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Green RP-HPLC Stability-Indicating Assay Method for Neomycin Sulfate in the Veterinary Formulation

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Abstract

The current work studied the stability of neomycin sulfate (NS) in its pharmaceutical dosage form. The ability of NS to withstand alkaline, acidic, and oxidative forced degradation conditions was evaluated. We developed a RP-HPLC method over a Kromasil C18(w) using a mobile phase of 0.1% aqueous phosphoric acid maintained at a flow rate of 1mL/min within a relatively short run time of 3.0 min. The UV-detector monitored the effluent at 210.0 nm, NS expressed a correlation coefficient approaching unity within the concentration range 40-200 µg/mL. The method achieved adequate specificity for NS in presence of the products of degradation and excipients of the dosage form. Validation parameter were calculated according to the USP guidelines. The stability study expressed poor NS stability in either of the three degradation conditions. The procedure fulfilled the greenness criteria of the four quadrants of the National Environmental Methods Index metric. The method represents a reliable green analytical solution for NS stability assessment in quality control laboratories.

Keywords: RP-HPLC; Neomycin sulfate; Forced degradation; Method validation.

1. Introduction

Neomycin sulfate (NS) chemically is (2R,3S,4R,5R,6R)-5-amino2-(aminomethyl)-6-{[(1R,2R,3S,4R,6S)-4,6-diamino-2-[(2S,3R,4S,5R)-4- {[(2R,3R,4R,5S,6S)-3-amino-6-(aminomethyl)-4,dihydroxyoxan-2-yl]oxy}-3-hydroxy-5-(hydroxymethyl)oxolan-2-yl]oxy}-3-hydroxycyclohexyl]oxy}oxane-3,4-diol (**Figure 1**). Neomycin is a bactericidal aminoglycoside antibiotic that binds to the 30s ribosome of susceptible organisms. Binding disrupts mRNA binding and acceptor tRNA sites, resulting in non-functional or poisonous peptides being produced [1, 2]. The neomycin is a variable mixture of two stereoisomers, neomycin B and C which are active components and

neomycin A is a degradation product [3, 4]. Neomycin Sulfate is an Aminoglycoside antibiotic ctopical application to the skin and eye [5, 6]. Neomycin sulfate is listed in the Indian and British pharmacopoeias [7]. Because neomycin sulfate has a chromophore group, it necessitates the use of a derivatizing reagent. So, $CuCl_2.2H_2O$ was used to derivatized neomycin sulfate.

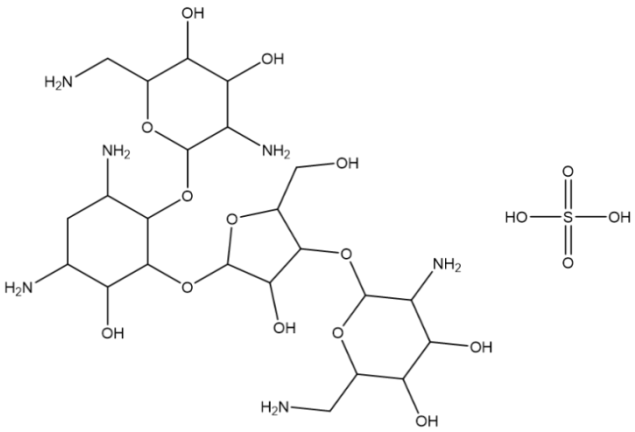

Figure 1. Chemical structure of neomycin sulphate.

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A review of the literature on neomycin sulfate reveals Derivative spectrophotometry [8], HPLC [9], RP-LC [10], RP-HPLC [11-14], normal phase high performance liquid chromatographic (NP-HPLC) [15], Capillary electrophoresis [16], Flow injection chemiluminescence [17], and TLC [18-21] have all been used for single and combined forms.

Greenness has recently become a major concern for chemists. Greenness concepts yearn to minimize the carbon footprint, reduce consumption of chemicals, and production of wastes.

This paper introduces a novel isocratic RP-HPLC method using a Kromasil C18(w) column that can withstand a green (100% aqueous) mobile phase for the determination of neomycin sulfate in presence of its degradation products. The method was used to investigate the stability of neomycin under forced acidic, basic, and oxidative degradation conditions. The method was validated according to the USP guidelines [22]. The method greenness parameters were assessed according to the National Environmental Methods Index (NEMI) [23]. This method is different from All methods used for neomycin sulfate as HPLC by tandem mass spectrometry (LC–MS/MS) is very high cost and not available in all Pharmaceutical factories. ELISA and immunochromatographic assay (these methods not used in Pharmaceutical and very high coast. HPLC by derivatized with 9-fluorenylmethychloroformate (not a direct method) has a long time and high error. antibiotic Assay (not used in combination manly with another antibiotic).

