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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF HUMAN NEUTROPHIL PEPTIDE-5

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ABSTRACT

Recent Peptide is promising therapeutics for many diseases. Human Neutrophil is a newer cytolytic broad spectrum antimicrobial peptide, having antibacterial, antiviral, antifungal and anticancer like activity. It has molecular weight of 4 kDa. It is mainly known for its antimicrobial and antiviral activity against huge range of gram-negative, gram-positive bacteria and enveloped, non-enveloped viruses. Peptides are complex bioactive molecule, so it is very challenging to analyse all the parameters for development of peptide. The present work describes analytical method development and validation of Human Neutrophil Peptide-5. An easy, accurate and right method has been developed and validated for estimation of Human Neutrophil Peptide-5 by using BSA as an internal standard, using Enable Q C18 column, water: acetonitrile (60:40 v/v) with Trifluoroacetic acid 0.1% as mobile phase and flow rate of 1 ml/min. Then detection was carried out at 280 nm. The retention time of BSA and HNP-5 was 2.342 and 5.279 min respectively. The linearity range was found to be 10-50 µg/ml, co-relation coefficient was found to be 0.999. The obtained validation data has been proved that possible utility of the developed method for the estimation of Human Neutrophil Peptide-5.

Keywords: Antimicrobial, Peptide, HNP, Antiviral, RP-HPLC, Validation

Abbreviations: HNP: Human Neutrophil Peptide; AMP: Antimicrobial peptide; HD: Human Defensin; Cys: Cysteine; BSA: Bovine Serum Albumin; IS: Internal Stock solution; ACN: Acetonitrile; TFA: Trifluoro acetic acid

INTRODUCTION

Some of the peptides have antiviral and antimicrobial properties from that Human Neutrophil Peptides (HNP) are the most popular peptide for their activity against various types of microorganisms **[1, 2]**. HNPs are small, cationically charged, cysteine-rich endogenous antibiotic peptides with antimicrobial and cytotoxic properties which contain 29 to 35 amino-acid residues, contain six in-variant disulphide linked cysteines moiety having a molecular weight of 4-5 kDa **[3, 4]**. In recent years considerable research has been started on HNPs because of their potential medical and pharmaceutical application.

Different human neutrophil peptides from that HNP-1 to HNP-4, known as myeloid Defensins, which found in myeloid neutrophil cells upto 5-7 %. Paneth cells include HNP-5 and HNP-6, two intestinal neutrophil peptides. Human neutrophil peptides also known as Human alpha Defensin (HD) peptides. According to the position of their disulphide bonds, The α, β and θ subfamilies are the three groups into which defensins are divided **[5, 6]**. Mammalian Leukocytes and epithelial cells both contain the defensins, but these cells are more commonly detected in neutrophils and tiny intestine Paneth cells **[7]**. The disulphide links Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5 are present in the cysteine residues that have in HNPs [8-10]. The structure has been shown in **(Figure 1) [11]**.

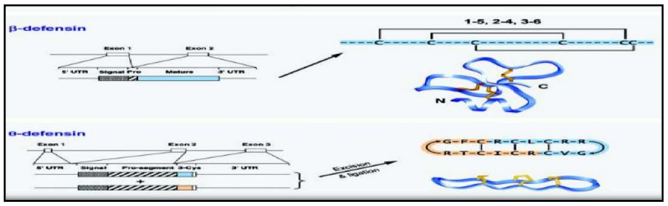

Figure 1: 3D Structure of different Human Neutrophil Peptides [11]

HNPs are antimicrobial peptides (AMP), which are active on membrane, they act by breaking negative charged target cells surface molecule on which electrostatically attraction produced by them **[12]**. The neutrophil peptides antimicrobial activity by hydrophobic face disrupts the lipid bilayer of the bacterial cell wall, leading in membrane disruption and cell death **[13]**. All HNPs have activity on gram negative bacteria and on gram positive bacteria **[14]**. Studies have suggested that HNPs may be used in conjunction with antitubercular medications **[15]**. Additionally, several studies have revealed that antimicrobial HNP-5 peptide play a crucial role in the host's defence against microbial invasion **[16]**.

