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DERIVATIZATION OF PROLINE FOR THE ENANTIOMERIC SEPARATION AND ESTIMATION OF D-PROLINE IN L-PROLINE BY NP-HPLC

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ABSTRACT

The core object of this exertion is to progress and validate a modest, competent and explicit method for the exodus of D and L isomers of proline in a racemic mixture and limit the content of D-Proline in commercial L-Proline. This has been technologically advanced in a chiral method using normal phase HPLC on CHIRALPAK-IA (250X4.6 mm) 5 µ column. This progress advanced with polar mobile phase ethanol encompassing modifier/additive TFA in 0.1% concentration. Due to the chromophore's deficiency in proline, proline's derivatization has been done using fluorescent reagent NBD-Cl. After derivatization, proline has made UV detectable at 465 nm. Within run time of 20 minutes, D and L isomers of proline were eluted at 6.72 and 9.22 minutes, respectively. The technologically advanced method was validated as per ICH guidelines. Linearity regression coefficients for both D and L-Proline are obtained as 0.999. Retrieval for D-Proline was obtained at 93 to 95% range for 4 levels. LoD and LoQ for both D- and L-Proline were detected as 0.6 and 2 ppm, respectively.

Hence this method is newer, modest, particular and explicit chiral method.

KEYWORDS: Proline, CHIRALPAK-IA, NBD-Cl, NP-HPLC and D- and L-Proline.

INTRODUCTION

A molecule is considered to have handedness if there is another molecule with the exact same atoms but arranged in a mirrored layout that cannot be aligned identically. The presence of an asymmetric carbon atom is frequently what causes handedness in molecules. Handed molecules are molecules whose mirror image versions cannot be matched up on top of each other. The two-handed forms of a racemic drug frequently exhibit different properties in how the drug works pharmacologically and is metabolized in the body. It is common for one-handed version of a drug to be therapeutic while the other-handed form is toxic to biological systems. The different chemical characteristics of the handed forms can change how the drug interacts with the body and where it has an effect. Proline is a non-essential amino acid that mammals produce. Proline is mostly involved in collagen formation at tendons and joints. Proline is non-polar, hydrophobic and heterocyclic amino acid; more precisely can be referred as, an imino acid, which is a part of a five-member ring in a molecule. Literature survey revealed that different methods have estimated it (Scheme 1).[1-7]

MATERIALS AND METHOD

Chemicals

The dextrorotatory (D) and Levorotatory (L) forms of the amino acid proline were obtained fr om Sigma Aldrich. The fluorescent probes NBD-Cl and NBD-F were obtained from Alfa-Aesar. Merck Life Science Pvt. Ltd in Mumbai, India, was contacted in order to get methanol, isopropanol, n-hexane, and trifluoroacetic acid as solvents.

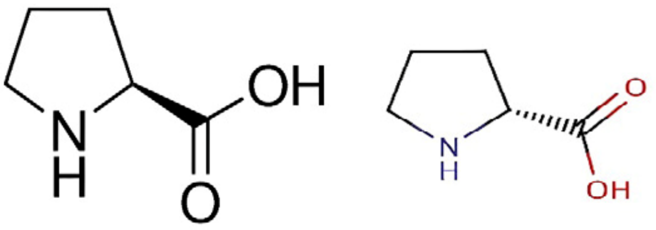
Equipment

The HPLC technique was created and tested using a Waters Alliance HPLC system equipped with a 2998 photodiode array detector. Columns used included a CHIRALPAK AD-H (250 mm × 4.6 mm) 5 µm column, a CHIRALPAK IC (250×4.6 mm) 5 µm column, and a CHIRALPAK IA (250 x 4.6 mm) 5µm column all obtained from Phenomenex India Pvt Ltd.

An ATX - 224 analytical balance manufactured by Shimadzu was used for pH measurements.

Derivatization

Both isomers of proline were reserved at 1-mg/mL concentration into a volumetric flask augment NBD-Cl (4-Choloro-7-nitrobenzofurazan) reagent at 3 mg/mL concentration.



