REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 25, No.1 (2024)
http://www.veterinaria.org
Article received- Revised- Accepted-

Qualitative Analysis of Neomycin in Virtue of HPLC Estimation

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ABSTRACT
Neomycin, an aminoglycoside antibiotic, is widely used in various pharmaceutical formulations for treating bacterial infections. Accurate determination of its concentration is critical for ensuring drug efficacy and safety. Preformulation studies play a critical role in understanding the physicochemical properties of drugs to ensure the development of stable and effective dosage forms. In this study, a comprehensive preformulation analysis of neomycin was conducted with an emphasis on its qualitative analysis and estimation through High-Performance Liquid Chromatography (HPLC). The study focuses on optimizing HPLC conditions, including mobile phase composition, flow rate, and detection wavelength, to achieve a robust analytical procedure for neomycin quantification. Standard neomycin solutions were prepared and analyzed using a reverse-phase C18 column. The method demonstrated excellent linearity over a specified concentration range, with minimal interference from other components in the formulation. Specificity, accuracy, and precision tests were conducted to validate the method, confirming its suitability for routine quality control in pharmaceutical applications. The results suggest that HPLC can provide a rapid and reliable method for neomycin estimation, aiding in both qualitative and quantitative analyses. Also, the results demonstrated the efficiency of HPLC in determining neomycin content with high sensitivity and precision. This preformulation study provides a reliable foundation for the development of neomycin-based pharmaceutical dosage forms.

Keywords: Neomycin, Preformulation, Qualitative Analysis, HPLC, Aminoglycoside Antibiotic

1. INTRODUCTION

Neomycin, a widely used aminoglycoside antibiotic, plays a critical role in the treatment of bacterial infections, particularly those caused by Gram-negative bacteria **(Harika *et al.*, 2022; Abasi *et al.*, 2021)**. Neomycin is a *Streptomyces fradiae* antibiotic used to treat bacterial infections, primarily affecting the gastrointestinal tract, skin, and wounds **(Seethala and Fernandes, 2001)**. It inhibits protein synthesis, leading to cell death. Despite its widespread use, there's a need for effective quantification methods. It is commonly administered topically or orally and exhibits broad-spectrum antimicrobial activity **(Dessai *et al.*, 2024)**. Due to its clinical importance, accurate quantification and analysis of neomycin are essential for ensuring the efficacy and safety of pharmaceutical formulations **(Pushpa and Rajeshwari, 2023)**. High-performance liquid chromatography (HPLC) has emerged as one of the most reliable and efficient methods for the qualitative and quantitative estimation of antibiotics, including neomycin, in various formulations **(Jhade *et al.*, 2021; Chauhan *et al.*, 2022)**. The preformulation stage of drug development involves studying the physicochemical properties of active pharmaceutical ingredients (APIs) to optimize drug formulation design. Preformulation studies are essential to evaluate drug stability, solubility, compatibility, and other factors critical for drug development **(Kowalski and Dargiewicz, 2005; Gaisford, 2021; Elena *et al.*, 2020)**. When coupled with HPLC, preformulation studies can provide in-depth qualitative and quantitative analysis of neomycin, ensuring its integrity, stability, and potency in pharmaceutical products. In recent years, HPLC has been favored for neomycin analysis due to its high sensitivity, selectivity, and reproducibility **(Setiawati *et al.*, 2020)**. High-Performance Liquid Chromatography (HPLC) is a powerful analytical technique for accurately detecting and quantifying neomycin, a drug with a narrow therapeutic index and potential for nephrotoxicity and ototoxicity. Various analytical techniques, such as microbiological assays **(Huang, 2020)**, spectrophotometry, and ELISA, have been used for neomycin detection but often lack sensitivity and specificity **(Mitchell and Reed, 1997)**. HPLC offers precise quantification and minimal interference from complex matrices. Studies have shown that HPLC methods can be effective in separating and quantifying neomycin even in the presence of other aminoglycosides or excipients in pharmaceutical formulations **(Salinas and Sanchez, 2003)**. Reversed-phase HPLC (RP-HPLC) has shown promise due to its better resolution and shorter analysis times. Key factors influencing HPLC performance include the choice of mobile phase, stationary phase, column temperature, and detector settings **(Olivieri *et al.*, 1998; Nakagawa *et al.*, 2000)**. Derivatization techniques have been employed to improve detection sensitivity and chromatographic behaviour, such as pre-column or post-column derivatization. HPLC estimation of neomycin is a reliable and effective approach for qualitative analysis, and its accuracy, sensitivity, and precision continue to evolve with new detection technologies and improved chromatographic methods **(Hamidi *et al.*, 2020; Liu *et al.*, 2020)**. The technique

