CHAPTER 3

ANALYTICAL METHODS

"Let our advance worrying become advance thinking and planning."

-Winston Churchill

Analytical methods

3. INTRODUCTION

High Performance Liquid Chromatography (HPLC) is one of the most important tools of analytical chemistry [1]. In the modern pharmaceutical industry, HPLC is the major and integral analytical tool applied in all stages of drug discovery, development, and production [2]. HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes in synthetic procedures or scale up, evaluating new formulations and carrying out quality control / assurance of the final drug products [3]. The goal of HPLC method is to separate and quantify the drug, any impurities, any available synthetic intermediates and any degradants [4]. Any sample that can dissolve in a solvent can be used with an HPLC to identify, separate, and measure the compounds that are present. HPLC is the most accurate analytical method widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability [5]. In HPLC, a sample solution is pumped into a porous column (the stationary phase), and a liquid phase (the mobile phase) is pushed through the column at high pressure. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase. HPLC represents an approach which may directly address the shortcomings of the bioanalytical methods and conveniently sidesteps the concomitant ethical issues [6]. The technique of HPLC has following features [7].

- High resolution
- Quick analysis
- Relatively high mobile phase pressure
- Mobile phase flow rate that can be controlled

Hence, HPLC method development was done to analyse drugs in the developed dosage form.

3.1 Materials and Instruments

The materials used in this study were selected to ensure accuracy and reliability in achieving the research objectives. The selection criteria for these materials were based on quality, compatibility with methodology, availability and cost-effectiveness. The details of materials which were used in the research work are portrayed in table 3.1. Additionally, all materials

were sourced from reputed suppliers and validated for quality to maintain the integrity of the experimental and analytical process. This careful selection ensured the study's outcomes were both consistent and replicated.

Table 3.1: Raw materials, chemicals and reagents employed in the current research work

Items	Source
Acetonitrile (HPLC)	Spectrochem, India
Acetic Acid Glacial	Merck, USA
Advantaflex tube	Advantapure, USA
Angiotensin-II	Sun Pharmaceutical Industries Ltd, Ahmednagar
Disodium hydrogen phosphate	Merck, USA
Hydrochloric acid	J T Baker, USA
Methanol	Merck India Ltd
Methanol (HPLC)	Spectrochem
Needles (Hypodermic-BD)	Beckton Dickinson and Co., USA
Octane	S.D. Fine-Chem Ltd
Orthophosphoric acid	Fisher Scientific, Mumbai, India
Oxytocin	Sun Pharmaceutical Industries Ltd, Ahmednagar
Potassium Dihydrogen Phosphate	Fuligins Fine Chemicals Ltd., Mumbai, India
Sodium Chloride	Merck, USA
Sodium Hydroxide	Merck, USA
Vasopressin USP	Polypeptide laboratories Pvt. Ltd., India
Water for Injection	Milli-Q
Water for HPLC	Merck Specialties Pvt. Ltd., Mumbai, India

- **Analysis of procured materials**

The procured materials underwent a through reanalysis to ensure their suitability for the research objectives and compliance with the required standards. The results of the reanalysis were compared with results provided by supplier in the certificate of analysis of the materials. In case of in-house material, reanalysis was done against reference standard or as per pharmacopeial monographs. This process involved various analytical techniques for the quality check (in term of appearance, physicochemical properties, purity analysis etc.), which were conducted to verify their consistency, reliability and compatibility with the experimental procedure. Any discrepancies or deviation identified during reanalysis was documented and material replacement or additional processing were implemented as needed. This step is essential to minimize variability, enhance the accuracy of the findings and maintain the

integrity of the research outcomes. The outcome of reanalysis and test results provided by the suppliers are compiled below for selected materials.

A. Oxytocin

Supplier: Sun Pharmaceutical Industries Ltd, Ahmednagar			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	White to almost white powder	White powder	White powder
Solubility	Very Soluble in Water	Very Soluble in Water	Very Soluble in Water
Water content (By Coulometer)	Not more than 5.0%	Mean: 2.31 % w/w	Mean: 2.29 % w/w
Clarity of Solution	Transmittance of 1 cm layer of 1 % w/v solution in water at 650 nm is not less than 95.0%	99.051%	99.052%
Colour	Absorbance of 1 cm layer of 1 % w/v solution in water at 420 nm is not more than 0.1 AU	0.008 AU	0.006 AU
pH	Between 3.0 to 6.0	4.60	4.55
Assay by HPLC	Between 93.0% to 103.0% (on anhydrous and acetic acid free basis)	On anhydrous and acetic acid free basis: 99.17 % As is basis: 87.33 %	On anhydrous and acetic acid free basis: 99.21 % As is basis: 87.39 %
Acetic acid content (by HPLC)	Between 6.0% and 10.0%	9.63 %	9.45 %

B. Vasopressin

Supplier: Polypeptide laboratories Pvt. Ltd., India			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	White to off-white powder	White powder	White powder
Solubility	Not reported in USP	Not applicable	Not applicable
Identification			
By HPLC	The retention time of the vasopressin peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.	Complies	Complies
Assay by HPLC	95.0%–105.0% on the anhydrous, acetic acid-free basis	99.00%	99.42%
Water Determination	NMT 8.0%	NMT 8.0%	4.94%
Acetic Acid in Peptides	NMT 15.0%	NMT 15.0%	11.43%
TFA content (by HPLC)	-	NMT 1.0 %	0.17%

C. Angiotensin-II

Supplier: Sun Pharmaceutical Industries Ltd, Ahmednagar			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	White to off white powder	Off white powder	Off white powder
Identification by HPLC	In the test for assay, the retention time of principle peak from the sample preparation matches with that of rom the standard preparation	Complies	Complies
Water content (By Coulometer)	NMT 10.0%w/w	Mean: 6.38 % w/w	Mean: 4.47% w/w
Solubility	Sparingly soluble in water	Sparingly soluble in water	Sparingly soluble in water
pH of solution	Not mentioned	3.54	3.59
Acetic acid content (by HPLC)	Between 5.0% and 15.0%	7.50%	9.34%
Clarity of Solution	% Transmittance of 0.05% w/v solution in 1% v/v acetic acid at 650 nm should not be less than 95.0 %	99.14%	100.09%
Colour of Solution	Absorbance of 0.05%w/v solution in 1% w/v acetic acid at 420 nm should be less than 0.1AU	0.006 AU	0.0004 AU
Content - TFA Content by IC	NMT 0.1%	0.01%	Nil
Peptide content	NLT 75.0%		87.38%

D. Glacial acetic acid

Supplier: Merck, USA			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	Clear, colorless liquid, having a pungent, characteristic odor	Clear, colorless liquid, having a pungent, characteristic odor	Clear, colorless liquid, having a pungent, characteristic odor
Assay by titrimetric	Between 99.5% and 100.5% (On as is basis).	100.00 %	Mean: 100.02 %
Congealing Temperature	Not less than 15.6°	17.2°C	17.7°C