L-Proline D- Proline

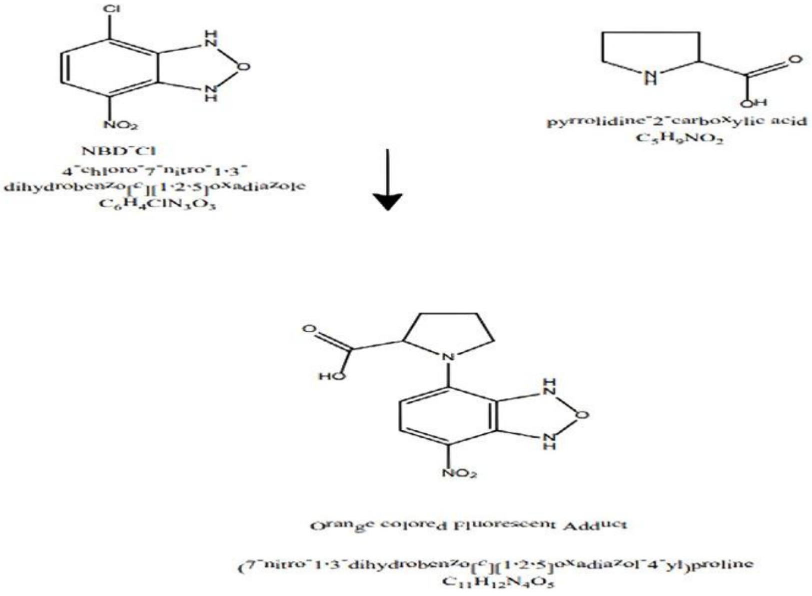
Scheme 1: Structure of L-Proline (2S)-2-Pyrrolidinecarboxylic acid and D-Proline (2R)-2-Pyrrolidinecarboxylic acid.

Table 1: Method validation results were summarized in the following table.

System suitability parameters	**Observed value**		**Acceptance criteria (As per *USP*)**
	D-Proline	**L-Proline**	
Tailing factor	1.25	1.11	NMT 2.0
Theoretical plate count	10906	10318	NLT 2000
Resolution between D-Proline & L-Proline		8.93	NLT 2.0

Reaction

Derivatisation reaction between NBD-Cl and Proline as shown in Scheme 2.



Scheme 2: Derivatisation reaction between NBD-Cl and Proline

Chromatographic Conditions Enantiomeric separation was achieved using a CHIRALPAK-IA (250 × 4.6 mm) 5 µm column. A solution of 0.1% trifluoroacetic acid in ethanol was used as the mobile phase. The conditions for the chromatographic separation used isocratic elution at a flow rate of 0.6 mL/min, a column temperature of 400℃, an injection volume of 10 L, and a detector wavelength of 464 nm.

Preparation of Samples

In 2 mg/mL DandL-Proline, 4 mg/mL NBD-Cl in ethanol was derivatized at 60°C for 10 minutes**.** In 2 mg/mL derivatized racemic mixture has been organized. From this stock series of standard solutions in the range of LoQ to 150% levels were prepared.

RESULTS AND DISCUSSION Method Validation

To validate a technique, one must first determine its performance characteristics and then identify the elements that may affect those features and to what extent. The goal of method validation is to provide confidence in the reliability of the data produced by the method. Data's ongoing reliability and comparability can be certain only through the execution of a quality assurance system with the application of method validation deliberating to internationally recognized procedures and performance criteria. Method validation consequences were summarized in Table 1.

Specificity

Specificity, as defined by the International Council for Harmonization (ICH), is defined as the capacity to reliably identify the analyte of interest in the presence of other predicted sample components, such as contaminants, degradants, or matrix components. The capacity of an analytical procedure to separate the analyte(s) of interest from other components of a sample is referred to as its "specificity." A

study evaluated potential interference from excipients used as a placebo. An equivalent amount of placebo as specified in the test method, was analyzed in duplicate. Solutions of D-Proline, L-Proline and blank solution were prepared with a 4 mg/mL concentration of the derivatizing reagent in ethanol. The derivatized blank, standards, and impurity were injected individually into the system to determine if either proline enantiomer interfered with the blank. Retention time, tailing factor, theoretical plates and resolution were summarized in Table 2.

