

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR NATAMYCIN IN BULK AND OPHTHALMIC DOSAGE FORMS.

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ABSTRACT

Objective: This study was aimed to develop stability indicating RP-HPLC method for analysis of Natamycin and its dosage form according to the ICH Q1A (R2) guidelines.

Methods: Successful separation of drug from degradation products formed under stress conditions was achieved by a gradient HPLC method, performed on Series 200 UV-visible detector by using Kromasil C₁₈(100 x 4.6 mm, 5 μm) column; the flow rate was 1 ml/min, injected volume 20 μL, the optimised mobile phases consist of Solvent A (Methanol) and Solvent B (Phosphate Buffer pH 3.5, adjusted pH with o-phosphoric acid) and UV detection was carried out at 304 nm.

Results: The retention time of Natamycin was 8.96±0.10 minutes. The method was validated with respect to system suitability, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness. Linearity (20–100 μg/ml), LOD and LOQ was found to be 1.2 and 3.66 μg/ml respectively. The RSD for intra-day (0.59-0.86) and inter-day (0.44-0.96) precision were found to be less than 1 %. The mean percentage recovery was 100±2% which shows good agreement with the labelled amount in the ophthalmic formulations. Natamycin was exposed to acid-base hydrolytic, oxidative, thermal and photolytic stress conditions. The forced degradation studies were performed by using 0.01 N HCl (8.62%), 0.01 N NaOH (9.18 %), 15 % H₂O₂(24.13 %), thermal (100 °C, 5.13%) and sunlight (29.41%).

Conclusion: Natamycin is susceptible towards acidic, basic, oxidative and photolytic conditions in comparison to unaffected by thermal degradations. The method is simple, specific, precise, robust and accurate for the determination of Natamycin in ophthalmic formulations.

Keywords: Natamycin, RP-HPLC, Stability Indicating Method, Validation, Forced degradation.

INTRODUCTION

Stability indicating methods have become an important aspect of any analytical method validation. Natamycin (figure 1) is amphoteric macrolide antifungal antibiotic drug from *Streptomyces natalensis* or *Streptomyces chattanoogensis*. It is chemically [1R,3S,5R,7R,12R,22R,24S,25R,26S)-22-[[[(3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyloxan-2-yl]oxy]-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.0^{5,7}]octacosane-8,14,16,18,20-pentaene-25-carboxylic acid, used for a variety of fungal infections, mainly topically.[1, 2] The Reverse-Phase High performance liquid Chromatography (RP-HPLC) is very useful for the determination of Natamycin drug substance in ophthalmic dosage form.

Stability-Indicating Method (SIM) for formulation is defined as a validated analytical procedure that accurately and precisely identifies active Pharmaceutical ingredients (API), free from interferences like degraded product, process impurities, excipients. SIM is a quantitative analytical procedure which is used for the detection of decrease in the amount of API present due to degradation by applying various stress conditions. For developing SIM, a forced degradation study is normally carried out under more severe conditions than those used in accelerated studies. Approximately 5-20% degradation of the drug substance has been considered as reasonable and acceptable for validation of chromatographic assays [3, 4].

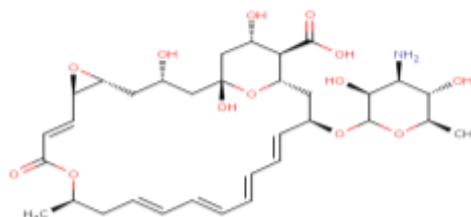
Literature survey revealed that very few analytical methods have been reported for the determination of Natamycin in Doogh, refreshing drink, rabbit and human plasma and wines by HPLC[5], LC MS/MS[6-8] and UPLC-tandem mass Spectrometry[9] method. The reported HPLC methods were considered to be uneconomical, time

consuming and having poor symmetry [10, 11]. Natamycin 5 % w/v eye drops is available in the market but the Natamycin has not been adopted by any official pharmacopoeia (USP, BP or EP etc.), there is no reported stability indicating method using HPLC in bulk drug or formulation. So in the present research work attempt was taken to develop and validate RP HPLC method which was cost effective and having short analysis time in order to increase efficiency and also performed forced degradation study of Natamycin for developing Stability Indicating Method.

