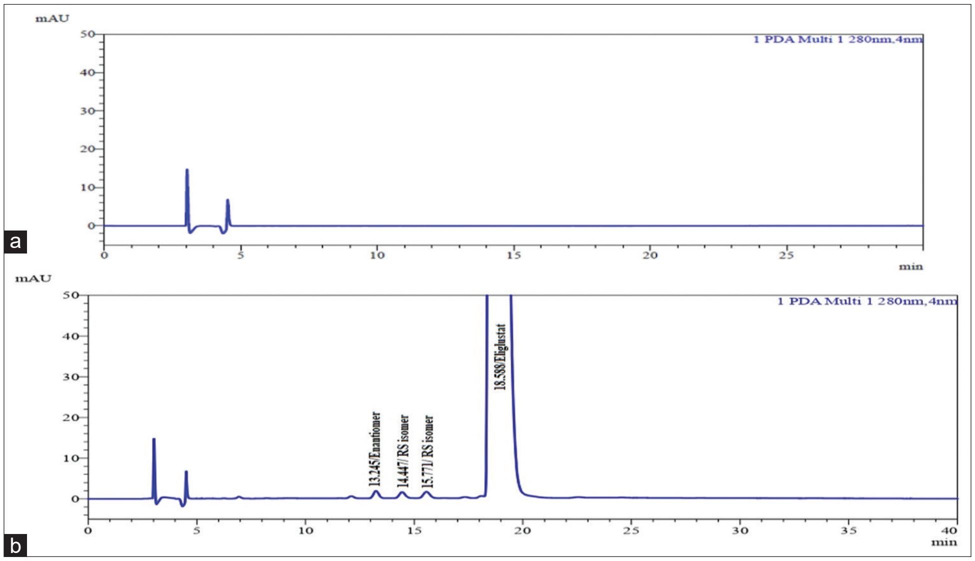

Figure 4: Representative chromatograms of ELGS (a) blank and (b) spiked at 0.15% test concentration

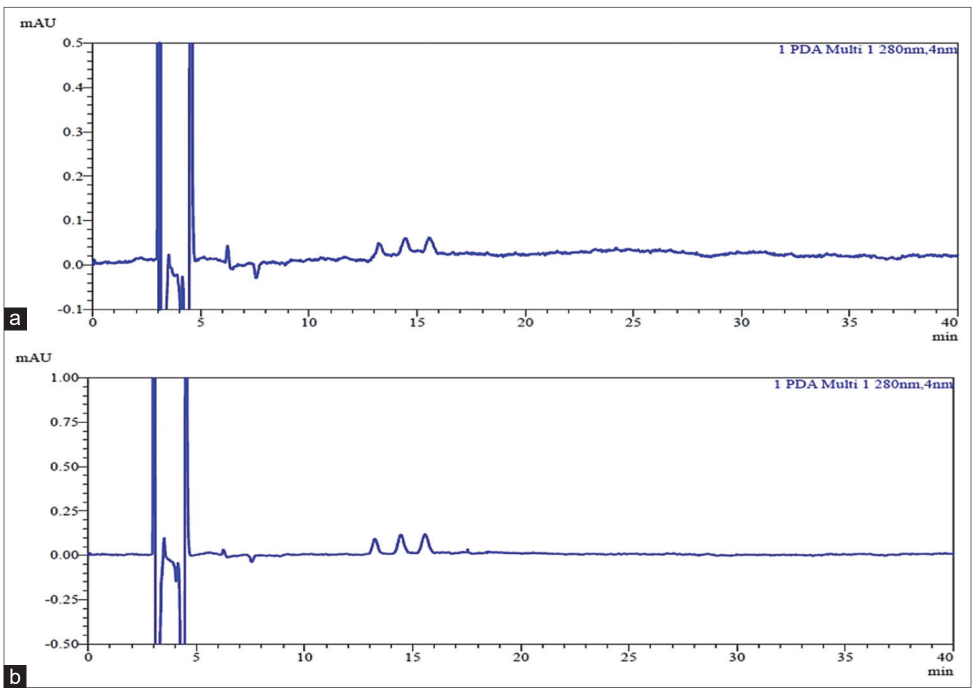

Figure 5: (a) Limit of detection and (b) limit of quantitation chromatograms of ELGS and its isomers

Table 4: Peak purity of ELGS and its isomers

Sample spiked	R.T (min)	Purity factor	Threshold
S, S-Isomer (Enantiomer)	13.2	999.992	999.718
R, S-Isomer	14.400	999.991	999.658
S, R-Isomer	15.5	999.986	999.497
Eliglustat API	18.5	999.985	999.122

R.T: Retention time; API: Active pharmaceutical ingredient

the developed method was determined as system precision and method precision. System precision was processed by infusing six individual standard preparations of ELGS spiked at 0.15% specification level. Method precision was processed by infusing both sample and standard spiked samples. The relative standard deviation (%RSD) was estimated for each isomer in the spiked and test preparation. The %RSD values for system precision and method precision were <1.94 and <2.33, respectively.