allows for the accurate detection and quantification of neomycin in complex matrices, such as ointments, creams, and solutions, making it a valuable tool for both research and quality control in the pharmaceutical industry **(Binns and Tsuji, 1984)**. The aim of this study is to perform a preformulation analysis of neomycin and to develop a robust HPLC method for its qualitative and quantitative estimation. This study contributes to the existing knowledge base by providing detailed insights into the HPLC method's efficacy for neomycin analysis and ensuring that the drug's therapeutic efficacy remains uncompromised in pharmaceutical formulations.

2. MATERIAL AND METHOD

2.1 Chemicals required

The materials required for the study include ethyl cellulose, which was obtained from CDH, and Neomycin. Polyvinyl alcohol was sourced from LobaChemie, while dichloromethane was procured from Merck. Additionally, distilled water was used as a solvent throughout the process.

2.2 Pre-formulation studies

1) Organoleptic Properties

The organoleptic properties of Neomycin were visually observed. Through this study the texture, color and state were observed.

2) Solubility study

Solubility study of Neomycin in different solvents was determined according to USP NF, 2007. Drug (1 mg) was accurately weighed and transferred into a 10 ml test tube; then, it was dissolved in the different solvents such as methanol, ethanol, DMSO, water, acetonitrile, methanol, chloroform etc **(Abbot *et al.*, 2022)**.

3) Melting Point

Melting point analysis was done using a digital melting point device in that the thread band was used to secure the capillary to the thermometer. Placed the sample in the capillary tube and with the help of thread tie it to the bottom of the thermometer above the mercury and placed at the port of the melting point apparatus and on the apparatus. Note the reading at which the melting of the drug occurs **(Hanko and Rohrer, 2007)**.

4) pH

Using a digital pH meter, pH was measured (EI). A digital pH meter was used to measure the pH after the medication (Neomycin) of 1-2 mg was dissolved in 10 ml of distilled water. The electrode should be carefully cleaned with deionised water and dried with scientific wipes to prevent dilution of the sample being analyzed. This can be done with the aid of a digital pH meter. The electrode should then be submerged in the solution before being read. pH meters should ideally be stored in an appropriate solution after use **(Chandira *et al.*, 2022)**.

5) Partition coefficients

Partition coefficient (oil/ water) is an indicator of drug lipophilicity. The partition coefficient was determined by the shake flask method using two immiscible solvents, the most common hydrophilic solvent was water and octanol as oil phase were taken for the study **(Wang *et al.*, 2000)**. A partition coefficient was calculated by the ratio of the concentration of a substance in one medium or phase (C1) to the concentration in a second phase (C2) when the two concentrations are at equilibrium; that is,

Partition coefficient = (C1/C2) equil

2.3 HPLC ANALYSIS

1) Instrumentation

The HPLC system (Waters, Milford, MA, USA) was consisted of a 600 controller pump, a multiple- wavelength PDA detector, an in-line AF 2489 series degasser, a rheodyne 7725i injector with a 20 ml loop with integrated Empower integration software. The separation was performed using C18 100 Å, 150× 3.9mm filled with 5 mm particles (Phenomenex, Torrance, CA, USA) column.