2. Experimental

2.1. Chemicals and reagents

o-H_3PO_4 85% was HPLC grade from SDFCL chemicals. Water, HCl fuming (37%), NaOH pellets, and H_2O_2 (10%), were bought from Merck (Germany). The mobile phase was filtered using a 0.45 µm nylon membrane filter, ChromTech, (UK).

2.2. Standards and samples

Neomycin sulfate working standard was obtained from Hetero Drugs, (Hyderabad, India). neomycin Adwia 20% water Soluble and neoxymart WSP (combination of 20% neomycin + 20 % Oxytetracycline HCl was acquired at a nearby market.

2.3. Instrumentation and chromatographic conditions

C18(w) (4.6 x 250.0 mm; 5-µm) Kromasil was used to analyze drugs on an Agilent 1200 with a UV detector. Peak areas were integrated using an Agilent LC solution software program (chemstation). LC-solution software was used to record the data. An ultrasonic cleaner from Skymen (JP-060S, China) Degassing the mobile phase was accomplished with this method.

The separation and quantification were accomplished using HPLC a 250.0 x 4.6 mm C18 (5.0 µm particle size) column. The mobile phase was prepared by adding one mL of phosphoric acid 85% result pH 3 as a buffer with Acetonitrile in was run isocratic. A flow rate of 1.0 mL/min was used to deliver the mobile phase to the system. At room temperature, all measurements were taken. The total volume injected was 20 l. The wavelength of the detector was set at 210 nm. 3.0 minutes was specified as the run time

2.4. Standard stock and standard solution Preparation

Stock Standard solutions: weight and dissolve 100 mg of neomycin sulfate standard in 100 mL volumetric flask using mobile phase. by sonicated for 5 minutes. Then, in a 50 mL volumetric flask, dilute 5 mL of Stock Standard solutions and fill to volume with Mobile phase (final concentration 100 g/mL). The concentration range of Neomycin Sulfate from 50.0 – 200.0 µg/mL was used in linearity.

2.5. Preparation of sample solution

Stock Sample solutions: weight and dissolve (eq. to 100 mg of neomycin sulfate) from Sample in 100 mL volumetric flask using Mobile phase. by sonicated for 5 minutes. then diluted 5 mL from Stock Sample solutions into 50 mL volumetric flask and complete to volume with Mobile phase (final concentration 100 µg/mL). The concentration range of Neomycin Sulfate from 80.0 – 160.0 µg/mL was used in linearity.

2.6. Specificity

It gives an indication of the procedure's selectivity and specificity. The method is selective if the main peak is well separated from any other peak by a resolution of at least 2. This was accomplished by injecting placebo and comparing it

to standard and placebo; spiked with standard and sample. Then the peak purity was determined using PDA.

2.7. Forced degradation study

ICH [24] given stress conditions such as acidic, basic, and oxidative stresses, which were carried out.

2.8. Standard Drug Stock Solutions

2.9. Under hydrolysis and oxidation conditions, the drug's forced degradation was investigated. 100 mg of neomycin sulfate, carefully weighed, was placed in a 100-mL volumetric flask and diluted with the Mobile phase to obtain a final concentration of 1000 g/mL of neomycin sulfate. For the forced degradation investigation, these stock solutions were used.

2.9. Acid hydrolysis

Forced degradation in acidic media was performed by weight and dissolve (eq. to 100 mg of neomycin sulfate) form Sample in 100 mL amber volumetric flask. Then add 10 mL of 1 N HCl and these mixtures were kept at reflux for 2 hours. This solution was neutralized with 1 N NaOH before analysis. then diluted 5 mL from Stock solutions into 50 mL volumetric flask and complete to volume with Mobile phase as shown in (**Figure 2a**).

2.10. Base hydrolysis

Forced degradation in basic media was performed by weight and dissolve (eq. to 100 mg of neomycin sulfate) form Sample in 100 mL amber volumetric flask. Then add 10 mL of 1 N NaOH these mixtures were kept at reflux for 2 hours. This solution was neutralized with 0.1N HCl. then. diluted 5 mL from Stock solutions into 50 mL volumetric flask and complete to volume with Mobile phase as shown in (**Figure 2b**).