Accuracy

Accuracy is the closeness of the agreement between the result of a measurement and a true value. It is measured as the percent of analyte recovered by assay. Three different concentrations of impurities were spiked into the sample extract in triplicate from LOQ to 150% level and recovery was estimated using the following formula:

$$\% \text{ Recovery} = \frac{\text{Recovery amount} - \text{Original amount}}{\text{Spiked amount}} \times 100$$

The average recovery values were calculated from the resulting chromatograms and are tabulated in Table 6. The average recovery values were found in between 94% and 110%.

Linearity

The linearity of an analytical method can be explained as its capability to show results that are directly proportional to the concentration of the analyte in the sample.[15] The linearity was determined by constructing a calibration graph with six different concentrations ranging from LOQ to 150% of specification level (0.15%). Two injections from each concentration were analyzed under the same conditions. Using least square analysis method, the linearity of the calibration graph was determined and the correlation co-efficient (r^2) was found to be 0.9996, 0.9979, 0.9994, and 0.9996 for ELGS API, R,S-isomer, S,R-isomer, and S,S-isomer, respectively. The regression equations of ELGS API, R,S-isomer, S,R-isomer, and S,S-isomers was y = 18841x – 438.01,y = 208227x–1588.7, y = 22369x–379.89, and y = 19144x–770.1, respectively. The findings of ELGS isomers linearity are represented in Table 7.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness of the method was performed by changing the chromatographic conditions such as flow rate (0.8–1.2 ml/min), temperature (±2%), and composition of mobile phases (±5%). Obtained

Table 5: LOD and LOQ of ELGS and its stereoisomers

Peak name	LOQ		LOD	
	Conc. in µg/mL	S/N ratio	Conc. in µg/mL	S/N ratio
S, S‑Isomer (Enantiomer)	0.0082	10.9	0.0027	4.0
R, S‑Isomer	0.1112	13.4	0.0367	4.6
S, R‑Isomer	0.1137	13.7	0.0375	4.6

LOQ: Limit of quantification, LOD: Limit of detection

Table 6: Accuracy data of ELGS isomers

Name of the isomer	% Recovery			
	Accuracy[a] 50%	Accuracy[a] 100%	Accuracy[a] 150%	Accuracy[a] at LOQ level
S, S isomer (enantiomer)	95	105	110	94
R, S isomer	96	104	108	108
S, R isomer	96	104	108	97

[a]average of triplicate solutions. LOQ: Limit of quantitation

Table 7: Linearity of ELGS isomers

Concentration level	R, S‑Isomer		S, R‑Isomer		S, S‑Isomer		ELGS	
	Conc. in µg/mL	Average area[a]	Conc. in µg/mL	Average area[a]	Conc. in µg/mL	Average area[a]	Conc. in µg/mL	Average area[a]
LOQ	0.0111	1984	0.0870	2141	0.0873	1334.0	0.0873	1370.5
50.0%	0.0725	12800	0.7418	16260	0.7437	13375.0	0.7437	13902.5
75.0%	0.1092	19634	1.1176	23585	1.1205	19993.0	1.1205	20235.0
100.0%	0.1450	28419	1.4835	32443	1.4874	27479.0	1.4874	27043.5
125.0%	0.1788	36185	1.8297	40835	1.8345	34696.0	1.8345	34255.0
150.0%	0.2175	44313	2.2253	49881	2.2311	42164.0	2.2311	41960.0
CC	0.9979		0.9994		0.9996		0.9996	
Intercept	−1588.72		−379.89		−770.10		−438.01	
Slope	208227		22368.59		19143.58		18841.48	
% Y Intercept	−5.6		−1.2		−2.8		−1.6	