HNPs have also known for their antiviral effects **[17]**. They are targeting the viral envelope, capsid or glycoproteind and disturbs the interaction to each other in viral. HNPs inhibits the replication of virus. So, there are also useful for different viral infections **[18, 19]**.

HPLC is most widely used of all the analytical separation techniques. It is widely applicable to a wide range of inorganic and organic chemicals, as well as amino acids, proteins, nucleic acids, carbohydrates, hydrocarbons, medicines, terpenoids, insecticides, antibiotics, and steroids **[20, 21, 22]**.

The most flexible and popular kind of elution chromatography is HPLC. Both the liquid-liquid partition chromatography and the liquid-solid adsorption chromatography, in normal or reversed phase, are employed with HPLC. Because polarity affects both adsorption and solubility, partition and adsorption chromatography both depend on changes in the polarity of the solute **[23]**. Partition chromatography works well for separating highly polar chemicals, while adsorption chromatography is ideal for separating very nonpolar materials. In reversed-phase HPLC, the mobile phase generally consists of water and one of the organic solvents, such as methanol, acetonitrile, tetrahydrofuran, or water, while the stationary phase is non-polar. As a modifier, the organic solvent is referred **[24, 25]**. Reverse phase chromatography is by far the most used HPLC method since a variety of organic compounds may dissolve and be readily separated **[26]**.

Main rationale for development and validation of RP-HPLC method for estimation of HNP-5 peptide is to produce reliable method for estimation of HNP-5 in formulations and purification, due to recent increase use of HNPs in market.

MATERIALS AND METHODS

Apparatus

HPLC measurements were made on Prominence-I, LC-2030 Plus, consisting of a column oven with a thermostat, a photo diode array detector, an auto sampler, and a gradient pump.

Reagents

HNP-5 was purchased from Abgenex Private Limited, Bhubaneshwar, Odisha. Acetonitrile(HPLC grade) and Trifluoroacetic acid(HPLC grade) was purchased from Fisher Scientific, Bovine Serum Albumin was purchased from Himedia.

Chromatographic condition

The analysis was performed by using HPLC instrument. The column used was Enable C18 Q (250 x 4.6 mm ,5µm, 300°A). The optimal conditions for separating BSA and HNP-5 were tested using various mobile phases. The ideal composition for optimized mobile phase was ACN: water: TFA (0.1%) 40:60 (v/v). UV detection was done at 280

nm with a flow rate of 1 ml/min. Before injecting the mobile phase and samples into the HPLC system, a 0.45 m membrane filter was used to filter them. For injection loop, 20µl sample was taken. Ultrasonicator degassed mobile phase. All determinations were be performed at ambient temperature **[27-29]**.

Preparation of standard stock solution

Preparation of standard stock solution of BSA:

Standard stock solution of BSA (1000 ppm): In a 10ml volumetric flask, 10 mg of BSA (IS) was weighed accurately before being diluted with mobile phase till the desired concentration.

BSA working standard stock solution (100 ppm): 1 ml of the stock solution is added to a 10 ml volumetric flask to prepare the preparation, then diluting it with the appropriate amount of mobile phase **[30]**.

Preparation of standard stock solution of HNP-5:

HNP-5 standard stock solution (1000ppm): Accurately weighed 10 mg of BSA was taken in 10ml volumetric flask and diluted with mobile phase up to the mark.

HNP-5 working standard stock solution (100 ppm): Prepared by transferring 1 ml from stock solution in 10 ml volumetric flask and diluted with mobile phase up to the mark **[31]**.

Preparation of sample solution

HNP-5 powder were accurately weighed. The 20 mg of HNP-5 was accurately weighed and transferred to a 10 ml appendroff tube. To this was added, 80 ml volume of mobile phase with sonication for duration time 15 minutes. The volume was prepared with mobile phase, mixed well and centrifuged at 4000 rpm for 10 minutes to get clear supernatant.