MATERIALS AND METHODS

Chemicals and Reagents

Natamycin working standard and Drug product (5 % w/v Ophthalmic Suspension) were procured from the market. Methanol procured from Merck India was of HPLC grade. GR grade potassium di-hydrogen orthophosphate from Molychem, o-phosphoric acid, hydrochloric acid (HCl), Hydrogen peroxide (H₂O₂) 15% w/v were from Merck India, sodium hydroxide (NaOH) pellets were from RFCL limited SQ grade. HPLC grade water was obtained through milli Q water purification system. All chemicals were of analytical grade and used as received.



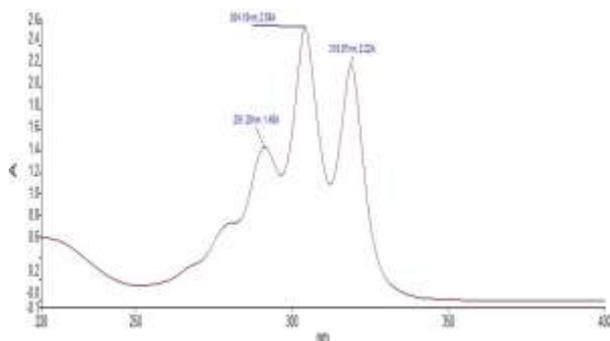


Fig.1: Chemical structure and UV spectrum (λ_{\max} = 304 nm) of Natamycin ((1R,3S,5R,7R,12R,22R,24S,25R,26S)-22-[[[(3S,4S,5S,6R)-4- amino-3,5-dihydroxy-6-methyloxan-2-yl]oxy]-1,3,26-trihydroxy-12- methyl-10- oxo-6,11,28-trioxatricyclo [22.3.1.0^{5,7}] octacos-8,14,16,18,20-pentaene-25-carboxylic acid)

Instrumentation

The HPLC System Analysis was performed with a Perkin Elmer (USA), aSeries 200 UV-visible detector using total chrome navigator software for data handling using C₁₈(100 x 4.6 mm, 5 μ m) column. Samples were injected through a Rheodyne injector valve with 20- μ L sample loop.

Chromatographic Conditions

The chromatographic separation was carried out at room temperature on a kromasil C₁₈(100 mm x 4.6 mm) analytical column under RP-HPLC conditions. In order to select a suitable mobile phase for the analysis of Natamycin, various combinations of solvents were tried on the basis of trial and error, considering the system suitability parameters like retention time, tailing factor, number of theoretical plates and HETP.

Preparation of Stock Standard Solution

Accurately weighed 10 mg of Natamycin was transferred to a 10 ml volumetric flask, sufficient amount of diluent (Methanol: Water, 1:1) was added to dissolve it and volume was made up to 10 ml (Stock A; 1000 μ g/ml). Aliquots of stock A were further diluted with diluent up to 10 ml to get concentration of 20, 40, 60, 80, and 100 μ g/ml for the linearity study.

Selection of Mobile Phase

The mobile phases consist of two solvents: Solvent A, Methanol and Solvent B, Buffer pH 3.5 was prepared by using 1.36 gm of potassium di-hydrogen phosphate dissolved in 1000 ml of water, pH was adjusted to 3.5 with o-phosphoric acid (HPLC Grade). Before proceeding for analysis the mobile phase was degassed by use of a sonicator (Oscar) and filtered through a 0.45 μ m HPLC filtration assembly. The diluents used to attain the final concentration consist of a mixture of water: methanol (1:1). Flow rate was 1 ml/min, injected volume 20 μ L, wavelength of detection is 304 nm. The system was equilibrated before each injection. Gradient elution system was performed which consists of solvent A and Solvent B. The program of gradient elution is shown in Table 1 below.