2) Chromatographic conditions

The assay of Neomycin was performed using externallystandardized isocratic conditions. The separation wascarried out using the mobile phase (pH 7.0) consistedmethanol and buffer (0.01M ortho- phosphoric acid) (60:40, v/v) which was degassed and filteredbefore run the column. The column temperature wasmaintained at 25°C and each injection volume was 20 ±l. The wavelength was set channel between 254 nm – 290 nm with a flowrate of 1 ml/min and the run time was set at 15 min. The peak identification and retention time (RT) of the sample.

3) Sample Preparation

Solvent Preparation

Buffer: (0.1%OPA): 1 ml of ortho phosphoricacid solution in a 1000 ml of volumetric flask added about 100 ml of milli-Q water and final volume make up to 1000 ml with milli-Q water.

Sample Preparation

Drug Neomycin weighed 4 mg and was dissolved in mobile phase solution to prepare 1 mg/ml solutions. The standard solution was subsequently diluted to prepareddifferent concentrations 10-60 µg /ml.Mobile phase and dilution primarily

REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
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filtered with the help of micron filter (0.42 micron) and syringe filter (0.22 micron). After preparation of sample degassed with the help of sonicater and this was performed before analysis.

4) Calibration curve

The calibration curve was established by analyzing the different concentrations of Neomycin standard solution ranging from 10-60 µg /ml. The calibration curve was plotted by linear regression analysis of the peak area against the respective concentration of Neomycin **(Zawilla *et al.*, 2006)**.

2.4 Method validation

The HPLC method was validated for system suitability, specificity, limits of detection and quantification, accuracy, precision, robustness and ruggedness. The method validation was performed according to the recommended guidelinesof International Conference on Harmonization (ICH) **(Harika *et al.*, 2022)**.

1) System suitability

The system suitability test was carried out to establish the parameters such as percentage relative standard deviations (% RSD) for RT, peak area response, resolution factor and capacity factor. The test was performed by analysing six replicates (n = 6).

2) Specificity

The method specificity was evaluated to minimize errors due to the presence of any other compounds. The method specificity was assessed by analyzing the chromatogram of standard puerarin. The peak purity of Neomycin was determined using multivariate analysis by comparison of RT and peak area.

3) Limits of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were assessed by determining thestandard deviation of the response and the slope (*S*) of the linear equation **(Shrivastava and Gupta, 2011)**. The following formulas wereused to determine the LOD and LOQ:

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where, σ = standard deviation of the response from thenumber of blank run and *S* = slope of the calibration curve

4) Accuracy

To ensure the accuracy of the analytical method,the recovery study of reference standard in the test sample was performed. The method was employed theaddition of known quantities of reference standard withthe pre-analysed extract sample (n = 3) followed by there-analysis of the contents by the proposed method. Therecovery of the standard was expressed as % RSD from mean recovery of the each theoretical concentration.

5) Precision

Precisions of the method were evaluated by analysingthe extract and different concentrations (10-60 µg / ml) of reference standard six times on the same day for intra-day and on six successive days(n = 6) for inter-day precision. The mean and % RSDwas calculated for intra-day and inter-day runs.

6) Robustness

Robustness study was carried out by analyzing the sample solution (20 µg /ml) under critical modifications of optimum conditions set for this method. The standard solution was analyzed with the small changes in the mobile phase ratio, flow rate, detection wavelength, pH, and column temperature to determine their effect on the RT, peak area responseand recovery. The % RSD of RT and peak area responseand percentage of mean recovery was calculated.

7) Ruggedness

The method ruggedness was performed by the analysis(n = 3) of different concentrations of sample solutionHPLC system. The HPLC system (Waters, Milford, MA, USA) was consisted of a isocratic pumpwith a photo diode array (PDA) detectorand a rheodyne 7725i injector with a 20 ml loop withintegrated Empower software. The separation wasachieved using, C 18, 3.9 ×150 mm column(Waters, Milford, MA, USA). The % RSD of RT, peak arearesponse and percentage of recovery was calculated.

