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Research Article

Direct Stereoselective Method Development and Validation of Elagolix on Zwitterionic Chiral Stationary Phase by High-Pressure Liquid Chromatography

Thirupathi Choppari^{1*}, Srinivasu Gunnam¹, Lakshmi Narayana Chennuru¹, Parameswara Murthy Cherla²

¹Daicel Chiral Technologies (India) Pvt. Ltd., IKP Knowledge Park, Hyderabad-500078, Telangana, India

²Department of Chemistry, Osmania University, Amberpet, Hyderabad-500007, Telangana, India

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ABSTRACT

A specific, sensitive, and accurate chiral technique has been developed for the quantification of elagolix enantiomer in a drug product. The present research work represents the development and validation of chromatographic technique for the separation of elagolix enantiomers on a chiral stationary phase (CSP) comprising a zwitterionic selector derived from cinchona alkaloid and sulfonic acid Chiralpak ZWIX(+) under isocratic condition containing 50 mM Acetic acid and 25 mM diethylamine in water and methanol in the ratio of 2:98 v/v with 0.5 mL min⁻¹ flow rate. The temperature of the column was monitored at 40°C with a resolving factor of more than 3.2. The eluted compounds were monitored at 275 nm. The optimized method has a high degree of selectivity with lower limiting values of detection and quantitation. It was found to be 2 and 7.5 ng/mL, respectively, for the enantiomeric chiral impurity. The developed method was subjected to validation as per the International Council for Harmonisation (ICH) guidelines, produced good findings regarding specificity, accuracy, precision, robustness, and linearity.

INTRODUCTION

Elagolix (ELGX), marketed as Orilissa brand name, is a gonadotropin-releasing hormone (GnRH) antagonist drug, which is utilized to treat pain in premenopausal women accompanying with endometriosis.^[1-8] This medicament is also under the development process to treat heavy menstrual bleeding and uterine fibroids in women.^[8] Endometriosis develops when tissue that is similar to the kind that is normally located in the uterus starts to grow outside of the uterus. Such growth leads to various symptoms, like pain during periods, pelvic pain between periods, and pain during sexual intercourse. The growths themselves are referred to as lesions and frequently develop on the ovaries, fallopian tubes, and other areas around the uterus, including the bowel or bladder. The growth of these lesions is dependent upon the estrogen hormone.^[4-6]

ELGX belongs to the class of non-peptide small molecule GnRH receptor antagonist that binds to GnRH receptors in the pituitary gland and prevents the endogenous GnRH-signaling. ELGX administration resulting in the dose-dependent suppression of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) leads to a decrease in blood ovarian sex hormones, progesterone, and estradiol concentration levels. IUPAC naming of ELGX is 4-[[[(1R)-2-[5-(2-Fluoro-3-methoxyphenyl)-3-[[2-fluoro-6-(trifluoromethyl)phenyl]methyl]-4-methyl-2,6-dioxypyrimidin-1-yl]-1-phenylethyl]amino]butanoic acid (Fig. 1) with molecular mass and formula of 631.590 g.mol⁻¹ and C₃₂H₃₀F₅N₃O₅, respectively. It has one chiral center and exhibits two enantiomeric (R and S) forms. Among these two, only R-isomer will produce the therapeutic action against the GnRH and useful in endometriosis, and the S-isomer will not produce the

*Corresponding Author: Thirupathi Choppari

Address: Associate Director, Daicel Chiral Technologies (India) Pvt. Ltd., IKP Knowledge Park, Hyderabad-500078, Telangana, India

Email ✉: thirrudr@gmail.com, thirupathi@chiral.daicel.com

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Fig. 1: Chemical structures of ELGX: **a)** S-isomer (enantiomer); **b)** R-isomer [Active Pharmaceutical Ingredient (API)]; **c)** Chiralpak ZWIX chiral stationary phases

same.^[9-11] If S-isomer is present in the final bulk or formulation, it will be considered as an enantiomeric impurity. The development of an analytical method for quantitating enantiomers in bulk and finished product is important for pharmaceutical quality.

The literature on ELGX discloses that no single analytical method was reported for the separation, identification, and quantitation of ELGX enantiomers. The present study focused on developing and validating a stereoselective analytical method for the separation and quantitation of ELGX isomers.

MATERIALS AND METHODS

Chemicals

ELGX, its enantiomer were received from Daicel Chiral Technologies India Pvt. Ltd., Hyderabad, India. Acetonitrile, ethanol (EtOH), n-hexane, methanol (MeOH), acetic acid, trifluoroacetic acid (TFA), diethylamine (DEA), and ammonium formate were obtained from Rankem, Thane, India. Ammonia (25% aqueous ammonia solution) and formic acid were bought from Merck, Mumbai, India. High performance liquid chromatography (HPLC) grade water was made from Milli-Q (Merck) water purification system Molsheim, France.