E. Sodium Chloride

Supplier: Merck, USA			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	Colorless, cubic crystals or white crystalline powder	White crystalline powder	White crystalline powder
Appearance of solution	The solution is clear and colorless.	Complies	Complies

Identification	Identification A: Sodium compounds impart an intense yellow color to a nonluminous flame	Complies	Complies
Acidity	Acidity or Alkalinity: NMT 0.5 mL of 0.01 N hydrochloric acid or 0.01 N sodium hydroxide is required to change the color of this solution	NA	0.1 mL of 0.01 N sodium hydroxide is required to change the color of this solution
Loss on drying	NMT 0.5% w/w	NA	<0.08%
Assay by titrimetric	99.0 % - 100.5 % W/W	99.25 % w/w	99.25 % w/w

F. Sodium Hydroxide

Supplier: Merck, USA			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	White, or practically white, fused masses, in small pellets, in flakes, or sticks, and in other forms.	White coloured small pellets	White coloured small pellets
Identification	Sodium: A dense precipitate is formed	Complies	Complies
Heavy metals	NMT 30 ppm	Complies	Complies
Limit	Potassium: No precipitate is formed	Complies	Complies
Assay	Between 95.0 % and 100.5% of total alkali; NMT 3.0% of sodium carbonate	97.5% total alkali; 0.6% of sodium carbonate	97.8% total alkali; 0.5% of sodium carbonate
Chemical Test	Insoluble substances and Organic matter: A solution (1 in 20) should be clear, and colorless to slightly colored.	A solution (1 in 20) is clear and colorless.	A solution (1 in 20) is clear and colorless.

G. Disodium hydrogen phosphate

Supplier: Merck, USA			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	White powder that readily absorbs moisture	White powder	White powder
Assay by titrimetric	98.0% to 100.5% (ODB)	99.82 % ODB	99.97 % ODB
Insoluble substance	NMT 20mg; NMT 0.4%	7.3mg; 0.15%	7.1mg; 0.13%
Solubility	Freely soluble in water, insoluble in alcohol	Complies	Complies

Supplier: Merck, USA			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Loss on drying - 130°C 3Hrs upto constant weight	Not more than 5.0%	0.38 %	0.31 %
Sulphate - USP	Shows no more sulfate than corresponds to 0.2 mL of 0.020 N sulfuric acid (NMT 0.2%)	Complies	Complies
Chloride	Shows no more chloride than corresponds to 0.42 mL of 0.020 N hydrochloric acid (NMT 0.06%)	Complies	Complies
Residual Solvents: Methanol	Not more than 3000ppm	5 ppm	4 ppm

H. Potassium Dihydrogen Phosphate

Supplier: Fuligins Fine Chemicals Ltd., Mumbai, India			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	Colorless crystals or white, granular or crystalline powder	White crystalline powder	White crystalline powder
Residual Solvents: Methanol	Not more than 3000ppm	5 ppm	4 ppm
Loss on drying - 105°C 4Hrs (USP)	Not more than 1.0%	0.22 %	0.20 %
pH - 2% in water	4.2 - 4.6 (Merck)	4.45	4.40
pH - 5% in water (Ph. Eur)	4.2 - 4.5	4.36	4.31
Loss on drying - 130°C 3 Hrs upto constant weight	Not more than 2.0%	0.29 %	0.26 %
Sulphate	Maximum 300 ppm	Complies	Complies
Insoluble substance	NMT 20 mg; NMT 0.2%	1.0 mg; 0.01%	1.0 mg; 0.01%
Reducing Substances	The colour of the permanganate is not completely discharged	Complies	Complies
Assay by titrimetric	98.0% to 100.5% (ODB)	Mean: 100.27 % ODB	Mean: 100.05 % ODB
Appearance of solution	Solution should be clear and colourless	Complies	Complies

Supplier: Fuligins Fine Chemicals Ltd., Mumbai, India			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Solubility	Freely soluble in water, practically insoluble in alcohol	Complies	Complies

I. Orthophosphoric acid

Supplier: Fisher Scientific, Mumbai, India			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Description	Clear, colorless, syrupy liquid, corrosive.	Clear, colorless syrupy liquid, corrosive	Clear, colorless syrupy liquid, corrosive
Assay (Mannual titration)	84.0 % w/w to 90.0 % w/w	85.5 % w/w	85.6 % w/w
Identification Test for Chemical	A) Dilute solution of Phosphoric acid is strongly acid	Complies	Complies
Heavy metals	NMT 10 ppm	Complies	Complies
Appearance of solution	Solution S is clear and colorless	Complies	Complies
Solubility	Miscible with water and with ethanol (96 %)	Complies	Complies
Relative Density	About 1.7	1.7	1.7
Identification	B) Yellow colored precipitates are formed on addition of silver nitrate in previously neutralized solution of phosphoric acid.	Complies	Complies

J. Methanol

Supplier: Merck India Ltd., Mumbai, India			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Appearance (Clarity)	Clear	Clear	Clear
Appearance (Colour)	Colourless	Colourless	Colourless
Appearance (Form)	Liquid	Liquid	Liquid
Assay (GC)	min. 99.8%	99.86%	99.99%
Density (g/ml) @ 20°C	0.790-0.792	0.790	0.790
Refractive Index (20°C)	1.328-1.329	1.328	1.328
Boiling Range	64-65°C	64-65°C	64-65°C
Non-Volatile Matter	max. 0.001%	0.0001%	0.0001%
Acidity (CH_3COOH)	max. 0.002%	0.0006%	0.0006%
Miscibility with water (1:3)	Clear & colourless liquid	Clear & colourless liquid	Clear & colourless liquid

Supplier: Merck India Ltd., Mumbai, India			
Test	**Specification**	**Supplier's Result**	**Retesting results**
Water (KF)	max. 0.05%	0.03%	0.02%

3.2 Instruments

The instruments employed in this study were meticulously selected to guarantee precision, reliability, and compatibility with the established research methodology. Each instrument was subjected to thorough validation and calibration, ensuring their appropriateness for the research objectives and enabling the provision of comprehensive and accurate insights into the study's focal points. The specifics of the instruments engaged in this research are detailed in Table 3.2.

Table 3.2: Details of various instruments employed during the current research work

Instruments	Make
pH meter	Mettler Toledo, Switzerland
Analytical balance	Mettler Toledo, Switzerland
HPLC	Waters, USA
UV-Visible spectrophotometer	Shimadzu, Japan
Overhead stirrer	IKA, Japan
Magnetic stirrer	IKA, Japan
Water bath	Julabo, Germany
Bath Sonicator	Borosil, India
Disc filters	Pall, USA

3.3 High Performance Liquid Chromatographic Method for Oxytocin

3.3.1. Instrumentation and chromatographic conditions

Instrumentation: The Shimadzu LC–2010CHT liquid chromatograph located in Japan is equipped with a system driver, a solvent delivery module, a low-pressure gradient pump, an online degasser, an auto injector (capable of injection volumes ranging from 5 to 100 µL), auto-samplers with a cooler, and a UV-Vis detector (refer to Table 3.3) [1].