8) Statistical analysis

The results were statistically analyzed using Graph Pad prism version 5.0. The results were calculated asthe mean ± SD/SEM

3. RESULTS AND DISCUSSION

3.1. Organoleptic properties

Table 1: Organoleptic properties of Neomycin

Drug	Organoleptic properties	Observation
Neomycin	Color	Off white
	Odor	Odorless
	Appearance	Powder
	State	Solid powder (hygroscopic in nature)

An evaluation of the API's organoleptic qualities, including appearance, color, odor, and state, were conducted. Neomycin was off white in color, odourless and has a solid state powder form, according to the observation. Neomycin exhibited the same appearance, color, odor and state as the I.P. requirements for these characteristics. Result show in Table1.

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3.2 Solubility study of Neomycin

Table 2: Solubility study of Neomycin

Drug	Solvents	Observation/Inference
Neomycin	Methanol	Slightly Soluble
	Ethanol	Insoluble
	DMSO	Insoluble
	Water	Freely soluble
	Chloroform	Sparingly Soluble
	Acetonitrile	Freely Soluble

The solubility of Neomycin was determined in various non-volatile or volatile liquid vehicles such as Methanol, ethanol, DMSO, water and chloroform shown in Table 2. Neomycin freely soluble in water and soluble in methanol but not in ethanol and DMSO.

3.3 Determination of melting point

Table 3: Melting Point

S. No.	Drug	Specification	Inference
1.	Neomycin	181°C~187°C	183°C±0.015

The capillary method was used to determine the melting point of a substance. The melting point of the Neomycin was found to be 183^0C, which was well within the limits of the drug specification range.

3.4 Determination of pH

Table 4: pH of the Neomycin

S. No.	Drug	Specification	Inference
1	Neomycin	5-7.5	7.2±0.019

The digital pH meter used to determination the pH of the Neomycin. These were found to be 7.2±0.019. These were well within the limits of the drug specification range.

3.5 Determination of Partition coefficient

Table 5: Partition coefficient of the Neomycin

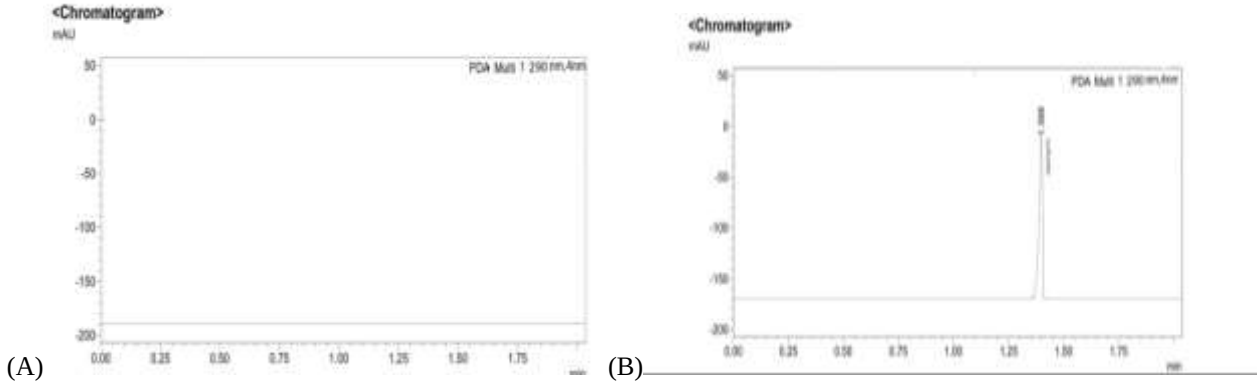
S. No.	Drug	Specification	Inference
1	Neomycin	2-3.75	3.3±0.024

The Partition coefficient of the Neomycin was 3.3±0.024 through this it was observed that the compound was hydrophilic in nature.