Instrumentation and Chromatographic Conditions

The liquid chromatographic (Shimadzu Corporation, Japan) system utilized with ultra violet (UV) detection system consisting of degasser: DGU-20A5, pumps: LC-20AD, column thermostat: CTO-20AC, autosampler: SIL-20A, coupled with photodiode array detector (SPD-M20A), and Valco column switching valve. The columns in use were Chiralpak ZWIX(+) (250 × 4 mm) 3 μ and Chiralpak QN-AX (150 × 4.6 mm) 5 μ, commercialized by Daicel Corporation (Tokyo, Japan) and manufactured at Chiral Technologies Europe (Illkirch, France). Other polysaccharide-derived columns (Chiralpak IA, IB, IC, ID, IE, IF, IG, IH, Chiralpak AD, Chiralpak AS, Chiralpak AY, AZ, CHIRALCEL

OD, OJ, OX, and OZ) took part in the preliminary research, commercialized by Daicel Corporation (Tokyo, Japan).

The composition of optimized mobile phasic system processed on Chiralpak ZWIX(+) (250 × 4 mm; 3 μ) column was 50 mM acetic acid and 25 mM DEA in MeOH and water in the proportion of 90:2 %v/v. It was subjected to filtration through a 0.45-micron membrane filter before utilization. The temperature of the column was monitored at 40°C with a 0.5 mL min⁻¹ flow rate. The eluted compounds were monitored at 275 nm with 10 μL injection volume and 20 minutes run time. MeOH of 100% was utilized as a diluent for the sample processing at the concentration of 0.5 mg/mL for injection.

Standard Stock Solutions

Weighed and transferred each 50 mg of ELGX and S-isomers separately into individual 10 mL volumetric flasks, added 5 mL diluent, and sonicated to dissolve. Further makeup to volume with diluent and mixed well. The obtained samples' concentration is ELGX (5 mg mL⁻¹) and enantiomer (5 mg mL⁻¹) stock solutions.

Sample Preparation

Weighed and transferred 50 mg of ELGX into 10 mL volumetric flasks, added 5 mL diluent, and sonicated to dissolve. Further, make up to volume with diluent and mixed well. Serial dilutions were made to obtain a working sample solution of a concentration of 0.5 mg mL⁻¹.

Analytical Method Validation

The developed method was subjected to validation in the present research work as per the ICH Q₂ (R₁) guidelines. Validation was executed for the parameter's specificity, accuracy, linearity, robustness, linearity, the limit of detection (LOD), the limit of quantification (LOQ), and precision.^[12-16]

Specificity

The analytical method specificity and selectivity were evaluated by infusing ELGX spiked with its isomer and its precursor at a 0.15% concentration level with respect to 0.5 mg mL⁻¹ sample concentration.

Precision

Precision was processed in terms of system precision and method precision. System precision was processed by infusing six individual standard preparations of ELGX spiked at 100% level with respective to 0.5 mg mL⁻¹ analyte concentration. Method precision was processed by infusing both sample and standard spiked samples. The % relative standard deviation (% RSD) was estimated for enantiomer in the spiked and test preparation.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

LOQ and LOD for ELGX enantiomers were assessed by signal-to-noise (S/N) ratios of 10:1 and 3:1, respectively, by infusing

a series of dilute solutions with known concentrations. The LOQ precision was executed by infusing six individual preparations, and the % RSD values were estimated for the peak responses of ELGX enantiomers.

Linearity

Method linearity is its capacity to get test outcomes, which are directly proportional to the drug concentration in the sample. Linearity solutions were processed by serial dilution of the stock solutions of enantiomers to a required concentration. Enantiomers solutions were processed at six different levels of concentration from LOQ level to 150% with respect to the enantiomer specification level of 0.15%. The linearity curves for the enantiomers were plotted by taking peak response on the Y-axis and respective concentrations on X-axis. The values of intercept, slope, and correlation coefficient of the linearity graph were assessed.

Accuracy

The closeness of agreement between true or reference value and the measured value expresses the accuracy of the analytical method. The recovery studies were processed to estimate the accuracy of the analytical liquid chromatographic technique for the quantitation of potential enantiomeric impurity of ELGX in drug samples. The experiment was executed in triplicate at three different concentration levels, i.e., 50, 100, and 150%, with respect to 0.5 mg mL⁻¹ sample concentration.