Chromatographic conditions: YMC Pack ODS-A column with dimensions of 150 × 4.6 mm and a particle size of 3 µm was utilized. The mobile Phase consisted of two parts wherein phase A consisted of 13.8 grams of sodium dihydrogen phosphate monohydrate dissolved in 1 liter of water and the pH was adjusted to 3.0 with orthophosphoric acid. This solution was filtered and transferred to reservoir, labelled as A. On the other hand, mobile phase B constituted of a mixture of water and acetonitrile in 50:50 ratio v/v. This solution was also filtered in the same

manner as that of mobile phase A and stored in mobile phase reservoir, designated as B. The flow rate was kept at 0.6 mL/min throughout the HPLC run and the column temperature was maintained at 40°C. The injection volume was kept as 500 µl. The mobile phase was utilized in a gradient mode and the ratio of mobile phase A and B with respect to time is represented in Table 3.3. The run time for a single HPLC run was kept 75 min.

Table 3.3: Chromatographic conditions for oxytocin

Column	YMC Pack ODS-A (150*4.6 mm), 3 µm		
Mobile Phase A	Sodium dihydrogen phosphate monohydrate pH (3.0)		
Mobile Phase B	Water: acetonitrile (50:50, v/v)		
UV Detector	220 nm		
Flow Rate	0.6 mL/min		
Injection volume	500 µL		
Column temperature	40°C		
Run time	75 min		
Gradient	Time	%A	%B
	0	82	18
	50	45	55
	55	40	60
	65	40	60
	65.1	82	18
	75	82	18

Standard Preparation: An accurately weighed amount of standard oxytocin powder that is 12.5 mg was placed into a 200 mL volumetric flask, and dissolved in 100 mL of water thoroughly. The flask was then filled to the mark with water and mixed again. Four milliliters of this solution was transferred to a second 200 mL volumetric flask, and water was added until the mark was reached, followed by thorough mixing. 4 mL of this solution was placed into a 50 mL volumetric flask and mobile phase-A was added till 50 mL mark. The solution was mixed well to make it as 0.10 ppm standard solution.

Sample solution preparation: 50 mL of sample was transferred from preconditioned strata C18-E cartridge on application of 150 mm of Hg water's Negative pressure processor and later the cartridge was washed each with 1.5 mL (500 µLx3) of Milli Q water. The Oxytocin was eluted from cartridge 12 times each with 200 µL of mobile phase-B and it was collected in a

clean and dry test tube. To this, 0.6 mL of sodium dihydrogen phosphate buffer solution was added and the solution was mixed well. The different concentrations of solutions ranging from 0.02 µg/mL to 0.2 µg/mL were prepared and were then injected into HPLC system. Table 3.3 illustrated the chromatographic conditions for detection of oxytocin.

3.3.2. HPLC method validation

The analytical method underwent comprehensive validation to ensure its reliability under established conditions, focusing on critical parameters such as linearity, accuracy, precision, limits of detection (LOD), limits of quantification (LOQ), and both intra- and inter-day precision [8, 9]. For the validation process, a stock solution was meticulously prepared at a concentration of 1 µg/mL and then carefully diluted to achieve a precise concentration range of 0.01-0.20 µg/mL. The resulting samples were analysed using HPLC at a wavelength of 220 nm, which corresponds to the maximum absorbance (λmax) for optimal detection.

- **Linearity**

Linearity serves as a key parameter that reflects how well the experimental data conforms to a straight line, mathematically represented by the linear equation (Equation 3.1) below:

$$y = mx + c \qquad \text{eq. 3.1}$$

In this equation, y represents the response measured, x denotes the concentration of the analyte, m is the slope of the correlation, and c is the intercept point of the best-fit line on the y-axis. To rigorously assess the linearity of the employed analytical method, a series of solutions were prepared, reflecting concentrations of 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, and 0.20 µg/mL of the drug. A detailed graph was plotted that showcased the relationship between these varying concentrations and the corresponding area under the curve (AUC) observed during the analysis. The collected data was then subjected to a regression analysis to thoroughly evaluate and confirm the linearity of the method.

- **Accuracy**

To thoroughly evaluate the accuracy of the proposed analytical method, a series of quality control solutions were meticulously prepared at three distinct concentration levels: a lower concentration (LC) of 0.02 µg/mL, a medium concentration (MC) of 0.1 µg/mL, and a higher

concentration (HC) of 0.2 µg/mL. These solutions were independently created from a stock solution and subjected to analysis, with each concentration tested three times (n = 3) to ensure reliability. The assessment of accuracy focused on determining how closely the observed concentrations matched the actual target concentrations, with the goal of achieving the lowest possible % relative error and the highest % recovery rates.

- **Precision**

- **Intra-and inter-day precision**

The consistency of three distinct OXT samples, each prepared at varying concentrations of 0.02, 0.10, and 0.16 µg/mL, was thoroughly evaluated at multiple time intervals on the same day to assess intra-day variations, as well as over three separate days to examine inter-day reliability. The collected data served to calculate the relative standard deviation (RSD) values, providing insights into the reproducibility of the measurements.

- **Limit of detection (LOD) and limit of quantification (LOQ)**

The LODin the context of analytical techniques is defined as the lowest concentration of an analyte that can be reliably recognized within a sample matrix. This threshold is significant as it marks the point at which the presence of the analyte can be confirmed, even if the concentration does not permit definitive quantification. On the other hand, the LOQ is characterized as the smallest concentration at which the analyte can be measured with a high degree of accuracy and reliability. This quantitative benchmark is particularly crucial when dealing with the assessment of contaminants and degradation products, where precise analytical identification is essential for safety and compliance.

The determination of both the LOD and LOQ involves a careful analysis that considers the slope of the calibration curve, which indicates the relationship between concentration and response, as well as the standard deviation of the observed AUC values derived from the sample. These two factors are pivotal in the estimation process, ensuring that the calculated limits are both statistically sound and representative of the analyte's behavior in the sample. The equations used for computation are expressed in Equation 3.2 and Equation 3.3, with LOD calculated as:

$$LOD = 3.3 * \sigma/S \quad \text{eq. 3.2}$$

$$LOQ = 10 * \sigma/S \quad \text{eq. 3.3}$$

In these formulas, σ symbolizes the standard deviation of the AUC values observed in the sample, while S represents the calculated slope of the calibration curve, underscoring the essential relationship between these variables in analytical chemistry.

3.3.3. Results and Discussion

3.3.3.1 HPLC method development

The earlier scientific literature on chromatographic techniques for OXT was examined in detail, and initial trials were carried out according to the described methodology [10, 11]. Most of these studies have employed a mix of organic and aqueous solvent systems, including phosphate buffers (modified with orthophosphoric acid) and acetonitrile, using various flow rates and column oven temperatures, while monitoring a wavelength close to λmax 220 nm (figure 3.1).