3.6 Standard graph of Neomycin by HPLC

3.6.1 Chromatographic condition

A mobile phase system consisting of 0.01M ortho- phosphoric acid and methanol was used in the ratio of 40: 60% v/v at a pH 2.5 adjusted with ortho phosphoric acid and it is also used as diluents for preparing the working solution of drugs. The separation was performed with the elution method and flow rate was1.0ml/min. The injection volume was 10 µL. The eluent was proctor by the photo diode array detector (PDA) from 200 to 400 nm, and chromatograms were gained at the wavelengths of 290 nm. The total run time was 5 min and all establishments were performed at 30 ºC.



(A) (B)

Graph 1: (A), and (B) showing blank graph of the mobile ratio, peak on multi channel at 290nm

HPLC (Waters, Milford, MA, USA) used to determine the concentration graph with peak area and retention time of the Neomycin. During the analysis by HPLC flow rate was 1.00 ml/min, mobile phase ratio (Methanol: buffer; 60:40) and the run time was 15 min. The retention time of the graph started from the 0 min and ended at 5 min. At 290 nm channel the

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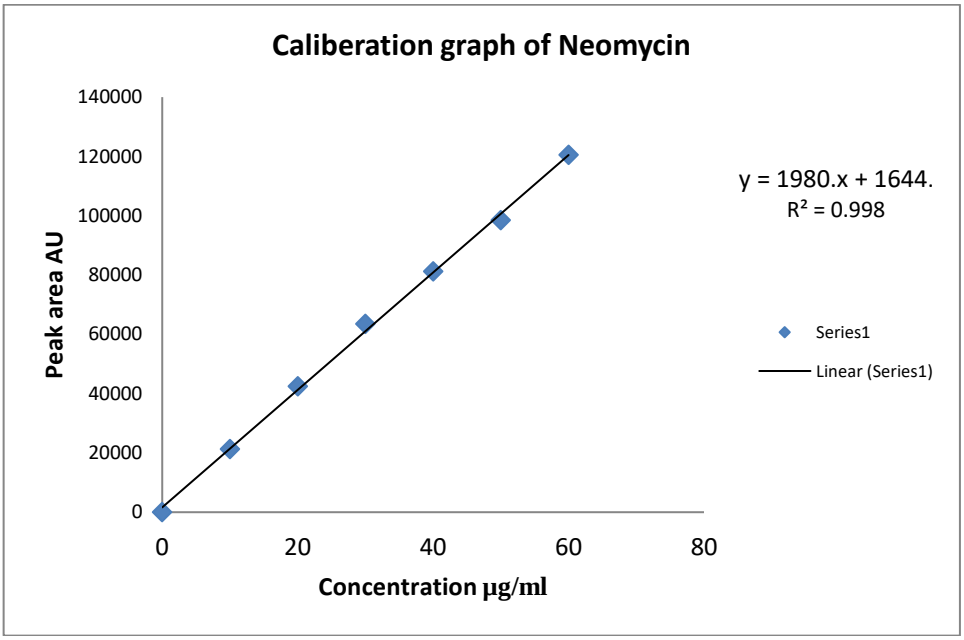
peak was observed. As shown in the Graph1**.** Before performing analysis baseline correction was done with the mobile phase for the saturation of the column. And then the sample of varied concentration checked at 290 nm.

Table 6 : showing concentration with peak area

CONCENTRATION (□g / ml)	RETENTION TIME	PEAK AREA
10	1.37	21298
20	1.33	42457
30	1.36	63481
40	1.39	81223
50	1.31	98451
60	1.38	120476
Mean	1.356667	71231
SD	0.030768	36461.35
%RSD	2.2	51



Graph 2: showing the calibration of Neomycin

The linearity of the proposed method was established by least squares linear regression analysis of the calibration curve. The regression equation for Neomycin was obtained by plotting Peak area (AU) versus concentration of Neomycin in the range of 10-60µg/mL. Six points calibration curve were obtained in a concentration range from 10-60µg/mL for drug. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation was y = 1980.x + 1644 and with correlation coefficient R^2 = 0.998**.**

3.6.2 System Suitability

Table 7: System suitability parameters

Parameters	Neomycin
Tailing Factor	1.15
Theoretical plates	468
USP Resolution	--
LOD(µg/ml)	0.04
LOQ(µg/ml)	0.065

After checking the system suitability it was found that the tailing factor , theoretical plate ranges were under the limit that means that the system were suitable for the analysis and also the limit of detection and limit of quantification were less than 1 this shows system was ready for the analysis.