Robustness

To estimate the developed analytical method's robustness, the LC-method conditions were purposely varied. A solution of ELGX drug spiked with its enantiomer at specification level (0.15%) was used in this study and analyzed six times at each experimental condition. The mobile phase flow of 0.5 mL/min to assess the effect of flow change on the resolution, varied to 0.45 and 0.55 mL/min. To assess the effect of variation in movable phase proportion of water varied to 5 and 10% level. The optimized column oven temperature (COT) of 25°C, also varied to 15 and 40°C.

RESULTS AND DISCUSSION

Preliminary Screening for Column Selection

In the present research work on the elution of ELGX enantiomers, different CSPs had been trialed with, together with polysaccharide-derived CSPs under polar, reverse, and normal phases with dual additives due to the amphoteric nature of the molecule. QN-AX-based CSPs, as well as, the cinchona-based CSPs under reverse phase. Preliminary screenings mainly focus on (i) selection of chiral column, (ii) role and strength of additives in the resolution of enantiomer, and (iii) optimization on the separation of enantiomers.

Selection of Chiral Column

A systematic screening study was executed on the immobilized and coated amylose, and cellulose derived CSPs (Fig. 2) in polar, normal, and reversed-phase modes.^[17,18] In polar mode with MeOH/DEA/TFA (100/0.1/0.1 %v/v/v) and MeOH/acetonitrile/DEA/TFA (50/50/0.1/0.1 %v/v/v/v) as mobile phases, there was no enantiomeric selectivity observed. In normal phase with n-hexane/EtOH/TFA/DEA (70/30/0.1/0.1 v/v/v/v) and n-hexane/IPA/TFA/DEA (70/30/0.1/0.1 v/v/v/v), partial separations with peak broadening on Chiralpak IC, Chiralpak IG, Chiralpak AD-H, and CHIRALCEL OX-H polysaccharide-derived CSPs, and no selectivity with peak broadening on rest of the columns. In the reverse phase mode, the chromatographic system was processed with two programs consisting of solvent A (10 mM ammonium bicarbonate) and solvent B (100% acetonitrile in one program and 100% methanol in another program). The gradient elution was monitored as time/B%, i.e., 0/10, 10/80, 15/80, 15.1/10, 20/10, at 1 mL/min. The separation of an enantiomer, as well as, rotamers were separated on CHIRALCEL OX-3R, in which drug product rotamer has been co-eluting at enantiomer retention time under both gradient programs and not possible for the quantification of enantiomer in analyte due to co-elution of rotamers.

ELGX has amphoteric in nature, so the Chiralpak ZWIX (Fig. 1c) column is the best choice for stereoselectivity. To suppress the intra-molecular counter ion effect of the selector, basic and acidic additives were utilized, like DEA, and formic acid or diethylamine was suggested to moderate the pairing of ions and ion-exchange mechanisms between the selectors.

Baseline separation was achieved in the primary screening mobile phase as 25 mM HCOOH and 25 mM ammonium formate (MeOH/water 90/10 %v/v); baseline separation was observed with partial rotamers separation. Favorable elution was noticed on Chiralpak ZWIX(+) column, and the same was chosen for optimization.

Role and Strength of Additives in Resolution of Enantiomers

As per the literature on ZWIX columns,^[19-21] MeOH was an important solvent system in the mobile phase composition. The addition of fewer proportions of water content was favorable for moderating, retaining, and improving the solubility of analytes in the mobile phase. Owing to the intra-molecular counter ion consequence of the chiral selectors, utilization of basic and acidic additives, like acetic acid, ammonium formate, DEA, and formic acid, is suggested to moderate the ion-pairing and ion-exchange mechanisms between the selector and the analyte molecules. In addition to the mobile phase composition, it was necessary to consider the detection viability and sensibility of the separation method. Further