3.3.3.2 HPLC method validation

In this current research, the process of validating the analytical method encompassed a comprehensive assessment of all crucial parameters. This thorough evaluation aimed to establish the trustworthiness and accuracy of the analytical procedure, which will be elaborated upon in the following sections:

- **Linearity**

31.552 ± 0.02 minutes was the observed retention time for OXT. Linear regression analysis was employed to evaluate the peak area data at various drug concentrations. The respective AUC values at different concentration of the drug have been provided in Figure 3.2 and Table 3.4 The line of equation has been shown as equation no. 3.4.

$$y = 4000000x + 3484 \quad \text{… 3.4}$$

OXT demonstrated a linear response within the concentration range of 0.02-0.20 µg/mL, achieving a coefficient of determination of $r^2 = 0.9989$. This strong r^2 value confirmed that the system complied with Beer's law and that the calibration graphs were linear. Throughout all examined drug concentration levels, the standard deviation values ranged from 98 to 352, while

the relative standard deviation remained below 2% [12]. The low RSD value indicated a high level of repeatability in the method.

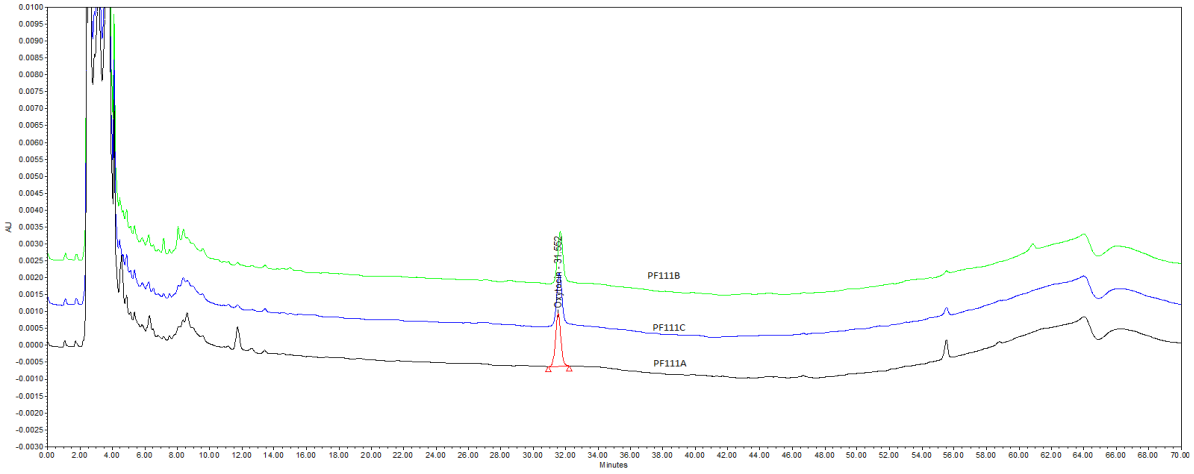

Figure 3.1: HPLC chromatogram for oxytocin standard and samples

Table 3.4: Peak areas at different concentrations of OXT for constructing the calibration plot

Standard conc. (µg/mL)	Mean peak area ± SD	Rt	% RSD	R^2
0	0	-	-	0.9989
0.02	73903.33±89.7	31.552±0.02	0.03	
0.04	151846.3±127.6	31.499±0.03	0.07	
0.08	301636.3±222.3	31.509±0.04	0.07	
0.12	456234.3±289.3	31.518±0.03	0.07	
0.16	609522±331.2	31.562±0.04	0.07	
0.20	729364±390.2	31.528±0.02	0.03	

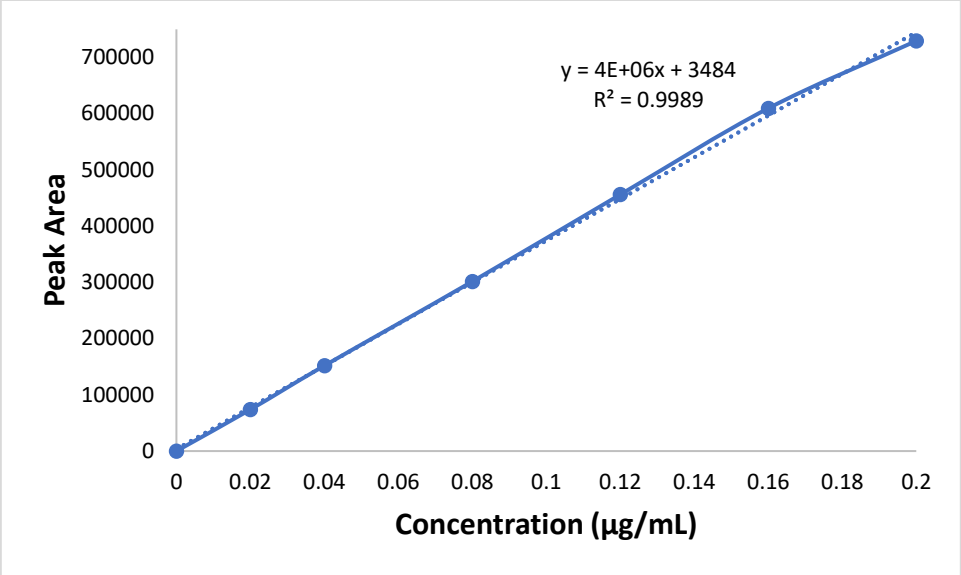

Figure 3.2: Linearity plot of oxytocin

Percent RSD of the selected concentration range was found be well within the limits [6].

- **Accuracy**

Table 3.5 gives the accuracy data that was recorded to validate the analytical procedure. The accuracy of the measured value was assessed by comparing it to the standard value in the current studies. The measured value was found to be highly accurate, with a percent accuracy range of 99.60% to 99.46% for drug concentrations studied (0.02, 0.10, 0.16 µg/mL).

- **Precision**

Precision, as defined by USFDA is "degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of homogenous samples" [13]. The developed analysis method was tested for accuracy within (Intra) and between days (Inter) in the following ways:

- **Intra-day and inter-day precision**

The works in Table 3.6 show intra and inter-day method validations. The % RSD values for intra-day studies ranged between 0.67 and 1.10, and those for inter-day studies ranged between 0.46 and 1.32. This showed that the analysis method can be used more than once. The numbers were well within the acceptable range [14, 15].