Table 8: System suitability parameters for Neomycin (n = 6)

Injection number	Retention time	Peak Area (AU)	Resolution factor(R_S)	Capacity factor(k')
1	1.4	68472	4.23	4.27

2	1.38	69714	4.24	4.28
3	1.25	69778	4.26	4.34
4	1.35	68998	4.32	4.43
5	1.29	69114	4.36	4.47
6	1.31	69974	4.39	4.58
Mean	**1.33**	**69341.6667**	**4.3**	**4.39**
SD	**0.056921**	**575.347431**	**0.066**	**0.120**
% RSD	**4.2**	**0.82**	**1.5**	**0.2**

Six replicate injections of standard solution were injected and the chromatograms were recorded. The system was suitable for analysis if the % relative standard deviation (%RSD) of area counts in six replicate injections should be not more than 2.0%. The resultsof system suitability parameters were given in above table and % RSD of the peak area were found to be less than 2% indicating the system suitability for the method.

3.6.3 Accuracy

Injected the standard solutions of accuracy 25%, 50% and 75% for neomycin.

Table 9: Accuracy of the Neomycin

Sample	**Neomycin**		
% Concentration	25%	50%	75%
Trail-I	100	99.14	98.11
Trail-II	99.93	98.72	99.07
Trail-III	99.05	98.92	98.53
AVG(%Recovery)	**99.95**	**98.93**	**98.79**
SD	**0.90254**	**0.211438**	**0.378347**
%RSD	**0.9**	**0.21**	**0.38**

The accuracy of neomycin was checked by the trails this results shows that the percentage RSD was less than 2% and system as well, as method was accurate for the study. The % recovery for neomycin at each level should be between 99 to 101%.The results was under the limit.

3.6.4 Precision

a) Intraday Precision (n=6)

Table 10: Intraday Precision of Neomycin (n=6)

Concentration (□g / ml)	**Retention time**			**Peak area (AU)**		
	Mean	**SD**	**%RSD**	**Mean**	**SD**	**%RSD**
10	1.33	0.026458	1.9893	21535.67	223.26	1.0367
20	1.34	0.017321	1.2926	42594.67	163.9217	0.3848
30	1.38	0.020817	1.5048	64148.67	1159.032	1.8068
40	1.4	0.01	1.01	84263.67	2815.715	2.3416
50	1.34	0.026458	1.9744	98572	132.7516	0.1347
60	1.36	0.028868	2.1174	121307.3	767.5235	0.6327

b) Interday Precision (n=6)

Table 21: Interday Precision of Neomycin (n=6)

Concentration (□g / ml)	**Retention time**			**Peak area (AU)**		
	Mean	**SD**	**%RSD**	**Mean**	**SD**	**%RSD**
10	1.2	0.1	0.083333	20323	925.3275	0.045531
20	1.22	0.110151	0.090042	40720	1528.947	0.037548
30	1.33	0.025166	0.018875	61732.33	1566.326	0.025373
40	1.35	0.036056	0.026708	78842.67	3037.94	0.038532
50	1.25	0.047258	0.037606	88912	566.2093	0.006368
60	1.356	0.020817	0.015344	48426.33	62397.01	1.288493

The standard neomycin solutions were injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six repeat injections was established to be within the specific limits. The precision of the developed method was expressed in terms of % relative standard deviation (% RSD). The intermediate precision was assessed by comparing the results obtained on three different days. The experimental values (%RSD) obtained for repeatability (intra-day precision) and intermediate precision (inter-day precision) were shown in the above tables. The % RSD of inter- and intra-day analysis of standard were found to be lower than 2% with a high repeatability in the RT. There was no significant difference in the inter- and intra-day analysis indicates the proposed method was very suitable for the analysis of Neomycin.

