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RESEARCH ARTICLE

Development and Validation of Chiral HPLC Method for the Identification and Quantification of Enantiomer in Posaconazole Drug Substance

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Abstract: A simple and sensitive normal phase isocratic high performance liquid chromatographic method was developed for the determination of trace level enantiomer in posazonazole drug substance. The separation was achieved using chiral pack, IC, 250 x 4.6 mm, 5 µm using mobile phase containing isopropyl alcohol, dichloro methane and diethyl amine (50:50:0.1 v/v/v). The flow rate was 0.6 mL/min and column temperature was 25 °C. Enantiomer was detected using UV detector at the wave length 262 nm. The retention time was 18.5 min for enantiomer and 28 min is for posaconazole. The optimized method was validated to prove its performance characteristic by demonstrating selectivity, sensitivity (Limit of detection and Limit of quantification), linearity, precision and accuracy. The limit of detection and limit of quantification of ennatiomer was found to be 0.06 µg/mL and 0.2 µg/mL for 10 µL injection volume. The linearity of the method is found to be 0.2 µg/mL to 1.5 µg/mL. Relative standard deviation of system precision, method precision is found to be 0.3% and 0.44% respectively. The accuracy of the method is found to be 96.0% to 98.1%.

Keywords: HPLC, Chiral chromatography, Normal phase, Posaconazole, Validation

Introduction

Posaconazole, chemical name 4-{4-[4-(4-{[(3R,5R)-5-(2,4-difluorophenyl)-5-(1*H*-1,2,4-triazol-1-ylmethyl)oxolan-3-yl]methoxy}phenyl)piperazin-1-yl]phenyl}-1-[(2S,3S)-2-hydroxypentan-3-yl]-4,5-dihydro-1*H*-1,2,4-triazol-5-one which is used a antifungal agent. The biological activity of chiral substances often depends up on their stereochemistry. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers and many of them show significant enantioselective difference in their pharmacokinetics and pharmacodynamics[1-3]. Analysis of the enantiomeric purity of chiral drug candidates has

become very important particularly in the pharmaceutical and biological fields. Because few enantiomers of racemic drugs have relatively different pharmacokinetics properties and divers pharmacological or toxicological effects[4-6].

Literature survey show some work related to posacoanzole assay in biological fluids applying mainly chromatographic methods[7-10]. Considering, the analysis in bulk or pharmaceutical products, there is no work published and no monograph available in pharmacopoeias. So, the objective of this work is to develop and validate the chiral HPLC method for the determination of trace level enantiomer in posaconazole drug substance. Hence no HPLC method is reported for the estimation of enantiomer in posaconazole drug subastance. In the present work, a successful attempt has been made to estimate the enantiomer in posaconazole drug substance. The posacoanzole empirical formula is $C_{37}H_{42}F_2N_8O_4$ and its molecular weight is 700.8 and the chemical structure is shown in (Figure 1).

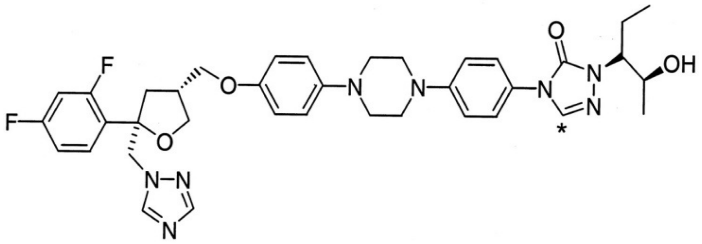

Figure 1. Molecular structure of Posaconazole (SCH) 56592

HPLC is a conventional effective analytical technique. The optimized HPLC Normal phase isocratic method was validated according to ICH guidelines[11-15]. To prove its suitability and reliability for the determination of enantiomer (Figure 2) content in posacoanzole drug substances during routine analysis.

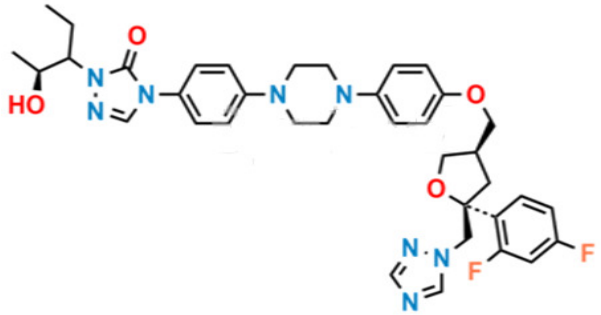

Figure 2. Molecular structure of enantiomer

Experimental

Analytical reagent grade of isopropyl alcohol, dichloro methane, diethyl amine procured from Merck chemicals, India. Analytical reagent grade of ethanol procured from china. Posaconzole, enantiomer and related impurities are gift samples from reputed Pharma company.