Table 3.5: Accuracy in measuring a known concentration of OXT

Standard conc. (µg/mL)	Calculated conc. (µg/mL)	% Accuracy	Mean ± SD
0.02	0.0199	99.60	99.60±0.1
	0.0198	99.70	
	0.0198	99.50	
0.10	0.1003	99.60	99.53±0.21
	0.0997	99.70	
	0.0989	99.30	
0.16	0.1588	99.25	99.46 ± 0.22
	0.1591	99.44	
	0.1595	99.69	

Table 3.6: Validation for intra- and inter-day precision

Standard conc. (µg/mL)	Mean predicted concentration (µg/mL) ± SD	% RSD
Intra-day		
0.02	0.0199 ± 0.001	1.10
0.10	0.0998 ± 0.001	0.77
0.16	0.159 ± 0.001	0.67
Inter-day		
0.02	0.0198 ± 0.001	0.46
0.10	0.0997 ± 0.001	1.32
0.16	0.158 ± 0.001	0.80

- **LOD and LOQ**

The LOD for oxytocin (OXT) was identified to be 0.00195 µg/mL. This measurement reflects the relationship between the signal produced by the analyte and the inherent noise of the analytical system, with the LOD conventionally defined as the lowest concentration at which the signal-to-noise ratio reaches a minimum threshold of 3:1 [13]. Moreover, the LOQ for OXT was measured at 0.00558 µg/mL. This parameter also pertains to the interplay of signal and noise within the system and is customarily recognized as the concentration at which the signal-to-noise ratio is established at no less than 10:1 [8].

3.4 High Performance Liquid Chromatographic Method for Vasopressin

3.4.1 Instrumentation and chromatographic conditions

- **Instrumentation**

The liquid chromatograph utilized in this study was the Shimadzu LC–2010CHT, a sophisticated instrument manufactured in Japan designed for precise and efficient chromatographic analysis. This system was equipped with a comprehensive setup including a system controller for overall operation, a solvent delivery module for managing solvent flow, a low-pressure gradient pump to facilitate smooth solvent transitions, and an online degasser to remove dissolved gases that could interfere with the analysis. Additionally, it featured an auto-injector, capable of handling injection volumes ranging from 5 to 100 µL, as well as an auto-sampling unit combined with a cooler to maintain sample integrity. The analysis was monitored using a UV-Vis detector to capture and quantify the light absorption of the analytes. Chromatographic separation was executed on a YMC Pack ODS-A column, measuring 250 mm in length and 4.6 mm in diameter, with a particle size of 5 µm, which played a crucial role in achieving optimal separation of components, as detailed in Table 3.7 [1,8].

Column Operation: The YMC Pack ODS-A column was meticulously operated at a controlled temperature of 40°C, ensuring consistent performance and reliable results.

Preparation of Mobile Phase Buffer: For the mobile phase buffer, precise measurements were taken: 6.6 g of di-basic ammonium phosphate was carefully weighed and then dissolved in 1000 mL of distilled water. To achieve the target pH of 3.0 ± 0.05 a critical parameter for the separation processes an appropriate amount of strong orthophosphoric acid solution was added. The resulting solution was then filtered through a 0.45 µm membrane filter to eliminate any impurities, and degassed to remove dissolved gases, utilizing Millipore-Durapore HVLP 0.45 µm filter paper (Cat No.: HVLP04700).

Composition of Mobile Phase: The mobile phase was formulated by mixing the prepared buffer with acetonitrile in an 87:13 volume ratio, creating an optimal environment for effective chromatography.

Diluent Preparation: The diluent was composed of a carefully crafted blend of the buffer solution and methanol in an 80:20 ratio, ensuring suitable conditions for sample dilution.

Standard Preparation: To create the working standard, 23.5 mg of normal vasopressin powder was accurately weighed and dissolved in 70 mL of the prepared diluent within a 100 mL volumetric flask. The flask was then filled to the calibration mark with additional diluent and thoroughly mixed to ensure homogeneity. A portion of this solution, specifically 6 mL, was subsequently transferred into a new 100 mL volumetric flask. Buffer was added until the mark was reached, and thorough mixing was performed to obtain a consistent standard solution.

Sample Preparation (1.887 µg/mL Strength): For this sample preparation, 10 g of the selected sample was placed into a 20 mL volumetric flask. Sufficient solvent was then added to fill the flask to the mark, followed by vigorous mixing to ensure the sample was fully dissolved, resulting in a final concentration of 0.94 ppm.

Sample Preparation (37.74 µg/mL Strength): In this preparation, 1.25 g of the desired sample was carefully measured and added to a 50 mL volumetric flask. The flask was filled with enough solvent to reach the calibrated mark, and the contents were mixed thoroughly to ensure a homogeneous solution was achieved for further analysis.

Table 3.7: Chromatographic conditions

Column	YMC pack ODS A (250 mm x 4.6 mm), 5 µ (YMC Co. Ltd Japan)
Flow rate	1.2 mL/min
Wavelength	220 nm
Column temperature	40°C
Sample temperature	10°C
Injection volume	200 µL
Run time	20 min
Needle Wash	Water: Acetonitrile (50:50)
Seal Wash	Water: Acetonitrile (50:50)

3.4.2 HPLC method validation

The analytical method underwent a thorough validation process in accordance with the recommended criteria, which included assessing linearity, accuracy, precision, and LOD and LOQ. Additionally, intra- and inter-day precision were evaluated to ensure reliability. A stock solution with a concentration of 1 µg/mL was meticulously prepared and subsequently diluted to create a comprehensive concentration range of 0.5 to 50 µg/mL. The samples were then carefully analysed at the wavelength of maximum absorption (λmax) of 220 nm using HPLC, ensuring precise and accurate results [1].

- **Linearity**

In order to confirm the linearity of the employed analytical method, a series of solutions containing the drug were meticulously prepared across a concentration spectrum ranging from 0.5 to 50 µg/mL. The specific concentrations included 0.5, 1, 2, 5, 10, 20, 30, 40, and 50 µg/mL. Each solution was subsequently analysed to determine the AUC a critical parameter in assessing the drug's response. Following the data collection, a graph was constructed to illustrate the correlation between the varying concentrations and their corresponding AUC values. This data was then subjected to regression analysis to evaluate the linearity of the method rigorously.

- **Accuracy**

To evaluate the effectiveness of the proposed method, three distinct quality control solutions were prepared from the stock solution and tested (n = 3). These solutions included a low concentration (LC: 0.5 µg/mL), a medium concentration (MC: 20 µg/mL), and a high concentration (HC: 50 µg/mL). The accuracy was determined by comparing the observed concentration to the actual concentration, aiming for the lowest relative error percentage and the highest recovery percentage.

- **Precision**

- **Intra-and inter-day precision**

A series of experiments was conducted to assess three varying concentrations of vasopressin: 0.5 µg/mL, 20 µg/mL, and 50 µg/mL. Each concentration was tested multiple times

specifically, three times within a single day to evaluate intra-day variability and three additional times over a span of three different days to analyse inter-day variability. The results obtained from these tests were then employed to calculate the relative standard deviation (RSD) values, providing insights into the precision and consistency of the measurements across different conditions.

- **LOQ and LOD**

The LOQ and LOD were determined as per previously described method (Section 3.3.2).

3.4.3 Results and Discussion

3.4.3.1 HPLC method Development

The previous scientific literature on chromatographic techniques for vasopressin was carefully reviewed, and initial trials were carried out following the described methodology [16, 17]. As illustrated in Figure 3.3, the majority of these investigations utilized a wavelength around λmax 220 nm and employed a combination of organic and aqueous solvents, including acetonitrile and ammonium phosphate buffers (adjusted with orthophosphoric acid), while varying flow rates and column oven temperatures.