Acceptance Criteria: The % RSD should not be more than 2%.

3.6.5 Ruggedness

Table 32: Ruggedness of the sample Neomycin (n=3)

Concentration (□g g / ml)	Retention time	%RSD	Peak area (AU)	%RSD	Recovery (%)
10	1.21	0.09531	20759.33	0.041037	99.2
20	1.24	0.112983	41207	0.033756	98.57
30	1.35	0.039196	62825.78	0.036877	99.21
40	1.3	0.011428	81835.89	0.052717	98.99
50	1.28	0.068108	92390	0.059377	99.65
60	1.30	0.055505	60987.11	0.91482	98.54

The method ruggedness was performed by the analysis(n = 3) of different concentrations of standard solution. The peak area, retention time, % RSD and recovery percentage were calculated and result shown in the above table. The method ruggedness was determined by comparing the results of RT, peak area response and the assay of Neomycin obtain from the different HPLC systems. The % RSD was found to be less than 1% which indicates the method was rugged.

3.6.6 Robustness

Table 43: Robustness of method (n = 3)

Parameter	Proposed	Variation	Retention time (RT)	% RSD	Response (AU)	% RSD	Recovery (%)
Mobile phase (v/v)	60:40	65:45	1.36	0.65	6952334.33	0.16	99.01
		70:30	1.32	0.40	6964648.33	0.17	98.63
Flow rate (ml/min)	1	0.9	1.39	0.24	69539.17	0.12	98.80
		1.5	1.30	0.49	69612.78	0.20	100.01
Wavelength (nm)	290	295	1.35	0.23	69524.64	0.16	97.25
		280	1.45	0.91	69455.43	0.29	99.01
Column temperature (°C)	25	30	1.34	0.56	69473.61	0.27	98.24
		26	1.38	0.82	69641.08	0.40	99.88
pH	7.0	7.2	1.25	0.67	69457.28	0.32	98.20
		6.8	1.34	0.34	694243.37	0.31	98.99

Robustness was estimated by making deliberate changes in the chromatographic conditions like change in temperature, flow rate, and mobile phase composition and evaluated for the impact on the method.
It was observed from the chromatograms that the results were within the limits. This indicates that the method developed was robust.
The results of method robustness were given in above table and no significant variation in RT, peak area responseand recovery of Neomycin were observed under modifiedcritical conditions and the % RSD was always less than 1% and this indicate the system was robust at the time of analysis. For the robustness mid of the concentration taken was 30 µg/ml.

3.7 FTIR

Table 54 : FTIR- Spectrum Frequency Range

Sr. No.	Drugs	Frequency Range	Group Absorption (cm^{-1})	Appearance	Group	Compound Class

1	**Neomycin**	3550-3200 (cm^{-1})	3432.63	Strong, Broad	O-H stretching	Hydroxyl Group
		2400- 2000 (cm^{-1})	2366.81	Strong	C-H stretching	Alkane
		2350-2340 (cm^{-1})	2345.70	Strong	O=C=O stretching	Carbon dioxide
		1650-1580 (cm^{-1})	1637.66	Medium	N-H bending	Amine
		1550-1500 (cm^{-1})	1541.42	Strong	N-O stretching	Nitro compound
		1400- 1100 (cm^{-1})	1102.79	Weak	C-C stretching	Alkane
		690-515 (cm^{-1})	618.91	Strong	C-Br stretching	Halo compound

4. CONCLUSION

In conclusion, the preformulation and qualitative analysis of neomycin through HPLC estimation demonstrated the effectiveness of this method for accurate and reliable quantification. The study successfully established a precise and reproducible chromatographic method, ensuring the purity, stability, and appropriate formulation of neomycin. The results confirmed the method's sensitivity, selectivity, and efficiency in detecting neomycin, thus providing a robust tool for future pharmaceutical applications. This approach can be invaluable for quality control in both the development and production phases, contributing to enhanced therapeutic outcomes and regulatory compliance.

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