RP-HPLC Method Optimization:

Different mobile phases were tried in order to find the best condition for separation of BSA and HNP-5. The ideal composition for optimized mobile phase was ACN: water: TFA (0.1%) 40:60 (v/v). At 280 nm, UV detection was done at a flow rate of 1 ml/min. Before introducing the mobile phase and samples into the HPLC system, they were filtered using 0.45 m membrane filters. By using an ultrasonicator, mobile phase was degassed. At room temperature, every determination was made.

System Suitability Test

System performance is examined to guarantee system appropriateness before or during the study of unknowns. For six times of 100% concentration of BSA and HNP-5, variables such plate count, tailing factors, resolution, and reproducibility in retention time were measured **[32]**.

VALIDATION OF RP-HPLC METHOD

Linearity and Range

Linearity: Volumes ranging from 1 to 5 ml of the HNP-5 working standard (100ppm)

were taken as aliquots and added into volumetric flask(10 ml) and 1ml aliquot was taken from BSA (IS) working standard (100 ppm), mixed them and diluted upto the mark with mobile phase (ACN:water:TFA). The peak area ratio was plotted against the concentration of HNP-5. For HNP-5, the method demonstrated high linearity in the concentration range of 10 to 50 µg/ ml.

Range: The range of an analyte concentration in a sample is the range for which it has been shown that the analytical technique has an adequate level of precision, accuracy, and linearity. For HNP-5, the linear response was shown throughout a range of 10-50 µg/ml.

Precision:

Precision of the method was verified by repeatability, Inter-day and intra-day precision **[33]**.

A) Repeatability:

By frequently injecting (n = 6) standard solutions of BSA and HNP-5 under the identical chromatographic conditions, the instrument's accuracy was assessed **[34]**.

Acceptance criteria should be less than 2% for % RSD or CV.

B) Intraday Precision:

Three replicates of three concentrations (20, 30 and 40µg/ml) of standards of BSA and HNP-5, nine determinations were analyzed at different interval and chromatogram was recorded at 280.0 nm. % RSD was estimated. Acceptance criteria should be less than 2% for % RSD.

C) Interday Precision:

Nine determinations were evaluated over three consecutive days using three duplicates of the standard BSA and HNP-5 at doses of 20, 30, and 40 g/ml. Chromatography was recorded at 280.0 nm. Calculated was % RSD. Acceptance criteria should be less than 2% for % RSD.

Accuracy:

Accuracy was carried out using a minimum of 9 determinations over a minimum of 3 concentration (20, 30 and 40µg/ml) covering the specified range.

Aliquots ranging from 2, 3 and 4 ml were added from HNP-5 working standard solution(100 ppm), transferred to 10 ml volumetric flask , added 1 ml aliquot of BSA taken from BSA working standard solution(100 ppm) constant in series of different solution, mixed it properly and make up the volume upto the mark with mobile phase.

LOD and LOQ:

The set of five calibration curves used to assess the method's linearity were utilised to estimate the LOD.

The equation for LOD as **[35]**,

$LOD = 3.3 \times (SD/Slope)$

The set of five calibration curves used to assess the method's linearity were utilised to estimate the LOQ.

The equation for LOQ as,

LOQ = 10 × (SD/Slope)

Where, SD = The 5 calibration curves of Y-intercepts standard deviation.

Slope = The 5 calibration curves's mean slope

Robustness:

A few factors were purposefully changed in order to assess the robustness of the established technique. These variables included the flow rate's variability, the organic phase ratio, and the detecting wavelength. The average value of % RSD less than 2 % revealed the robustness of the method.

RESULTS AND DISCUSSION

RP-HPLC method optimization:

The HPLC process was improved with the goal of creating a technique. Injecting BSA (IS) and HNP-5 simultaneously into an HPLC system and running them in several solvent solutions, these trials data are shown in **(Table I – V)** and chromatograms were shown in **(Figure 2-6)**.