2.11. Oxidative hydrolysis

Degradation with H_2O_2 was achieved by weight and dissolve (eq. to 100 mg of neomycin sulfate) form Sample in 100 mL amber volumetric flask, then add 20 mL of 10% (w/v) hydrogen peroxide, These mixtures were kept for 2.0 hours, then diluted 5 mL from Stock solutions into 50 mL volumetric flask and complete to volume with Mobile phase as shown in (Figure 2c).

2.12. Linearity

For neomycin sulfate, linear calibration plots of the proposed approach were obtained at concentration ranges of (40-200µg/mL). Each solution was made three times.

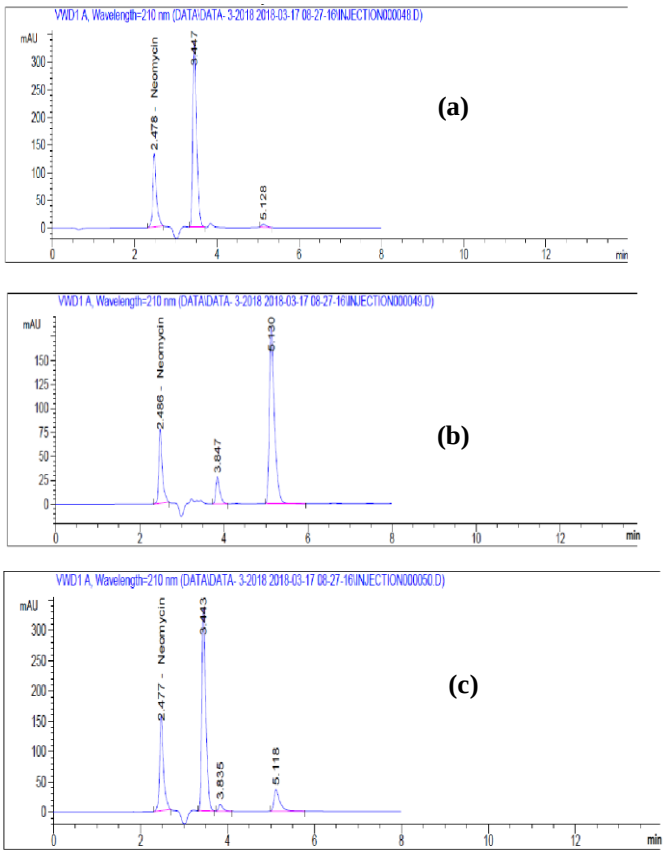

Figure 2. HPLC chromatogram of forced degradation in (a) acidic media, (b) basic media, and (c) hydrogen peroxide.

2.13. Accuracy

Spiking test samples with standard NS was used to determine accuracy. The measurements were taken at concentration levels that were in the middle of the target concentration's range. Three determinations over three concentration levels covering the specified range were used to spike the test samples with known quantities of neomycin sulfate. The peak area of standard neomycin sulfate utilised in the standards was compared to the peak area obtained using the calibration curve equation.

2.14. System suitability

To verify the accuracy of the chromatography system, we achieved system compatibility by repeatedly injecting the standard solution 6 times under 100% level test conditions. Through the use of distinct retention times, the purposed RP-HPLC method allows for the simultaneous assessment of neomycin sulfate in sample medication. **Table 1** shows the data on system appropriateness.

Table 1. System Suitability Parameters for Neomycin Sulfate

Parameters	Value
Tailing factor (T)	1.18
Retention time (min)	2.4
Retention factor (k)	1.67
Number of theoretical plates (N)	2811

2.15. Ruggedness

It is described as the consistency of test findings produced by testing the same samples under various situations such as different analysts, columns, dates, and , etc.

Day to day: For each determination, five replicates of a single powder material sample (100 percent) were employed. Five duplicates were examined on the first day. The following day, the same analyst assessed additional five replicates of freshly generated tests from the same sample.

Analyst to analyst: It determines ruggedness between different analysts. Five replicates of a single sample were analyzed. Then, a second person analyzed five replicates from the same sample, prepared by him.

Column to column: The same analysis method was performed on columns with the same filler and length but different lot numbers.

