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Enantioselective method development and validation of proline by using high performance liquid chromatography

Nagamalleswari Gampa*, Prachet Pinnamaneni, Naga Thirumalesh Chevala, Geervani Pamidmarri, Arulsamy Elphine Prabahar, Venkata Suresh Ponnuru and Rama Rao Nadendla

Department of Pharmaceutical Analysis, Chalapathi Institute of Pharmaceutical Sciences, Lam, Guntur, Andhra Pradesh, India, Pin code: 522034

Abstract

Chirality is a major concern in the modern pharmaceutical industry. The separation of chiral compounds has been of great interest because the majority of bioorganic and synthetic molecules are chiral. Aim of the present investigation was to develop a stereo specific, simple and precise normal phase high performance liquid chromatography (NP - HPLC) method for the separation and enantiopurity of Dextro (D) & Levo (L) enantiomers of proline (PRO) by using Lux 5µm Amylose – 1 LC column (250×4.6mm) by using n- Hexane: Iso propyl alcohol (IPA)as mobile phase in the ratios of 90:10 v/v at flow rate of 1.2 ml/ min. D & L forms of PRO was detected at 210nm with retention time of 8.1min and 9 min respectively with correlation coefficient (R^2) of 0.999.The method was validated with reference to International conference of harmonization (ICH) in terms of linearity, accuracy, precision (Inter - day and intra - day precision), limit of detection (LOD), limit of quantification (LOQ), stability of test solutions, specificity, system suitability, robustness and ruggedness.

Keywords: Enantiomers, enantioselective, enantiopurity, high performance liquid chromatography.

1. Introduction

Enantiomers are the two identical chemical molecules which differ each other by non-superimposable mirror images. Chirality of molecule depends upon the presence of one or more number of chiral centers or chiral plane or chiral axis etc. Different types of enantiomers were designated by various designations, it is done in accordance to Cahn – Ingold – Prelog for R and S systems. Octahedral structured molecules were designated by using Delta – Lambda. D and L Fisher – Ransoff should be used for the designation for amino acids and sugars. During 1980s: interest in the quantitative and qualitative estimation of enantiomers of the chemical moieties irrespective of their use was found to be important, due to variation in their activities. Enantiomers differ in their pharmacological activity, thereby estimation and separation of enantiomers got importance in the field of pharmaceutical analysis. Among all chromatographic techniques used HPLC is one of the best methods which can be used for the enantiopurity estimations and also for their separation with immobilized stationary phase. In the previous scenario enantiopurity was estimated by using achiral technique, by using chiral additives or chiral mobile phases and other method involves usage of auxillary chiral reagents which helps in the conversion of enantiomers into diastereomers there by enantiopurity can be determined by using reverse phase HPLC method. In the present scenario by using chiral stationary phase enatiopurity was estimated. Among all the chiral stationary phases, amylose and cellulose packed columns were most commonly used, due to wide range of applications. Resolution of peaks and separation of the enantiomers depends on the composition of mobile phase, pH and temperature of the column used. Proline is a natural nonessential amino acid which is synthesized by mammalian tissues it helps in the formation of collagen at joints and tendons. It is a nonpolar hydrophobic, hetero cyclic amino acid which is referred as imino acid which is part with 5 membered ring structures. Proline is available in 2 enantiomeric forms D and L forms. Among two forms L form is found to have key role in protein synthesis and also helps in normal physiological functioning of humans.

2. Experimental Conditions:

2.1 Materials Procurement:

Dextro (D) and Levo (L) forms of proline (PRO) was purchased from Sigma Aldrich and Isopropyl alcohol, n- Hexane and alcohol HPLC grade were purchased from Merck life science Pvt. Ltd, Mumbai.

2.2 Equipment:

Method development and validation was performed on HPLC, Mfg. by Agilent, model: 1200 infinity LC, with UV detector, amylose- 1 column (250mm × 4.6mm, 5µ) Phenomenex India Pvt Ltd was used for the separation and quantitative estimation. ATX - 224 analytical electronic balance was used for weighing Mfg. by Shimadzu, for pH measurement Titrasys - 352 model Mfg. By Systronics was used.

2.3 Chromatographic Conditions:

Enantiotropic separation was carried on Amylose-1 column, which is initially flushed with Iso Propyl Alcohol for 6 hrs at a flow rate of 0.5ml/min.

Corresponding Author*: malleswari.naga2011@gmail.com

Then the column was saturated with n-hexane and Iso Propyl Alcohol mixture for 1hr. A mixture of n-Hexane: Iso Propyl Alcohol at 90:10(v/v) ratio was taken as mobile phase and which is previously filtered by passing through 0.45 micron nylon membrane using vacuum filtration technique. Then it was sonicated for 30 min using water bath sonicator for degassing.

2.4 Separation of samples:

Approximately 10 mg of D and L forms of PRO were weighed and transferred to 10 ml volumetric flasks containing 5ml of mobile phase, shake for few minutes until drug gets dissolved. Finally volume was made up to the mark.