Trial-1:

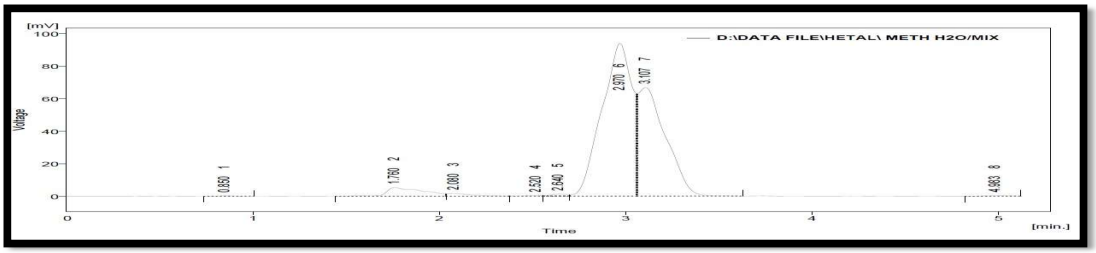

Figure 2: Chromatogram showing retention time of Bovin Serum Albumin (IS) and HNP-5 with mobile phase Water: Methanol with 0.1%TFA (90:10 v/v)

Table I: Data for BSA and HNP-5 with Water: Methanol with 0.1% TFA (90:10 v/v)					
Protein	**Retention Time**	**Area**	**Theoretical Plates**	**Tailing Factor**	**Resolution**
BSA(IS)	**2.970**	**1048.601**	**1144**	**0.458**	**1.226**
Defensin	**3.170**	**697.416**	**1850**	**4.857**	**0.427**

Trial 2:

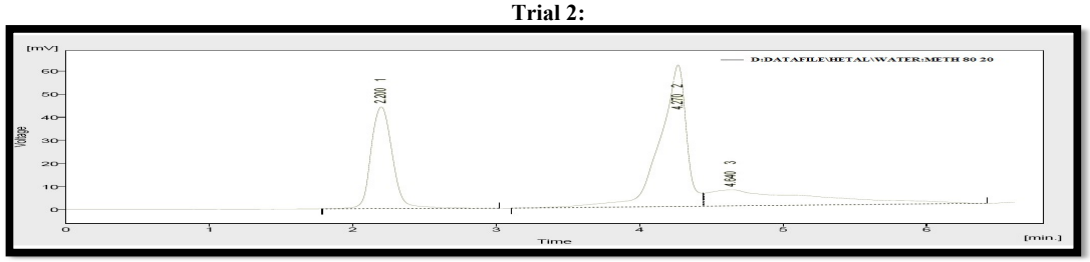

Figure 3: Chromatogram showing retention time of BSA (IS) and HNP-5 with mobile phase ratio Water: Methanol with 0.1% TFA (80:20 v/v)

Table II: Data for BSA and HNP-5 with Water: Methanol with 0.1% TFA (80:20 v/v)					
Protein	**Retention Time**	**Area**	**Theoretical Plates**	**Tailing Factor**	**Resolution**
BSA(IS)	**2.200**	**460.251**	**1005**	**1.184**	**-**
Defensin	**4.270**	**794.254**	**3118**	**0.538**	**7.095**

Trial 3:

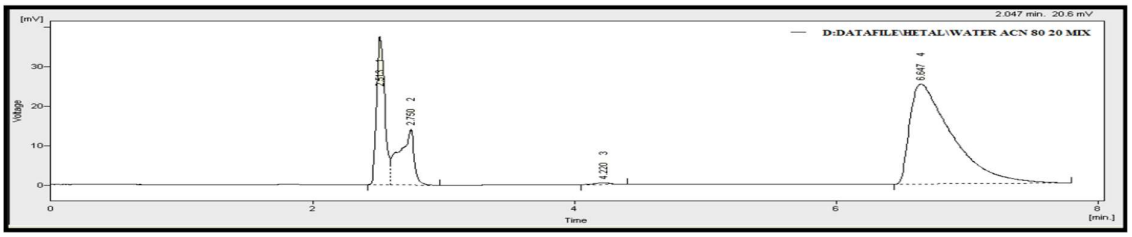

Figure 4: Chromatogram showing retention time of BSA (IS) and HNP-5 with mobile phase ratio Water: ACN with 0.1% TFA (80:20 v/v)