2.16. Robustness

The robustness of a method is measured by looking at how it reacts to slight changes in its typical operating settings. For example, in RP-HPLC, a small change in sonication time or aliquot stability could change this.

2.17. Limit of detection (LOD) and limit of quantitation (LOQ)

The limits of detection and quantification were determined by the signal-to-noise (S/N) method. Various concentrations of solutions were prepared by adding a known amount of neomycin sulfate to confirm quantitation and detection limits. The signal-to-noise ratio was calculated after each solution was made according to a particular methodology. The limits of quantitation and detection were calculated using the average signal-to-noise ratio of all experiments at each concentration level. A concentration level that provides a signal-to-noise ratio of 10:1 for accurate and accurate quantitation of the analyte is specified as the limit of quantitation. Concentration levels that provide a signal-to-noise ratio of 3:1 for easy detection of the analyte are indicated as the detection limits.

3. Results and Discussion

Chromatographic HPLC separation of the studied component was achieved on a Kromasil C18(w) 250x4.6 mm (5.0 µm particle size) column. The mobile phase was prepared mix of 98% of 0.1% phosphoric acid 85% and 2% Acetonitrile. The mobile phase was deliver mobile phase to the system. All tests were carried out at room temperature. The total volume injected was 20 µl. The wavelength of the detector was set at 210 nm. The run time was set for 3.0 min. Sharp, separated, and symmetric peaks are shown in (**Figure 2)**.

In the case of the proposed method, a linear calibration curve was obtained for neomycin sulfate in the concentration range of 40-200 µg/ml illustrated in (**Figure 3)**.

Each concentration was injected three times to obtain a reproducible response. A calibration curve was constructed by plotting concentration versus peak area. Each reading had an average of 3 definitions. They were expressed by the linear regression equation.

$Y_{\text{Neomycin sulfate}} = 17.61296x + 2.71178$, $r^2 = 0.99996$

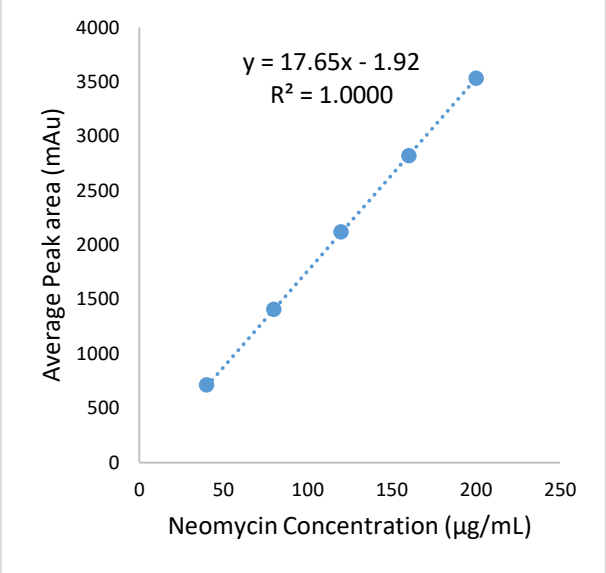

Figure 3. Calibration curve of neomycin sulphate in the linear concentration range 40 – 200 µg/mL.

Slopes and intercepts were obtained by using regression equation (Y = mx + c) and least square treatment of the results used to confirm the linearity of the method developed.

LOD LOQ was determined by making serial dilutions. LOD was found to be 10.0µg/mL for neomycin sulfate (signal to noise ratio of 3:1). LOQ was found to be 30.0µg/mL for neomycin sulfate (signal to noise ratio of 10:1).

Accuracy was calculated by adding the standard drug to pre-analyzed samples at three concentration levels and calculating recovery. The standard limit of %recovery study is 98 - 102%. Results of accuracy were proven by **Table II**.

Table II. Accuracy and Recovery Results for Determinations of Neomycin Sulfate

Working conc. (mg/mL)	Peak area	Found conc. (mg/mL)	% Recovery
80	1410.33	79.92	99.9
80	1416.33	80.26	100.33
80	1413.89	80.12	100.15
100	1750.33	99.22	99.22
100	1753.33	99.39	99.39
100	1752.25	99.33	99.33
160	2816.27	159.74	99.84
160	2820.6	159.99	99.99
160	2816.64	159.76	99.85
	Mean		99.78
	SD		0.383
	%RSD		0.384

The specificity of the method is its suitability for analyzing compounds in the presence of potential contaminants. To demonstrate the specificity of the optimized method, placebo, standard, and sample test solutions were administered at a single wavelength of 210 nm. A comparison of the retention times of neomycin sulfate in sample solutions and in the standard solutions was exactly the same. As demonstrated in the table, there was no interference from the placebo at the retention times for neomycin sulfate **(Figure 4)**. As a result, the proposed method is appropriate for quantifying active components in dose formulation. The validation parameter of the proposed method is summarized in Table **III**.