2.5 Method Validation:

Enantioselective method was developed; method was validated with reference to ICH guidelines. Method was validated for the linearity, precision, accuracy, LOQ, LOD, robustness and ruggedness. Details of validated parameters were mentioned in the results and discussion.

2.6 Linearity

Regression curve was constructed by detector response linearity assessment. Three sets of standard solutions were prepared in the linearity range of 25 to 125 µm/ ml containing racemate mixtures of PRO in mobile phase. 20µL standard solution was injected into the column, RT and peak area of the response were calculated. Regression curve was obtained by plotting concentration *(x- axis)* Vs peak area *(y- axis)*. Regression (R^2) and slope were calculated from the plot.

2.7 Precision and accuracy

Mean concentration was selected from the linearity, inter- day and intra- day precision was calculated from 6 replicate successive runs (n= 6) by different analysts. Accuracy for the developed method was also studied. % RSD was calculated for the precision and accuracy.

LOD and LOQ

LOD and LOQ were calculated by using following formulas:

LOD = 3.3σ/S

LOQ = 10σ/S

S – Slope of the standard graph.

σ - *y* intercept of the regression line

2.8 Robustness

Robustness of the method was determined based on the resolution of peak of the racemate mixture and separation of racemate mixture of PRO by altering chromatographic conditions. Purposefully flow rate was changed by increment and decrement of 0.2 ml to that of actual flow rate and peak resolution was calculated, similarly mobile phase composition altered to determine the effect of mobile phase on peak resolution and separation of racemate mixture.

2.9 Ruggedness

Ruggedness of the developed method was determined by calculating inter day and intraday precision for the mean standard concentration.

3. Results and discussion:

3.1. Linearity:

Table 1: Data of linearity

S. No	Concentration (µg/ ml)	Peak Area
1	25	5836489
2	50	11176389
3	75	16935268
4	100	22083764
5	125	26994726

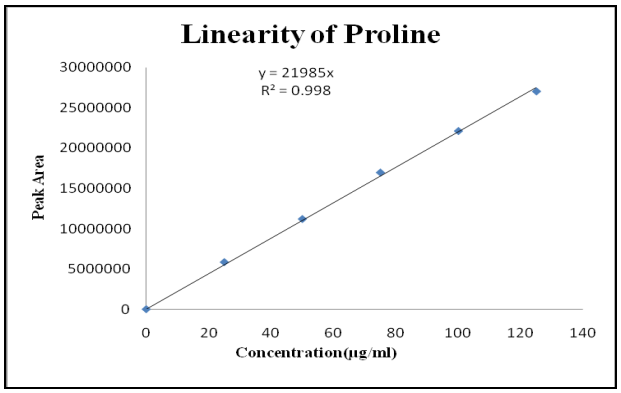


Fig 1: Linearity plot of Proline

3.2. Precision:

3.2.1 Interday:

Table 2: Data of interday precision

Concentration(µg/ml)	Trail	Peak Area
100	t1	21346522
100	t2	21380512
100	t3	21462461
100	t4	21542158
100	t5	21649125
100	t6	21125435
SD		180509.8
%RSD		0.842807

3.2.2 Intraday:

Table 3: Data of intraday precision

Concentration(µg/ml)	Trail	Peak Area
100	t1	21380512
100	t2	21465325
100	t3	21215421
100	t4	21354921
100	t5	21245582
100	t6	21345215
SD		91488.07
%RSD		0.428827

3.3. Accuracy:

Table 4: Data of accuracy

Nominal Concentration	% Accuracy	Amount spiked (µg/ml)	Final Concentration	Trails	Amount recovered (µg/ml)	% Recovery
100	80%	100+80	180	t1	179.4	99.66
				t2	179.6	99.77
				t3	180.1	100.05
				AVG		99.833
100	100%	100+100	200	t1	199.82	99.91
				t2	201.25	100.625
				t3	200.61	100.305
				AVG		100.28
100	120%	100+120	220	t1	220.6	100.272
				t2	221	100.454
				t3	220.4	100.181
				AVG		100.303

3.4. Robustness:

Table 5: Data of robustness

Parameters	USP resolution between (S)- Proline and (R) – Proline	% RSD
Flow rate		
	0.3	0.441
	0.5	0.145
	1.7	0.434
Mobile phase ratios (n- Hexane:IPA)		
	85:15	0.612
	90:10	0.425
	95:05	0.299

3.5. Ruggedness:

Table 6: Data of ruggedness

Concentration (µg/ml)	Trail	Peak Area
100	t1	21380512
100	t2	21475124
100	t3	21682512
100	t4	21542152
100	t5	21642158
100	t6	21284215
SD		152805.9
%RSD		0.710688

3.6. Limit of Detection and Limit of Quantification:

The limit of detection (LOD) for L enantiomer was found to be 2.784 for 10µl of injection volume. The limit of quantification (LOQ) for L enantiomer was found to be 8.436 for 10µl of injection volume.