Table III: Data for BSA and HNP-5 with Water: ACN with 0.1% TFA (80:20v/v)					
Protein	**Retention Time**	**Area**	**Theoretical Plates**	**Tailing Factor**	**Resolution**
BSA(IS)	**2.531**	**160.951**	**7142**	**1.438**	**--**
Defensin	**6.647**	**571.399**	**2203**	**3.795**	**6.300**

Trial 4:

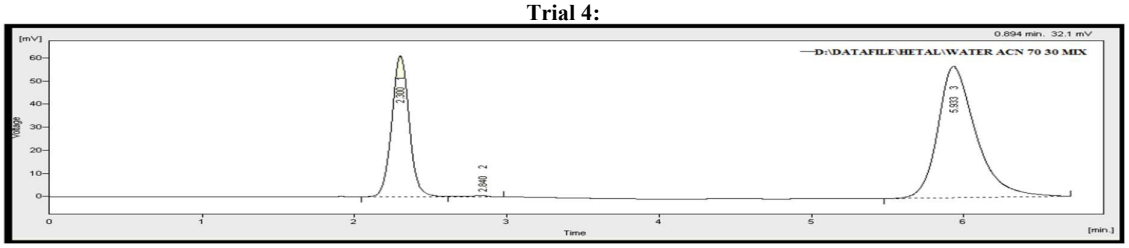

Figure 5: Chromatogram showing retention time of BSA (IS) and HNP-5 with mobile phase ratio Water: ACN with 0.1% TFA (70:30 v/v)

Table IV:Data for BSA and HNP-5 with Water: ACN with 0.1% TFA (70:30v/v)					
Protein	**Retention Time**	**Area**	**Theoretical Plates**	**Tailing Factor**	**Resolution**
BSA(IS)	**2.300**	**480.043**	**1927**	**1.091**	**-**
Defensin	**5.993**	**996.814**	**2885**	**1.565**	**7.429**

Trial 5:

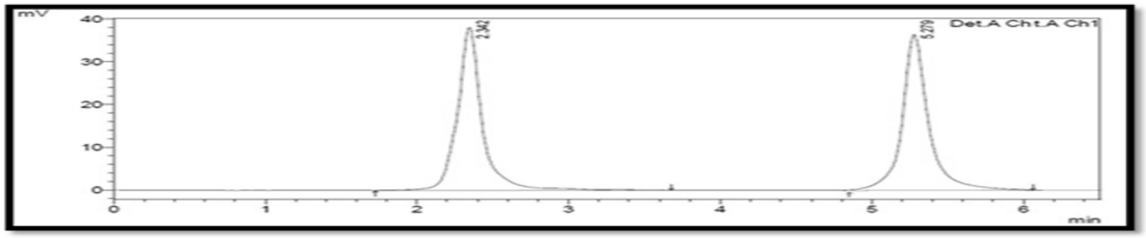

Figure 6: Chromatogram showing retention time of BSA (IS) and HNP-5 with mobile phase ratio Water: ACN with 0.1% TFA (60:40 v/v)

Table V: Data for BSA and HNP-5 with Water: ACN with 0.1% TFA (60:40v/v)					
Protein	**Retention Time**	**Area**	**Theoretical Plates**	**Tailing Factor**	**Resolution**
BSA(IS)	**2.342**	**484533**	**2190**	**1.221**	**-**
Defensin	**5.279**	**145717**	**3222.586**	**1.063**	**13.313**

Optimized Chromatographic conditions:

For optimization of chromatographic condition Enable C18 Q (250 x 4.6mm, 5µm, 300ºA) were selected as stationary phase. The mobile Phase ratio was Water: ACN (60: 40 v/v) optimized., with flow rate was maintained 1.0 ml/min, injection volume was taken 20 µl and detection was carried out at 280 nm. Summary of effect of different mobile phase compositions on the separation of IS and HNP-5 was shown in **Table VI**.