Instrument

The method development and method validation was performed in Agilent 1200 series liquid chromatographic system with diode array and variable wavelength detector. The data were collected and processed using Empower3 software. The peak homogeneity was studied by using Agilent 1200 series DAD Detector.

Chromatographic conditions

Chromatography was carried out by using Agilent technologies 1200 series instrument equipped with column oven, UV detector and the data was processed using Empower3 software, the chromatographic conditions were optimized using chiral stationary phase, chiral pack IC column (250x4.6 mm, 5 µm, Daicel, Japan). The isocratic mobile phase composition was a mixture of isopropyl alcohol, dichloro methane and diethyl amine (50:50:0.1v/v/v). It was pumped at flow rate of 0.6 mL/min, the temperature of the column was mentioned at 25 °C and the diluent was monitored at wavelength 262 nm. The injection volume was 10 µL. Ethanol is used as a diluent.

The chromatographic parameters including retention factor(k), separation factor(α) and the resolution(RS) were selected to evaluate the separation of compounds. All the chromatographic results are reproducible.

Preparation of mobile phase

500 mL of isopropyl alcohol and 500 mL of dichloromethane were mixed and 1 mL of diethyl amine was added.

Preparation of enantiomer impurity standard stock solution

Accurately 10 mg of enantiomer impurity standard was weighed and taken into 100 mL volumetric flask 50 mL of diluent was added and sonicated to dissolve and diluted to volume with diluent (Stock-I).

Prepartion of posaconazole standard solution

Accurately 10 mg of posaconazole standard was weighed and taken into 100 mL volumetric flask and 50 mL of dilunet was added and sonicated to dissolve and diluted to volume with the diluent (Stock-II).

Preparation of Retention Time (RT) identification solution

1.0 mL of stock-I and stock-II solution were pipetted out into 100 mL volumetric flask with the diluent. Resulting solution contains 0.10% of enantiomer impurity and posaconazole with respect to the sample concentration of 1.0 mg/mL (Figure 3).

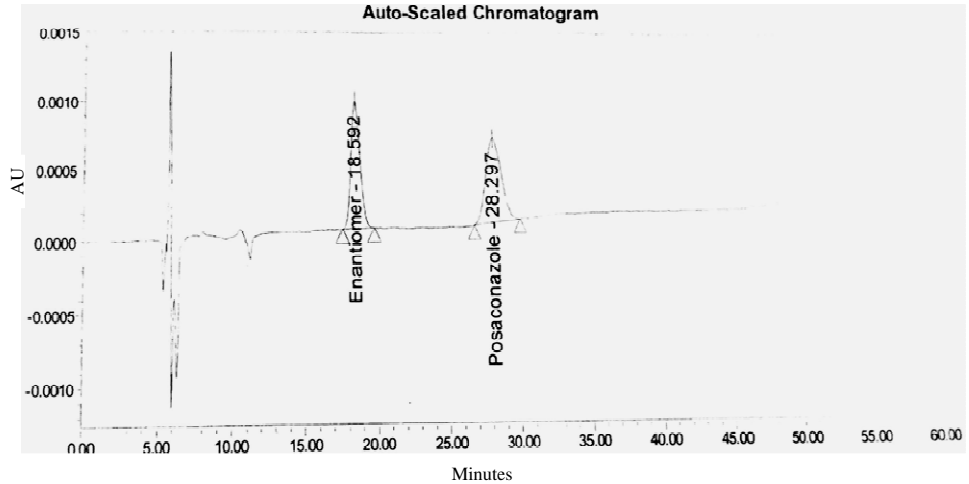

Figure 3. Chromatogram of RT identification solution

Preparation of diluted standard solution

1.0 mL of the stock-II solution was pipetted out into 100 mL volumetric flask with diluents Resulting solution contains 0.10% posaconazole with respect to the sample concentration of 1.0 mg/mL.

Sample preparation

About 50 mg of posaconazole sample was weighed and taken into a 50 mL volumetric flask and 30 mL of diluent was added, sonicated to dissolve and made up to the mark with the diluent and mixed well. Final concentration of the sample is 1.0 mg/mL.