Linearity

To determine the linearity of an assay, five or six standards are injected at various doses spanning from the LoQ to 150% of the assay's dynamic range. A series of five to six injections of five or more standards with concentrations ranging from the lower LOQ to 150% of the predicted concentration range should be performed. to ensure that the analytical reaction is proportionate to the concentration The concentration of the analytes should have some kind of linear relationship with the response, or the relationship should be well-defined mathematically. When applying linear regression to the data, the intercept shouldn't be too far from zero. If a statistically significant nonzero intercept is found, it must be shown that this does not affect the procedure's reliability. The derivatized racemic mixture was dissolved in water at 2 mg/mL. Standard solutions were created from this stock solution, spanning the LOQ to 150% of the predicted range. The regression line's slope, intercept, and correlation coefficient were calculated after plotting the average peak area against concentration.

Table 2: Summary of development results by NP-HPLC method.

Validation	**Parameters**	**D-Proline**	**L-Proline**
Specificity		No interference	No interference
System suitability	Tailing factor	1.25	1.11
	Resolution		8.93
	Theoretical plates	10906	10318
	% RSD	0.40	1.53
Limit Of Detection (LOD)		(0.03%) 0.6ppm	(0.03%)
	Limit Of Detection (LOD)		0.6ppm
Limit Of	Limit Of Quantification	(0.1%) 2ppm	(0.1%) 2ppm
Quantification			
(LOQ)			
Linearity	Correlation coefficient	0.999	0.999
	(R2)		
	Slope (m)	762.05	771.96
System Precision	%RSD	0.40	1.53
Method Precision	%RSD	0.61	1.00
Accuracy/Recovery	20	93.33	93.33
	50	92.00	92.00
	100	90.00	90.00
	150	95.11	95.11

Limit of Detection and Limit of Quantitation

The LOD of a technique is the minimum detectable concentration of an analyte in a sample, albeit this concentration may not be quantitatively determined. The LOQ is the lowest concentration of an analyte that can be measured with adequate precision and accuracy using the analytical technique under its intended use. The concentration distinguishes whether an analyte is above or below a certain threshold. The LOQ for D-Proline and L-Proline was determined by spiking 0.1% solutions of the enantiomers into blank solution and injecting them into the system. The LOQ was estimated based on the signal-to-noise ratio. The LOD was calculated by diluting a 3.3 mL portion of the 0.1% enantiomer solutions into 10 mL of ethanol diluent.

Precision

Precision is the degree to which numerous analyses of the same homogenous material under the same analytical circumstances provide the same result. It is a metric for assessing the consistency of results across several measurements taken under identical circumstances. The standard deviation or relative standard deviation (coefficient of variance) is a common way to quantify precision. It is a measure of how well the analytical procedure re can be replicated under standard laboratoryy circumstances.

System Precision

The repeatability of the system can be assessed by multiple injections ($n \geq 5$) of a homogeneous sample or standard solution. Six replicate injections of 0.5% D-Proline spiked solution of L-Proline were injected into the system with technologically advanced method conditions.

Method Precision

By injecting multiple preparations of standard, repeatability will limit the precision of method. To determine the method’s repeatability, 6 different preparations of D-Proline solutions spiked into derivatized L-Proline at 0.5% level. Each preparation is injected into the system.

Accuracy and Recovery

Accuracy is defined as the degree to which a result acquired by an analytical procedure agrees with a commonly used standard. There are a variety of routes to the real worth. If the uncertainty of the reference technique is known, one strategy is to compare the findings from the method of interest to those from the reference method. Analyzing samples with known concentrations, such control samples or certified reference materials, and comparing the observed result to the specified correct value is another way to assess accuracy. At least three replicates at three distinct concentrations should be used to establish the reliability of a quantitative technique. The mean value should be within ± 20% of the true value. The precision may be calculated as the deviation between the estimated mean and the actual value.