Fig 2: Blank Chromatogram

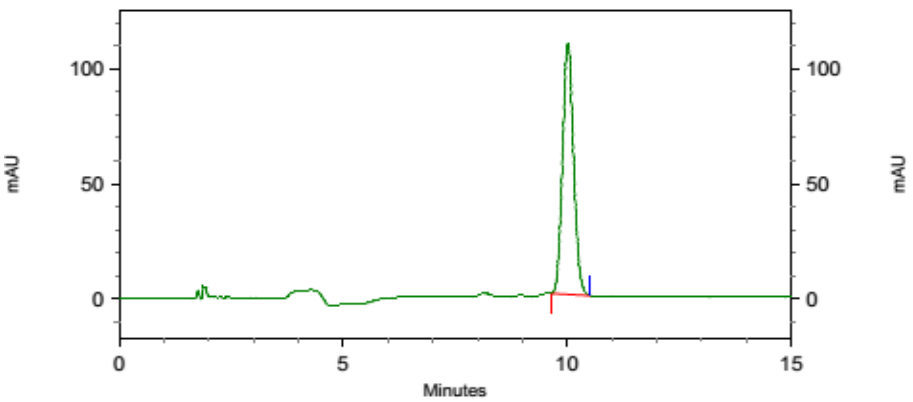


Fig 3: Z Proline Chromatogram

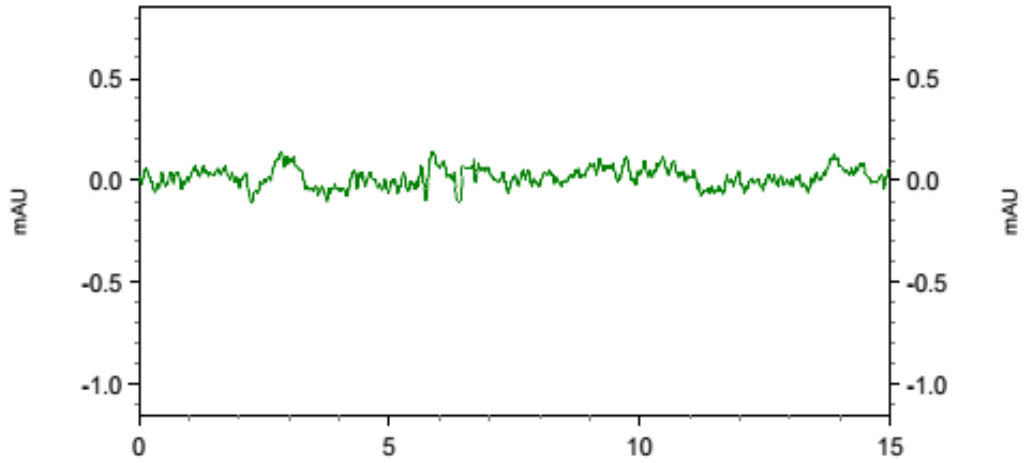
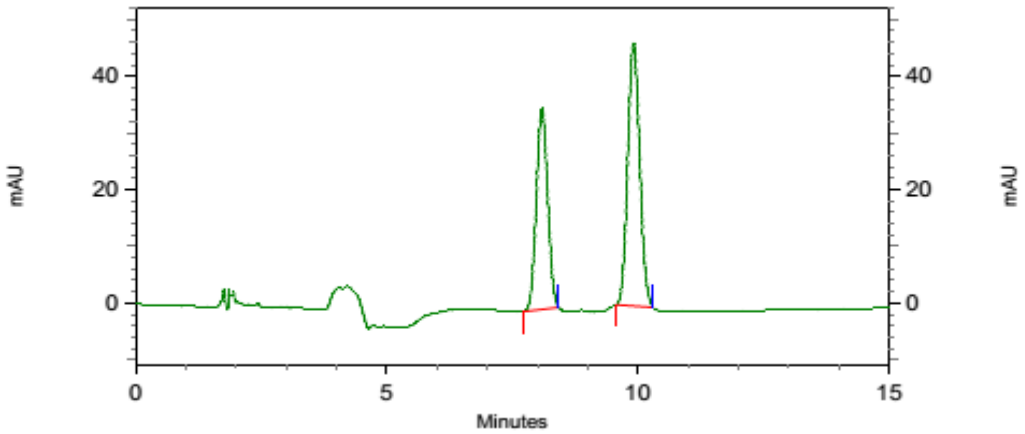


Fig 4: Mixture of L and D Proline Chromatogram



4. Conclusion

Stereo specific, simple and precise method was developed and validated for the enantiopurity estimation of Proline. Developed method was found to have stereo specificity with acceptable linearity, precision and accuracy. So the developed method can be used for the separation and enantiopurity estimation.

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Conflict of Interest

This article does not contain any studies with human and animal subjects performed by any of authors. All authors declare that they have no conflict of interest.

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