3.4.3.2 HPLC method validation

In the current research, the validation of the analytical method involved a comprehensive assessment of all critical parameters. This thorough evaluation aimed to establish the reliability and consistency of the analytical procedure, ensuring its effectiveness for accurate measurements. The detailed exploration of these factors is discussed in the following sections:

- **Linearity**

Vasopressin demonstrated a retention time of 10.056 ± 0.01 minutes, indicating the duration the substance remained in the analytical system before being measured. To assess the relationship between the peak area and various drug concentrations, a linear regression analysis was performed, allowing for a detailed investigation of the data. The AUC values corresponding to different concentrations of the drug are clearly depicted in Figure 3.4 and meticulously detailed in Table 3.8. The equation representing the calibration line is articulated as equation 3.5.

$$y = 5041.9x + 129.52\ldots \quad 3.5$$

Within the analysed concentration range of 0.5 to 50 µg/mL, vasopressin exhibited a remarkable linear response, as evidenced by a coefficient of determination (r^2) of 0.9999. This exceptionally high r^2 value validates the linearity of the calibration graphs, confirming the system's adherence to Beer's law, which states that absorbance is directly proportional to concentration. Throughout all tested concentration levels, the standard deviation measurements ranged from 2 to 6149, while the relative standard deviation (RSD) consistently remained below 2% [12]. Such a low RSD value signifies a high level of repeatability in the method, ensuring reliability in the results obtained.

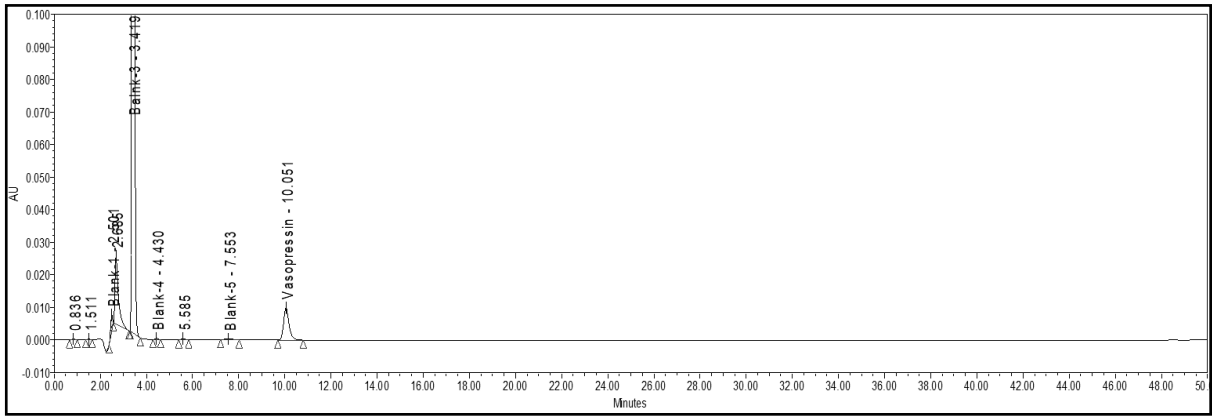

Figure 3.3: HPLC chromatogram for vasopressin standard

Table 3.8: Peak areas at different concentrations of Vasopressin for constructing the calibration plot

Standard conc. (µg/mL)	Mean peak area ± SD	Rt	% RSD	R^2
0	0	-	-	0.9999
0.5	2627.3±2.5	10.50±0.01	0.02	
1	5261.7±33.3	10.51±0.02	0.04	
2	10600.0±100	10.52±0.01	0.03	
5	25716.3±26.6	10.49±0.01	0.05	
10	50755.0±188.6	10.50±0.02	0.04	
20	100583.3±236.3	10.49±0.01	0.05	
30	150653.3±584.3	10.51±0.01	0.04	

40	200366.7±57.7	10.50±0.01	0.02	
50	253874.0±6148.9	10.48±0.01	0.06	

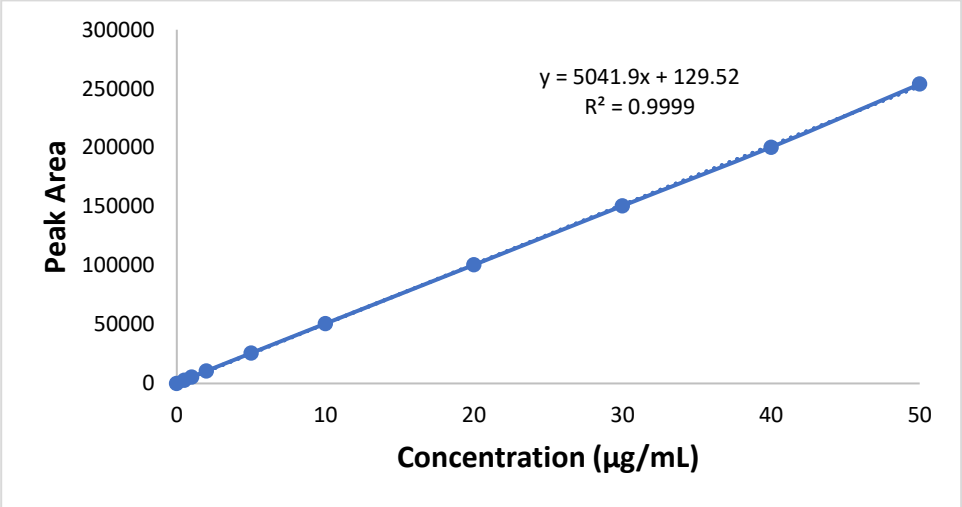

Figure 3.4: Linearity plot of vasopressin

Percent RSD of the selected concentration range was found be well within the limits [6].

- **Accuracy**

In Table 3.9, you can see the accuracy data that was collected to make sure that the analytical process worked. In these studies, the recorded value was compared to the standard value to find out how accurate it was. For the three drug concentrations that were looked at 0.5, 20, and 50 µg/mL, the percent accuracy ranged from 99.60% to 99.46 %, which means that the measured value was very close to the true value.

- **Precision**

Precision, as defined by USFDA is "degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of homogenous samples" [13]. The developed analysis method was tested for accuracy within and between days in the following ways:

- **Intra-day and inter-day precision**

Table 3.10 shows method evaluation studies that were done within and between days. The % RSD values for intra-day studies ranged between 0.78 and 0.97, and those for inter-day studies

ranged between 0.88 and 1.19. This showed that the analysis method can be used more than once. The values were well within the permissible limits [14, 15].