Procedure

10 µL of diluent (blank) in duplicate, RT identification solution, diluted standard solution (6 injections), diluent and sample preparation (2 injections) were separately injected as per Table 1 and the chromatograms were recorded.

Table 1. Sequence table

Sample Name	Number of injections	Type of testing
Diluent	1	Blank
RT Identification solution	1	RT Identification
Diluted standard solution	6	Diluted standard solution
Diluent	1	Blank
Sample solution	2	Sample
Diluent	1	Blank
RT Identification solution	1	RT Identification
Diluted standard solution	1	Bracketing diluted standard solution

Results and Discussion

Method development

Racemic mixture solution of posaconazole and enantiomer (1000 µg/mL) prepared in dilute was used in method development. To develope a rugged chiral HPLC method for the separation of 2 enantiomers. Diffrent stationary phases and mobile phases were attempted.

To develop a normal phase HPLC method for the separation of each enantiomer and drug substance, different stationary phases and mobile phases were used. Diffrent stationary phase like chiralpak IA, IB, IC, ID and IE are tried and different combinations of mobile phases consisting *n*-hexane, methanol, ethanol, isopropanol, dichloro methane, diethyl amine. Finally well separation is achieved in CHIRALPAK IC (250x4.6 mm, 5 µm) column with mobile phase consists of isoproply alcohol, dichloromethane and diethyl amine in the ration of (50:50:0.1v/v/v). With this mobile phase optimum resolution and selectivity for two enantiomers. Based on the data obtained from method development and optimization activities the flow rate of final method was 0.6 mL/min with injection volume 10 µL and the column temperature was 25 °C. The detection wavelength was 262 nm sample temperature was 5 °C under these conditions the two enantiomers were separated well and the peak of enantiomer and posaconazole were 18.5 and 28 min.

Validation results of the method

The HPLC chiral method was evaluated for its specificity, sensitivity, LOD (limit of detection), LOQ (Limit of quantification), linearity, accuracy, precision and solution stability.

Specificity

The specificity of the method was determined by using peak purity. The purity angle is less than purity threshold. There is no interference of blank at enantiomer RT and posaconazole RT. From the above two considerations the methods are said to be specific (Table 2).

Table 2. The specificity data of the method

Compound	Purity angle	Purity threshold	Peak purity
Enantiomer	0.212	0.516	Pass
Posaconazole	0.312	0.426	Pass

Sensitivity

The limit of detection (LOD) (Figure 4) and limit of quantification (LOQ) (Figure 5) were predicted using slope (S) and residual standard deviation that obtained from linear regression, is being one of the three approaches described in ICH guidelines. The formula used for the prediction of LOD and LOQ were 3.3xSD/S and 10xSD/S respectively. The predicted LOD and LOQ values were found to be 0.06 µg/mL and 0.2 µg/mL and analysed these predicted LOD and LOQ values. The %RSD was found to be 3.6 and 6.45 respectively. Thus the LOD and LOQ values were established to determine the content of enantiomer in posaconazole drug substance (Table 3 and 4).

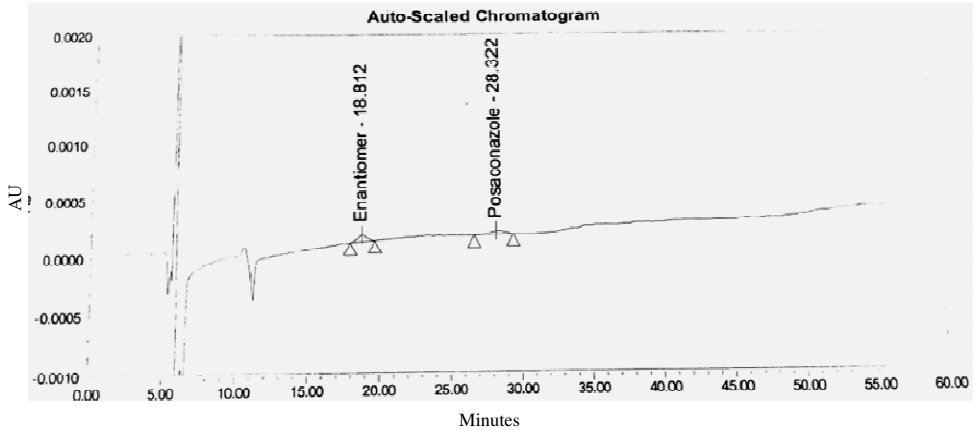

Figure 4. Chromatograph for the determination of LOD of enantiomer and posaconazole