Sr. No	Mobile Phase ratios	observation	Fig. No
1	Water:Methanol with 0.1% TFA (90:10)	No peak separation,Tailing of HNP-5 peak was high	2
2	Water:Methanol with 0.1% TFA (80:20)	Peak separated but theoretical plate of BSA (IS) was less.	3
3	Water: ACN with 0.1% TFA (80:20)	Resolution was improved but BSA peak was splited and tailing of HNP-5 peak was high	4
4	Water: ACN with 0.1% TFA (70:30)	Better resolution but tailing of HNP-5 was high	5
5	Water: ACN with 0.1% TFA (60:40)	Better resolution and obtained satisfactory results	6

System Suitability Testing:

System performance is checked to ensure it is suitable for use before or during test analysis. Tailing factors, Plate count, repeatability and resolution (retention time, area and % RSD for six repetitions) are some of the parameters that were measured, this was shown in **Table VII** and compared to the criteria established for the approach **[35]**.

Table VII: Result of System Suitability Testing		
Parameters	BSA ± SD	HNP-5 ± SD
Retention Time (min)	2.351 ± 0.001265	5.284 ± 0.00098
Tailing Factor	1.222 ± 0.00454	1.169 ± 0.0124
Theoretical Plates	2061 ± 1.41	6532.33 ± 1.86
Resolution	13.356 ± 0.055	

Validation of RP-HPLC Method:

Linearity and Range:

The proposed method showed good linearity for BSA (IS) and HNP-5 with correlation co-efficient, slope and intercept 0.999, 0.028, 0.008 respectively. The calibration curve was prepared by peak area ratio of peptides to concentration of analyte. It was shown in **Table VIII**.

Table VIII: Data For Linearity and Range					
Sr. No.	BSA (IS)		HNP-5		Ratio
	Concentration µg/ml	Area	Concentration µg/ml	Area	
1	10	484533	10	145717	0.300
2		495014	20	279206	0.5640
3		495275	30	428268	0.8647
4		495298	40	556367	1.123
5		495327	50	711504	1.436

Precision:

A. Repeatability:

Based on six measurements of the same protein solution for both BSA (IS) and HNP-5, the repeatability of peak area ratio data was estimated and which shown in **Table IX**.

Table IX: Repeatability Data of BSA and HNP-5				
Sr. No.	Peak area Ratio of HNP-5 to BSA(IS)	Mean Area ratio	S.D	%RSD
1	0.864	0.856	0.0070	0.82
2	0.847			
3	0.864			
4	0.851			
5	0.853			
6	0.859			

Intraday precision

Three fresh sample solutions containing BSA (IS) and HNP-5 in different ratio were prepared and analysed on same day to check intermediate precision, is shown in **Table X**.

Table X: Intraday Data of BSA and HNP-5								
Sr. No.	Concentration µg/ml		Peak area ratio			Mean	S. D.	%RSD
	BSA(IS)	HNP-5	1	2	3			
1	10	20	0.864	0.859	0.863	0.862	0.0026	0.30
2	10	30	1.112	1.119	1.123	1.118	0.0055	0.49
3	10	40	1.433	1.421	1.410	1.421	0.0115	0.80

Interday precision

Three fresh sample solutions containing BSA (IS) and HNP-5 in different ratio were prepared and analysed on 3 consecutive days to check intermediate precision, it shown in **Table XI**.

Table XI: Inter Day Precision data of BSA and HNP-5								
Sr. No.	Concentrationµg/ml		Peak area ratio			Mean	S.D	%RSD
	BSA(IS)	HNP-5	1	2	3			
1	10	20	0.864	0.870	0.867	0.867	0.003	0.34
2	10	30	1.112	1.117	1.126	1.118	0.0070	0.62
3	10	40	1.433	1.435	1.419	1.429	0.0087	0.60

Accuracy:

Accuracy was carried out using a minimum of 9 determinations over a minimum of 3 concentration (20, 30 and 40µg/ml) covering the specified range.