Table 3.9: Accuracy in measuring a known concentration of vasopressin

Standard conc. (µg/mL)	Calculated conc. (µg/mL)	% Accuracy	Mean ± SD
0.5	0.499	99.80	99.85±0.09
	0.499	99.80	
	0.500	99.96	
20	19.90	99.50	99.63±0.32
	19.90	99.40	
	20.00	100.0	
50	49.90	99.72	99.65±0.12
	49.90	99.72	
	49.80	99.52	

Table 3.10: Validation for intra- and inter-day precision

Standard conc. (µg/mL)	Mean predicted concentration (µg/mL) ± SD	% RSD
Intra-day		
5	4.98 ± 0.01	0.97
20	19.989± 0.002	0.91
50	49.991 ± 0.03	0.78
Inter-day		
5	4.91 ± 0.01	1.19
20	19.979 ± 0.003	0.98
50	49.982 ± 0.04	0.88

- **LOD and LOQ**

The LOD for vasopressin was identified to be 0.049 µg/mL, indicating the smallest concentration that can be reliably detected by the analytical system. This measurement is influenced by both the signal strength and the noise inherent in the system and is conventionally defined as the lowest peak with a signal-to-noise ratio of at least 3:1, ensuring that the signal can be distinguished from the background noise. In addition, the LOQ for vasopressin was determined to be 0.147 µg/mL. This value reflects the minimum concentration that can not only be detected but also quantified with reliable precision, and it is typically

characterized by a peak that has a signal-to-noise ratio of no less than 10:1, indicating a clear and measurable signal in comparison to the noise. [8].

3.5 High performance Liquid chromatographic method for Angiotensin II

3.5.1 Instrumentation and chromatographic conditions

Buffer solution: 2.72 g of potassium dihydrogen orthophosphate were mixed with 1000 millilitres of water and sonicated to break up the solids. It was given 30 mL of triethylamine. With orthophosphoric acid, the pH of the solution was brought down to 2.0 ± 0.05. Through 0.45-inch filter paper (Filter: MILLIPORE-Durapore HVLP 0.45 µm 047 mm, CAT No: HVLP04700) (Table 3.11).

Mobile Phase-A: buffer solution and acetonitrile were mixed in a 900:100 ratio to make it. The mixture was degassed prior to use.

Mobile Phase-B: was made by mixing 300 parts buffer solution to 700 parts acetonitrile. The mixture was degassed prior to use.

Diluent: HPLC grade water

Standard solution: Carefully measured approximately 5 mg of Angiotensin-II WS/RS and placed it into a 50 mL volumetric flask. Added around 10 mL of diluent and sonicated the mixture to ensure it was dissolved. Then, added more diluent until the solution reached the fill line. Transferred this solution into a 20-mL volumetric flask using a syringe, diluted it further with diluent to the mark, and mixed thoroughly, resulting in a final concentration of 10 ppm.

Sample preparation

Injected as such sample (10 ppm)

3.5.2 HPLC method validation

The analytical method was validated as per the [8] guidelines, and USFDA recommended conditions such as linearity, accuracy, precision, LOD and LOQ, intra- and inter-day precision [9]. For validation, stock solution of 1 µg/mL was prepared and further diluted to fetch the concentration range of 2-20 µg/mL. The samples were analysed at λ_{max} of 210 nm using HPLC.

- **Linearity**

To establish linearity of the used method, different solutions in the concentration range of 2, 4, 6, 8, 10,12, 14, 16, 18 & 20 µg/mL solution of the drug were prepared and analysed. A graph

was plotted between different concentrations and AUC observed, and the data was fitted into regression equation to check the linearity.

Table 3.11: Chromatographic condition for Angiotensin-II

Column	X-SELECT CSH Phenyl Hexyl (150 x 4.6) mm 2.5 µ (Make: Waters) Part No:186006735
Flow rate	0.6 mL/min
Wavelength	210 nm
Injection volume	100 µL
Column temperature	50°C
Sample cooler	5°C
Run time	50 min
Retention Time	About 17.46 min.

- **Accuracy**

To test how well the suggested method works, three separate quality control solutions were made: one with a lower concentration (LC) of 2 µg/mL, one with a middle concentration (MC) of 10 µg/mL, and one with a higher concentration (HC) of 20 µg/mL. These solutions were then tested (n = 3). Accuracy was measured by how close the concentration that was noticed was to the concentration that was actually there, with the lowest percentage of relative error and the highest percentage of recovery.

- **Precision**

- **Intra-and inter-day precision**

Three different samples of vasopressin were tested at different times on the same day (intra-day), and at different times on three different days (inter-day). The amounts were 2, 10, and 20 µg/mL. The numbers were used to figure out the RSD values.

- **LOQ and LOD**

The LOQ and LOD were determined as per previously described method (Section 3.3.2).

3.5.3 Results and Discussion

3.5.3.1 HPLC method Development

The initial scientific investigations into chromatographic techniques for analysing angiotensin were conducted with great attention to detail, and preliminary trials were executed following the specified protocol [15, 16]. In the majority of these studies, researchers employed a wavelength of approximately 210 nm, utilizing a carefully selected combination of organic solvents and aqueous solutions. This blend included acetonitrile and phosphate buffers, which were precisely adjusted using orthophosphoric acid. Throughout these experiments, variations in flow rates and column oven temperatures were systematically altered to optimize the separation process (see Figures 3.5 and 3.6 for visual reference).

3.5.3.2 HPLC method validation

In the current research, the validation studies for the analytical method involved a comprehensive evaluation of all crucial parameters. This thorough assessment was conducted to confirm the reliability and robustness of the analytical procedure, as elaborated upon in the sections that follow:

- **Linearity**

The retention time for vasopressin was observed to be 16.46 min. Linear regression analysis was used to look at the data of the peak area seen at different drug concentrations. The respective AUC values at different concentration of the drug have been provided in Figure 3.7 and Table 3.12. The line of equation has been shown as equation no. 3.6.

$$y = 4071.6x - 98.432 \quad \ldots 3.6$$

There was linearity in the concentration range of 2–20 µg/mL for angiotensin, with a r2 value of 0.9989. This high number of r^2 proved that the calibration graphs were linear and that the system could follow Beer's law. At all drug concentration levels studied, the values of standard deviation were in the range of 2-1230 and the relative standard deviation was < 2% [12]. The RSD value was quite low, indicating high degree of method repeatability.