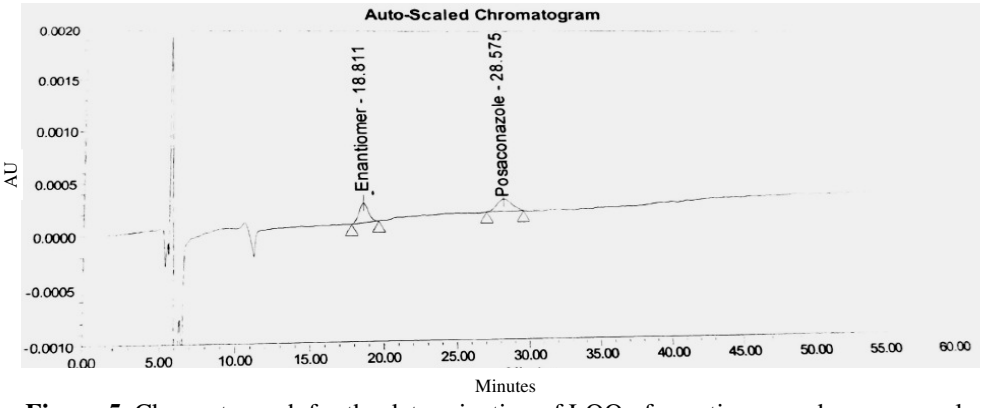

Figure 5. Chromatograph for the determination of LOQ of enantiomer and posaconazole

Table 3. Limit of detection for enantiomer and posaconazole

Compound	Enantiomer area	Posaconazole area
Injection-1	2455	2466
Injection-2	2549	2893
Injection-3	2780	2634
Mean	2595	2664
SD	167.24	215.11
%RSD	6.45	8.07

Table 4. Limit of quantification of enantiomer and posaconazole

Compound	Enantiomer area	Posaconazole area
Injection-1	8690	8794
Injection-2	8033	8705
Injection-3	8513	8766
Injection-4	8337	8910
Injection-5	7981	8831
Injection-6	8637	8791
Mean	8365	8800
SD	303.31	68.35
%RSD	3.63	0.78

Linearity

Good linearity of enantiomer was evaluated over 6 levels of enantiomer solutions from 0.2 µg/mL to 1.5 µg/mL. With the linear regression equation y=mx+c, where x is concentration in µg/mL and y is corresponding peak area of undesired enantiomer. We observed linear results with respect to concentration of enantiomer *vs.* area of enantiomer.

To establish correction factor we performed linearity of posaconazole drug substance. A series of solutions of posaconazole were prepared from 0.2 µg/mL to 1.5 µg/mL and based on that correction factor was established (Table 5) (Figure 6 and Figure 7).

Table 5. Linearity of enantiomer and posaconazole

n	Enantiomer concentration, µg/mL	Enantiomer area	Posaconazole concentration, µg/mL	Posaconazole area
1	0.205	8412	0.199	8755
2	0.513	20984	0.499	21755
3	0.822	32979	0.798	35242
4	1.027	42327	0.997	46123
5	1.232	51757	1.197	55649
6	1.540	62886	1.496	67641
CC	0.999		0.999	
R^2	0.999		0.999	
Slope	41304.673		46277.209	
Y-Intercept	-202.8882		-798.9401	
STEYX	708.0472		935.70625	
LOD, µg/mL	0.06		0.06	
LOQ, µg/mL	0.20		0.20	

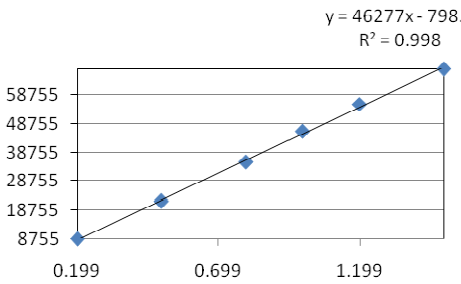

Figure 6. Linearity curve of posaconazole

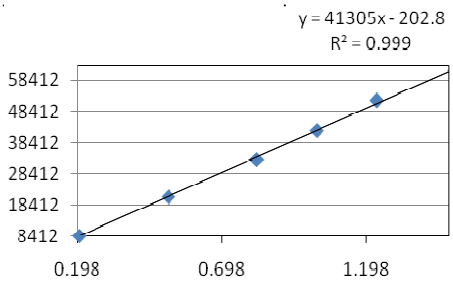

Figure 7. Linearity curve of enantiomer

The correlation coefficients were greater than 0.999, which meet the method validation acceptance criteria and hence the method is said to be linear in the range of 0.2-1.5 µg/mL.