Amylose-based				
		Chiralpak IA	Chiralpak ID	Chiralpak IE
		Tris(3,5-dimethylphenylcarbamate) immobilized on silica gel	Tris(3-chlorophenyl carbamate) immobilized on silica gel	Tris(3,5-dichlorophenylcarbamate) immobilized on silica gel
		Chiralpak IF	Chiralpak IG	Chiralpak IH
		Tris(3-chloro-4 methyl phenyl carbamate) immobilized on silica gel	Tris(3-chloro-5-methyl phenyl carbamate) immobilized on silica-gel	Tris[(S)-α-methylbenzyl carbamate] immobilized on silica gel
Cellulose-based				
		Chiralpak IB	Chiralpak IC	
		Tris(3,5-dimethyl phenyl carbamate) immobilized on silica-gel	Tris(3,5-dichlorophenylcarbamate) immobilized on silica-gel	
Amylose-based				
		Chiralpak AD	Chiralpak AS	Chiralpak AY
		Tris(3,5-dimethyl phenyl carbamate) coated on silica gel	Tris[(S)-α-methyl benzyl carbamate] coated on silica gel	Tris(5-chloro-2-methyl phenyl carbamate) coated on silica gel
		Chiralpak AZ		
		Tris(3-chloro-4-methyl phenyl carbamate) coated on silica gel		
Cellulose-based				
		Chiralcel OD	Chiralcel OJ	Chiralcel OX
		Tris(3,5-dimethylphenylcarbamate) coated on silica gel	Tris(4-methylbenzoate) coated on silica-gel	Tris(4-chloro-3-methyl phenyl carbamate) coated on silica gel
		Chiralcel OZ		
		Tris(3-chloro-4-methyl phenyl carbamate) coated on silica gel		

Fig. 2: Types of polysaccharide-based chiral stationary phases

investigations were conducted by changing the additive strength and composition of acid-bases. Better efficiency parameters were produced by the additive combination of 50 mM acetic acid + 25 mM diethylamine.

Baseline separation and the good resolution were obtained with Chiralpak ZWIX CSPs, with a reversed separation order detected between the ZWIX(-) and ZWIX(+) CSPs, which contain, respectively, the zwitterionic chiral selectors behaving as pseudo-enantiomers.^[19,20] Due to the encouraging elution order (minor enantiomers separated initially) found on the Chiralpak ZWIX(+) stationary phase, such a column was elected for further examination.

Optimization of Temperature

Temperature plays a vital role in the separation of enantiomers by utilizing CSPs. In most cases, the impurity peaks will co-elute with the enantiomers with low-resolution efficiency. In the present work, the optimized chromatographic parameters were subjected to variation in column oven temperature and processed at 40°C (Fig. 3a), and 25°C (Fig. 3b). The resulting overlaid chromatograms were shown in Fig. 3. At 25°C, the impurity peaks were eluting by merging with the enantiomeric peaks, compared with the chromatogram at 40°C temperature, 3a and 3b present in Fig. 3 overlaid. At 40°C, unknown impurities were well resolved, and no interference with enantiomeric peak

Fig. 3: Effect of temperature on chromatographic separation of enantiomers [3(a) at 40°C and 3(b) at 25°C]

Fig. 4: Effect of water ratio in mobile phase on chromatographic separation of enantiomers

and no loss of resolution and was selected as optimized temperature.

Optimization of Water Ratio in Mobile Phase

Water ratio in the mobile phase composition also alters the resolution, retention time, and total run time of chromatogram. To optimize the water ratio in the mobile phase, the study was further investigated at variation in water ratio at 2, 5, and 10% levels. Among three levels, at 2% water level, the finest resolution was observed and optimized (Fig. 4).

Validation

Specificity and Selectivity

The method specificity and selectivity of the developed chiral method were assessed in the presence of its enantiomer from products. Peak purity and the threshold were calculated and represented in Table 1, and Figs 5 and 6, and were within the limit.

Linearity

The linearity of the present technique was estimated in a concentration range from LOQ to 150% with respect to analyte concentration at 0.5 mg mL⁻¹ for the enantiomers by processing six standard solutions. The values of intercept, slope, and correlation coefficient of the linearity graph were reported and represented in Table 2, and Figs. 7 and 8.

Fig. 5: Representative chromatogram of ELGX blank

Fig. 6: Representative chromatogram of ELGX spiked sample at 100% level

Table 1: Peak purity of ELGX and its enantiomer

Spiked sample result	RT (min)	Purity factor	Threshold
ELGX enantiomer	8.46	999.99	999.625
ELGX API	9.702	999.987	999.245

RT: Retention time

Table 2: Linearity of ELGX enantiomers

Concentration level	ELGX API		ELGX enantiomer	
	Conc. in $\mu\text{g/mL}$	Average area ^a	Conc. in $\mu\text{g/mL}$	Average area ^a
LOQ	0.0075	948	0.0075	843
50%	0.0748	8,170.5	0.0786	7,805
75%	0.1122	12,255.75	0.1179	12,557.5
100%	0.1496	17,325.5	0.1571	17,413.5
125%	0.187	21,656.875	0.1963	21,766.88
150%	0.2244	26,772	0.2357	26,113.5
CC	0.9993		0.9991	
Intercept	1,497.1		113,782	
Slope	125,145		684.18	