Recovery describes how well an analytical procedure was able to extract the target analyte. Although complete recovery is unnecessary, the analyte and the internal standard should be recovered consistently, accurately, and repeatable. Samples extracted at low, medium, and high concentrations are compared to 100% recovery standards that were not extracted.

Procedure

Initially, four individual preparations of 2 mg/mL L-Proline standard has prepared in ethanol with derivatization. Similarly single preparation of 2 mg/mL D-Proline standard also prepared. D-Proline has spiked into L-Proline standards individually at four different levels by pipetting 0.1, 0.25, 0.5 and 0.75 mL of D-Proline. i.e., 20, 50, 100 and 150% levels Figure 1. Then, solutions were injected into the system. Recovery in Figure 2.

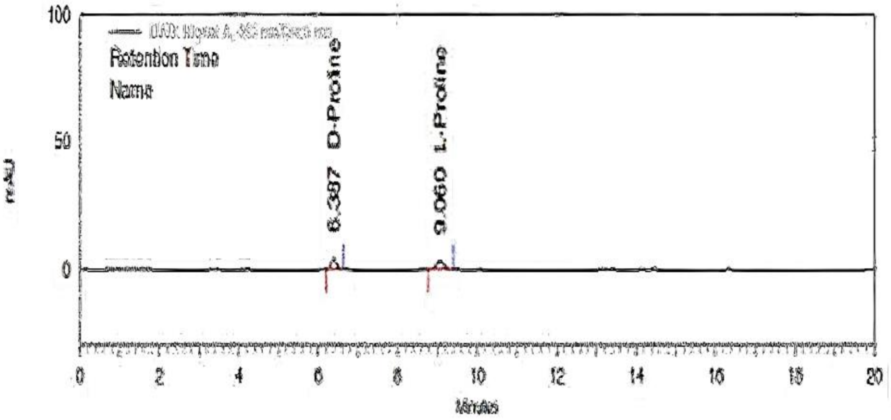


Figure 1: Mixture of D-Proline and L-Proline.

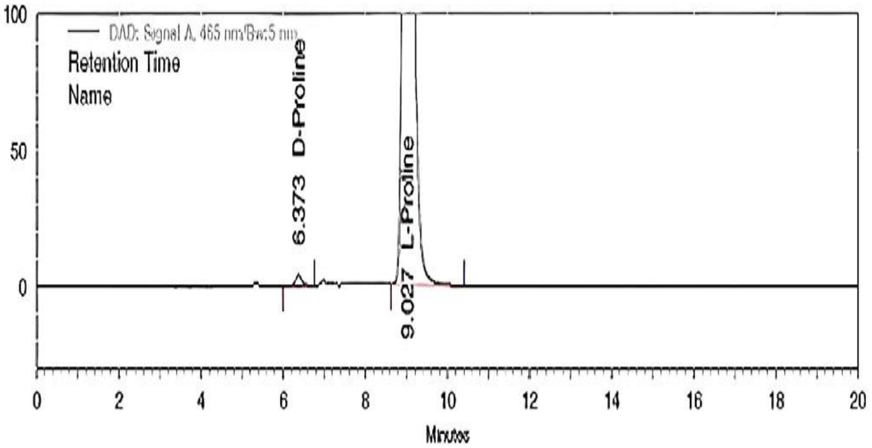


Figure 2: 100% level D-Proline recovery in L-Proline

CONCLUSION

Since proline is hydrophobic, a solvent blend of non-polar n-hexane and midpolar isopropyl alcohol was used. Due to the lack of a chromophore in the proline's structure, its native detection wavelength could not be identified. Therefore, derivatization was performed using the fluorescent derivatizing reagent, NBD-Cl. According to the literature, the detection wavelength for derivatized proline is 464 nm. After derivatization, the mobile phase and flow rate were selected based on the eluted peak parameters. A mobile phase of 0.1% trifluoroacetic acid (TFA) in ethanol with a 0.6 mL/min flow rate was robust. TFA was added as a modifier to obtain a better peak shape. The retention time for D-Proline and L-Proline were 6.72 minutes and 9.22 minutes, respectively. Based on the enantiomer retention times, run time was limited to 20 minutes.

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