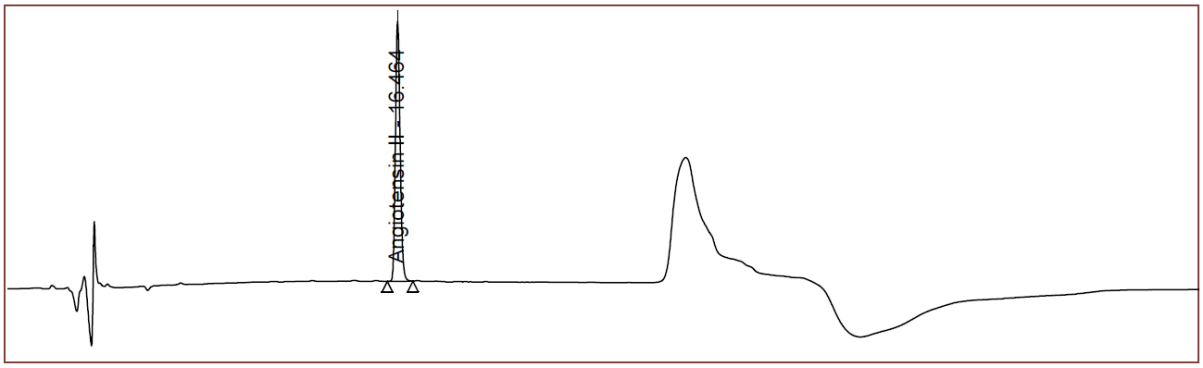

Figure 3.5: Blank-diluent chromatogram of angiotensin-II standard

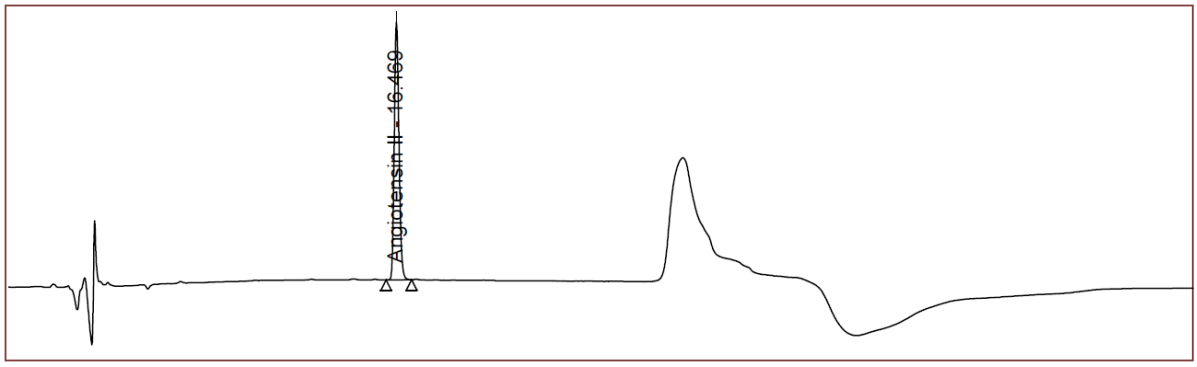

Figure 3.6: Blank-diluent chromatogram of angiotensin-II API

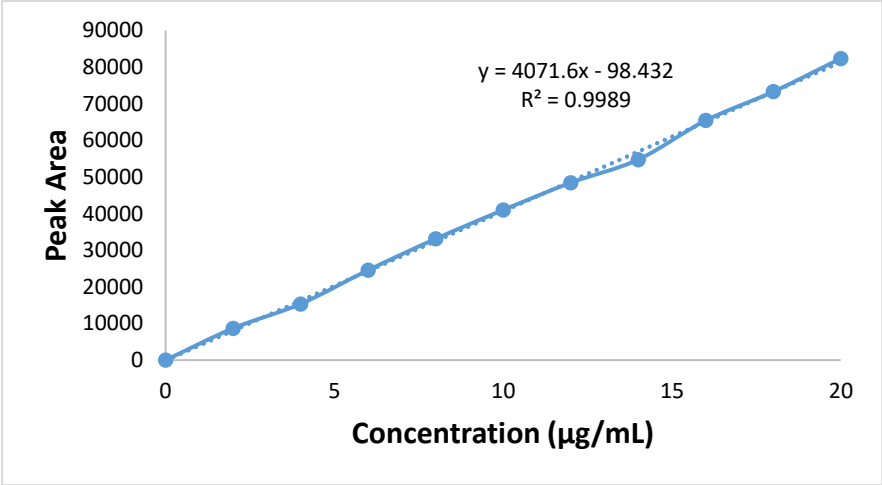

Figure 3.7: Linearity plot of angiotensin II

Table 3.12: Peak areas at different concentrations of Angiotensin for constructing the calibration plot

Conc. (µg/mL)	Mean peak area ± SD	Rt	% RSD	R^2
0	0	-	-	0.9989
2	8688.3±67.9	16.49±0.058	0.78	
4	15342.0±39.3	16.53±0.133	0.26	
6	24558.0±51.4	16.56±0.173	0.21	
8	33103.3±604.2	16.41±0.081	1.83	
10	40971.7±576.7	16.46±0.006	1.41	
12	48408.7±580.2	16.59±0.031	1.20	
14	54731.3±575.0	16.43±0.058	1.05	
16	65404.7±554.8	16.41±0.087	0.85	
18	73261.3±574.7	16.42±0.064	0.78	
20	82324.0±1229.8	16.48±0.040	1.49	

Percent RSD of the selected concentration range was found be well within the limits [6].

- **Accuracy**

The accuracy numbers that were recorded to make sure the analytical procedure worked are shown in Table 3.13. In these studies, the recorded value was compared to the standard value to find out how accurate it was. For the three drug concentrations that were looked at 2, 10, and 20 µg/mL the percent accuracy ranged from 99.29% to 100.10 %, which means that the measured value was very close to the true value.

- **Precision**

Precision, as defined by USFDA is "degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of homogenous samples" [13]. The developed method was tested for accuracy within and between days in the following ways:

- **Intra-day and inter-day precision**

The works in Table 3.14 show intra-day and inter-day method validations. The % RSD values for intra-day studies ranged between 0.41 and 0.48, and those for inter-day studies ranged between 0.10 and 0.78. This showed that the analysis method can be used again and again. The values were well within the permissible limits [14, 15].

Table. 3.13: Accuracy in measuring a known concentration of angiotensin II

Standard conc. (µg/mL)	Calculated conc. (µg/mL)	% Accuracy	Mean ± SD
2	1.998	99.90	99.87±0.03
	1.997	99.85	
	1.997	99.85	
10	10.010	100.10	99.60±0.70
	9.880	99.80	
	9.990	99.90	
20	19.859	99.29	99.13 ± 0.29
	19.859	99.29	
	19.759	99.79	

Table 3.14: Validation for intra- and inter-day precision

Conc. (µg/mL)	Mean predicted concentration (µg/mL) ± SD	% RSD
Intra-day		
2	1.989 ± 0.01	0.43
10	9.937 ± 0.04	0.41
20	19.881 ± 0.10	0.48
Inter-day		
2	1.984 ± 0.02	0.78
10	9.985 ± 0.01	0.13
20	19.913 ± 0.02	0.10

- **LOD and LOQ**

The LOD for angiotensin was identified as 0.09 µg/mL. This measurement reflects the balance between the signal produced by the substance and the background noise inherent in the system. It is conventionally defined as the minimum concentration at which a peak can be observed with a signal-to-noise ratio of at least 3:1 [13]. In a similar context, the LOQ for angiotensin II was determined to be 0.186 µg/mL. This figure is also associated with both the signal and noise levels of the system, and it is usually characterized as a peak that exhibits a signal-to-noise ratio of no less than 10:1 [8].

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