CC: Correlation coefficient; ^a: Two injections**Table 3:** System precision data of ELGX enantiomer

S. No.	Peak area of ELGX enantiomer
1	12,237,348
2	12,222,142
3	12,223,425
4	12,212,120
5	12,200,196
6	12,188,579
Average	12,213,968.3
Standard deviation	17,553.10761
% RSD	0.143713387

RSD: Relative standard deviation

Fig. 7: Linearity of ELGX API**Limit of Detection (LOD) and Limit of Quantification (LOQ)**

LOD and LOQ were assessed as the concentration of the analyte, giving rise to an S/N ratio of 3 and 10, respectively. The LOD/LOQ values of ELGX enantiomer were found to be 2 and 7.5 ng mL⁻¹, respectively (Figs 9 and 10). The findings of the technique prove the sensitivity of the proposed method for the quantification of ELGX enantiomer.

Precision

The precision of the analytical procedure was assessed in terms of system and method precision. The % RSD values

Table 4: Method precision data of ELGX enantiomer

Sample name	Enantiomeric impurity in $\mu\text{g/mL}$
Method precision-1	0.146
Method precision-2	0.149
Method precision-3	0.152
Method precision-4	0.147
Method precision-5	0.154
Method precision-6	0.14
Average	0.148
Standard deviation	0.005
% RSD	3.32

RSD: Relative standard deviation

Fig. 8: Linearity of ELGX enantiomer **Fig. 9:** Representative chromatogram at LOQ level

for system and method precision were found to be 0.14 and 3.32. The findings are shown in Tables 3 and 4.

Table 5: Accuracy data of ELGX enantiomer

Sample name	ELGX enantiomer		
	Spiked conc. in $\mu\text{g/mL}$	Measured conc. in $\mu\text{g/mL}$	% recovery
Accuracy 50%-1	0.076	0.086	113.2
Accuracy 50%-2	0.076	0.083	101.3
Accuracy 50%-3	0.076	0.083	101.3
Accuracy 100%-1	0.151	0.147	93.4
Accuracy 100%-2	0.151	0.149	94.7
Accuracy 100%-3	0.151	0.15	95.4
Accuracy 150%-1	0.227	0.245	105.3
Accuracy 150%-2	0.227	0.24	103.1
Accuracy 150%-3	0.227	0.253	108.8

Table 6: Robustness data of ELGX enantiomers

Variation in parameter		Chromatographic efficiency				
		t_1	t_2	R_s	N_1	N_2
Temperature	15°C	0.963	1.374	4.059	3,578	5,245
	25°C	0.966	1.263	3.816	4,990	7,112
	40°C	1.104	1.259	3.203	8,184	9,941
	0.45	1.121	1.251	3.265	8,411	10,396
Flow rate (mL/min)	0.5	1.097	1.258	3.205	8,317	9,888
	0.55	1.132	1.259	3.096	7,705	9,398
	2%	1.114	1.01	3.2	7,981	10,043
	5%	1.201	1.21	2.9	8,641	11,178
% water	10%	1.101	1.014	2.5	9,418	12,412

R_s : Resolution; N: Theoretical plates; t: Tailing factor

Fig. 10: Representative chromatogram at LOD level

Accuracy

Method accuracy was processed, evaluated, and the respective results were shown in Table 5. The percentage recoveries of the enantiomer were found between 93.4 to 113.2%. The findings of the accuracy data concluded that the developed analytical method was accurate.

Robustness

The method robustness was processed and evaluated for resolution and % RSD by varying the optimized chromatographic conditions, like mobile phase composition, flow rate, and COT. The resolution between isomers was more than two, and the % RSD values were found between 2 to 2.7. The chromatographic efficiency results are shown in Table 6.

CONCLUSION

A specific, sensitive, and accurate stereo selective chiral technique has been developed for the quantification of ELGX enantiomer in drug products and drug substances by chiral HPLC. The enantiomers were eluted on a CSP comprising a zwitterionic selector derived from cinchona alkaloid and sulfonic acid [Chiralpak ZWIX(+)] under isocratic condition containing 50 mM acetic acid and 25 mM diethylamine in water and methanol in the ratio of 2:98 v/v with a flow rate 0.5 mL min⁻¹ at the column oven temperature 40°C with a resolution factor more than 3.2. The eluted compounds were monitored at 275 nm. The percentage recoveries of the enantiomer were found between 93.4 to 113.2%. The method was found to be stereoselective with LOD and LOQ values of 2 and 7.5 ng/mL, respectively. The validated method can be successfully applied in routine analysis of ELGX enantiomers in bulk and formulations by pharmaceutical organizations.

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