Table XII: Accuracy of HNP-5							
Sr. No.	Concentration µg/ml		Peak area ratio			Mean	%Recovery
	BSA(IS)	HNP-5	1	2	3		
1	10	20	0.864	0.847	0.851	0.854	100.71
2	10	30	1.112	1.123	1.129	1.126	99.82
3	10	40	1.433	1.434	1.436	1.434	101.85

LOD and LOQ:

Five times the calibration curve was used before the standard deviation (SD) of the intercepts was determined and displayed in Table XII. LOD and LOQ were then determined using the formulas below: -

LOD=3.3 * SD/ slope,

LOQ=10 * SD/ slope,

SD = Standard deviation of intercepts.

Table XIII: Data of LOD and LOQ	
Parameter	BSA (IS) and HNP-5
S.D of the Y- intercepts of the 5 calibration curves	0.00851
Mean slope of the 5 calibration curves	0.0278
LOD = 3.3 × (SD/Slope) (µg/ml)	1.010
LOQ = 10 × (SD/Slope) (µg/ml)	3.062

Robustness:

The change was done in flow rate (±2ml/min) and in detection wavelength (±2nm). %RSD for area was calculated which should be less than 2%. The robustness was shown in **Table XIV, XV & XVI**.

Table XIV: Area ratio of BSA and HNP-5 at Different Flow Rate					
Sr. No.	Different Flow Rate (ml/min)	Peak area ratio of HNP-5 to BSA (IS)	Mean Area Ratio	S.D	%RSD
1	0.9	0.853	0.861	0.0073	0.847
2	1.0	0.864			
3	1.1	0.867			

Table XV: Area ratio of BSA and HNP-5 at Different Wavelength					
Sr. No.	Different Wavelength (nm)	Peak area ratio of HNP-5 to BSA (IS)	Mean Area Ratio	S.D	%RSD
1	276	0.848	0.853	0.00550	0.64
2	278	0.853			
3	280	0.859			

Table XVI: Area ratio of BSA and HNP-5 at Different mobile phase ratio					
Sr. No.	Different mobile phase ratio	Peak area ratio of HNP-5 to BSA (IS)	Mean Area Ratio	S.D	%RSD
1	58:38	0.856	0.863	0.0070	0.81
2	60:40	0.864			
3	62:42	0.870			

Using BSA as an internal standard, a straightforward, accurate, and exact RP-HPLC technique was created to quantify the HNP-5. Enable Q C18 column with ACN: water with TFA (0.1%), (40:60 v/v) as the mobile phase and identify at 280 nm were used to quantify HNP-5. According to **(Figure 8)**, the linearity range for HNP-5 was shown to be 10–50 g/ml. It was discovered that the correlation coefficient was 0.999. For repeatability, intraday, interday accuracy, and robustness criteria, the %RSD was determined to be less than 2%, indicating that the devised approach was accurate and robust.

CONCLUSION

A simple, accurate and precise RP-HPLC method for estimation of HNP-5 using BSA as an internal standard has been developed and validated and all validation parameter meets within the range as per ICH guideline. HNP-5 was estimated using Enable Q C18 column with water: ACN with TFA (0.1%) (60:40 v/v) as mobile phase and detection was carried out UV detection at 280nm. The 10–50 µg/ml linearity range and 0.999 co-relation coefficient were both determined. The recovery rate was found to be 101.79% and the repeatability rate was determined to be less than 2%. LOD, LOQ, and robustness for interday measurements were determined to be 1.01 and 3.062 µg/ml. So, the developed method was accurate, precise and robust. The novel trend in drug development is the design and development of biopharmaceuticals, thus, the pharmaceutical industry has focused on biomolecules method development and validation, which is successfully carried out in this research.

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