CC: Correlation coefficient; [a]Two injections, LOQ: Limit of quantitation

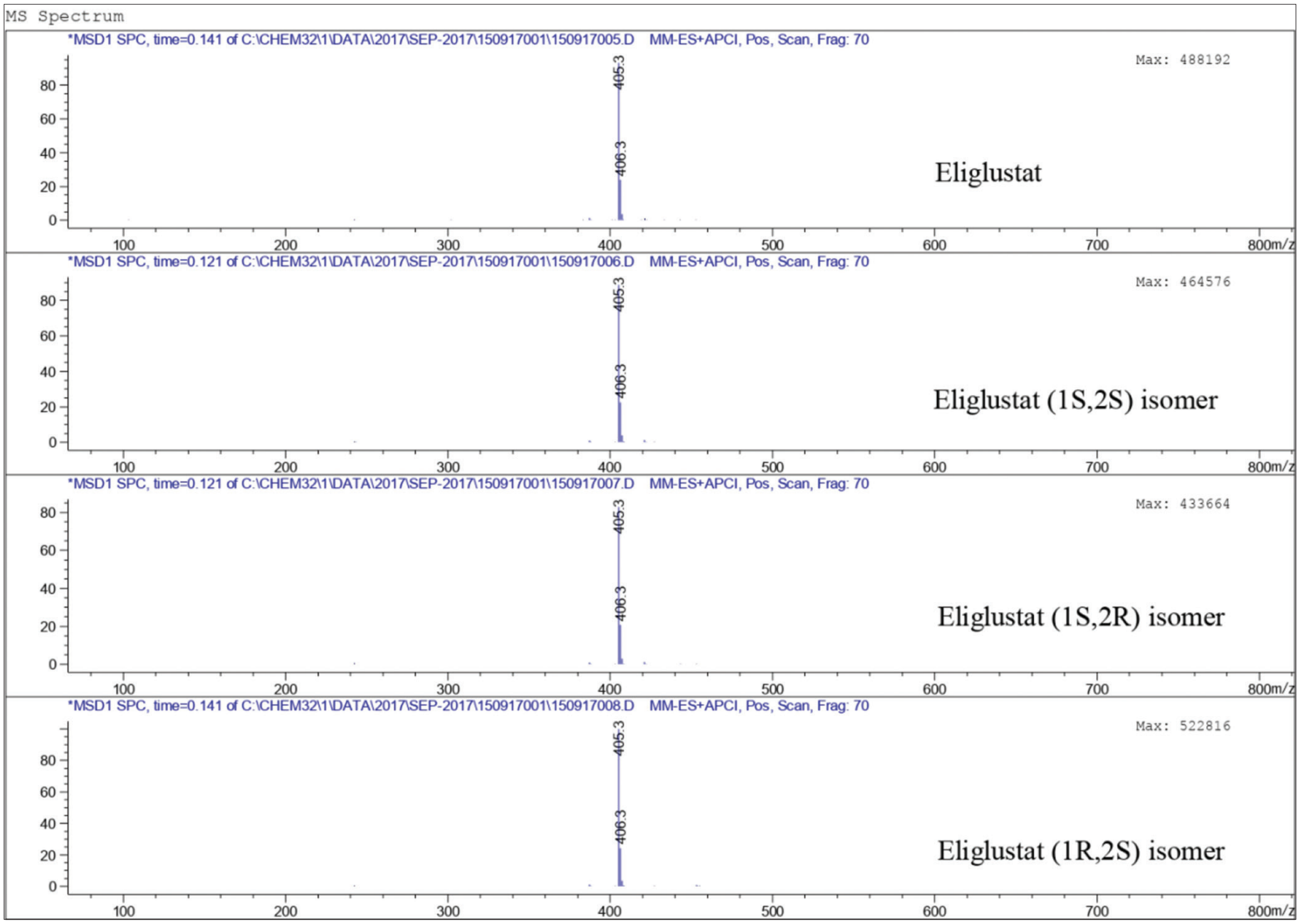

Figure 6: Mass spectrum of ELGS and its isomers

data for each case were evaluated by calculating %RSD and percent of recovery. The mixture of ELGS and its stereoisomers was injected in each varied conditions and the resolution between the isomers and ELGS was within the acceptable limits and the resolutions between ELGS and its enantiomer under various conditions were more than 2 and the %RSD values were found between 2.0 and 2.5.

Detection of isomers by direct insertion probe-mass spectrometry

The developed method was applied for the mass detection system to identify the m/z values of ELGS and its isomers by direct insertion probe-mass spectrometry. Sample solution was processed with ES+APCI +ve scan. From the resulting mass spectrum, it was confirmed that all the stereoisomers given same m/z values and the value was found to be 405.3 [Figure 6].

CONCLUSION

A new chiral HPLC method was successfully developed and validated for the quantification of EGLS and its stereoisomers in Eliglustat bulk drug sample API and formulation. An isocratic chromatographic elution was processed for the separation of ELGS and its stereoisomers on Chiralpak IF-3 (4.6 mm × 250 mm, 5 μm) chiral stationary column by utilizing n-hexane/EtOH/MeOH/DEA as a mobile phase in the proportion of 90:05:05:0.1 v/v/v/v. All the isomers were monitored at the detection wavelength of 280 nm. The analytical column was thermostated at 40°C and the mobile phase flow rate was set to 1.0 mL/min. As per the ICH guidelines, the developed method has been shown to be linear, accurate, precise, robust, and sensitive. The method is also considered QC friendly as it is robust, uses isocratic mobile phase, and employs commonly used solvents as mobile phase.

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