$$\text{Correction Factor} = \frac{\text{Slope of the drug}}{\text{Slope of the Enantiomer}} = \frac{46277.209}{42940.501} = 1.08$$

System precision

System precision was demonstrated by analyzing six replicate injections of standard solution (Enantiomer with respect to 1 mg/mL sample concentration). This indicates the acceptable reproducibility and there by the precision of the system. The relative standard deviation (RSD) should be less than 5% (Table 6).

Table 6. System precision of enantiomer (Diluted standard area)

n	Area of Enantiomer
1	45974
2	46198
3	46197
4	46338
5	46202
6	46067
Mean	46163
SD	126.12
%RSD	0.30

Method precision

Repeatability of the test method (method precision) was demonstrated by analyzing six separate sample solutions were prepared using single batch of posaconazole drug substance with known amount of enantiomer spiked in sample solution and the percentage relative standard deviation of enantiomer was found to be less than 5.0% (Table 7 & Figure 8).

Accuracy

The accuracy of the test method was demonstrated by preparing sample solution spiked with known amount of enantiomer at three different levels from LOQ, 100% and 150% of specification level and calculated the enantiomer content (Table 8). The recovery value of enantiomer ranged from 96.7 to 98.1 and the average recovery of three levels (nine determinations). The accepted limits of recovery are 95-99% and all observed data are within the required range which indicates good recovery values and hence the accuracy of the method developed.

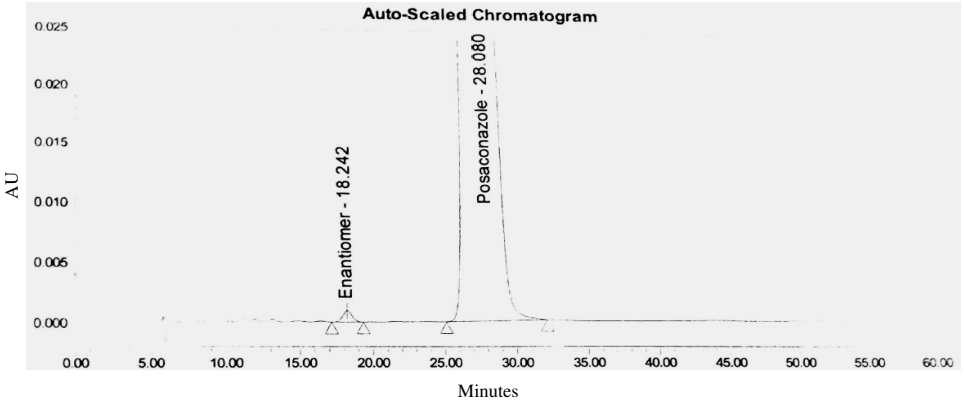

Figure 8. Enantiomer spiked to posaconazole at 0.1% level

Table 7. Method precision results of enantiomer (0.10%) spiked in posaconazole

n	Enantiomer content, %w/w	Enantiomer content, %area
1	0.10	0.10
2	0.10	0.10
3	0.10	0.10
4	0.10	0.10
5	0.10	0.10
6	0.10	0.10
Mean	0.10	0.10
SD	0.00	0.00
%RSD	0.44	0.54

Table 8. Recovery results from spiking of posaconazole with enantiomer

Accuracy (Average of triplicates)	Level-I (LOQ)	Level-II (100%)	Level-III (150%)
Amount added, µg/mL	0.199	0.997	1.496
Amount found, µg/mL	0.196	0.970	1.447
Recovery, %	98.1	97.2	96.7
RSD, %	2.15	1.60	1.56

Results reporting

Enantiomer counter was performed area normalization method and diluted standard method. The comparison results are shown in the above Table 7. There is no results variation in area normalization method diluted standard method. We can report area normalization method for routine estimation of enantiomer in posaconazole drug substance.

Conclusion

A sensitive normal phase HPLC method was developed, optimized and validated for the quantitative determination of enantiomer in posaconazole drug substances as per ICH guidelines.

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