The method demonstrated four green quadrants in the NEMI pictogram (**Figure 5**), which reveal the absence of persistent, bio-accumulative, toxic, corrosive, and hazardous chemicals and minimal wastes.

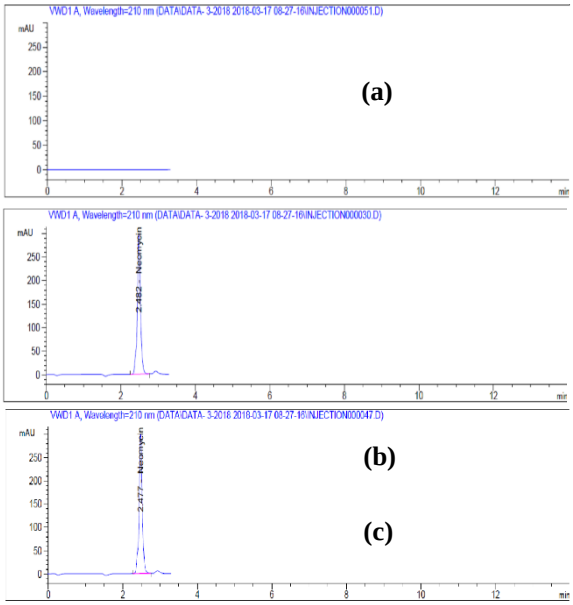

Figure 4. HPLC chromatogram of (a) placebo, neomycin sulphate (b) standard, and (c) test drug.

Table III. Assay validation sheet of the proposed method.

Parameters	Value
Calibration range (µg/mL)	40 - 200
Slope	17.65 ± 0.01
Intercept	-1.92
Correlation coefficient	1.000
Accuracy (Mean recovery%)	99.78
Precision:	
Repeatability (%RSD)[a]	0.004
Intermediate precision (%RSD)[b]	0.015
LOD (µg/mL)	13.3
LOQ (µg/mL)	40.0

[a]The intra-day precision (n = 9), average SD of three different concentrations repeated three times within one day.

[b]The inter-day precision (n = 9), average SD of three different concentrations repeated three times on three successive days.

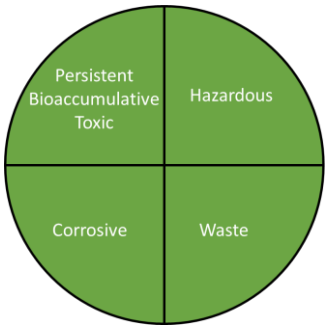

Figure 5. The National Environmental Methods Index metric for the developed chromatographic method.

The suggested RP-HPLC method uses fewer reagents and ingredients, is easier to use, and takes less time to complete. In the pharmaceutical sector, this procedure can be utilised for quality control testing. The chromatogram of neomycin sulfate was shown in **(Figure 3)**. There was a clear resolution between neomycin sulfate with a retention time of 2.4 minutes. Validation parameters include the limit of detection, and quantitation linearity, precision, accuracy, robustness, and specificity. All methods for determination of neomycin sulfate in pharmaceutical forms using by reversed-phase high-performance liquid chromatography with mass spectrometry & by ELISA and immunochromatographic assay and by liquid chromatography by derivatized with 9-fluorenylmethychloroformate with fluorescence detection. So, all methods for the determination of neomycin sulfate have different parameters as HPLC mass spectrometry & HPLC by derivatized.

The eco-friendly character of the method---proven by the NEMI greenness metric—guarantees minimal environmental impact. The method consumed minimal resources and produces fewer wastes.

4. Conclusion

A novel green RP-HPLC method was developed, validated, and employed to investigate neomycin stability in powder formulation. The drug is labile to acidic, basic, and oxidative degradation conditions. The method employed a novel 100% aqueous compatible column and a completely aqueous mobile phase. The four green quadrants of

the NEMI pictogram proved the greenness of the method.

5. Conflict of Interest

There are no conflicts to declare.

6. References

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