Table 1: Gradient Elution (Solvent Programming) Runs

Time (min)	Solution A (%)	Solution B (%)
0.5	25	75
8	90	10
8	25	75
3	25	75

Method Validation

The proposed method is validated according to the ICH guideline.

System Suitability:

The stock solution of the drug was prepared at strength of 100 μ g/ml. It was diluted to prepare solutions containing 40, 60 and 100

μ g/ml of the drug Natamycin, and 3 replicate injections of each concentration were inserted into the HPLC system.

Linearity Curve (Calibration Curve):

Aliquots of standard Natamycin stock solution were taken in five different volumetric flasks and diluted up to the mark with the diluents such that the final concentrations of Natamycin are 20, 40, 60, 80 and 100 μ g/ml. Each of these drug solutions was injected 20 μ L into the injector and recorded the peak area.

Accuracy

The accuracy of the method was determined by calculating percentage recovery of Natamycin. Recovery studies were carried out by applying the spiking method in which known amount of Natamycin corresponding to 80, 100 and 120% of label claim was added (standard addition method) to the placebo. At each level of the amount three determinations were performed and fitting these values to the linear equation of the calibration curve.

Precision:

Precision study of sample (Natamycin) was carried out on Intraday and Interday by estimating corresponding responses 3 times each for 40, 60 and 80% target concentration.

LOD and LOQ

The limit of detection (LOD) is defined as the smallest quantities of Natamycin clearly distinguishable from baseline, which has a signal to noise ratio of 3:1. LOD was calculated using following formula, $LOD = 3.3(SD)/S$, where SD = standard deviation of response (peak area) and S = average of the slope of the calibration curve. Limit of Quantification (LOQ) was determined at a signal to noise ratio (S/N), which was experimentally verified by diluting known concentrations of Natamycin until the average responses were approximately 10 times the standard deviation of the responses for six replicate determinations, LOQ was calculated using following formula, $LOQ = 10(SD)/S$.

Robustness

Robustness is the measure of method capacity to remain unaffected by deliberate small changes in the chromatographic conditions like detection wavelength and flow rate. Detection wavelength was changed from 304 nm to 304 ± 2 nm and flow rate was changed from 1 ml/min to 1 ± 0.2 ml/min. Effect of these changed parameters was studied by injecting the sample into the system.

Assay for Ophthalmic Suspension

For the assay of marketed formulation, 1 mL of the marketed sample (5% w/v Natamycin Ophthalmic Suspension USP - NATACIN) was pipetted out using a volumetric pipette and transferred to a 100 mL of volumetric flask and diluted with methanol-water mixer (50:50) to get the concentration of 500 μ g/ml. Again diluted it to prepare 50 μ g/ml solution. Sample was injected into column and peak areas of the bands were measured at 304 nm. The amount of drug present in sample was estimated from the calibration curve. Procedure was repeated six times for the analysis of homogeneous sample.

Forced Degradation study of Natamycin

Forced degradation studies of Natamycin were carried out under conditions of acid-alkali hydrolysis, dry heat, oxidation and photolysis. A minimum 10% degradation of the initial concentration is permissible for various stress degradation studies. Natamycin was weighed (10 mg) and transferred into 10 ml volumetric flask and added 10 ml of methanol-water mixture of equal ratio (1000 ppm stock solution). Three such sets were prepared for acid, alkali and peroxide degradation.

Acid Degradation

Forced degradation in acidic media was performed by adding 9 ml of 0.01 N HCl to 1 ml above stock solution and mixed it in one set of flasks and these mixtures were heated for up to 4h at 80°C. Then the solution was cooled and diluted with 1:1 ratio of methanol-water

mixture to prepare 50 ppm sample solution. Samples were collected at 30 min, 1hr, 2hr and 4hr time interval respectively.

Base Degradation

Forced degradation in basic media was performed by adding 9ml of 0.01 N NaOH to 1 ml above stock solution and mixed it in one set of flasks and these mixtures were heated for up to 4h at 80°C. Then the solution was cooled and diluted with 1:1 ratio of methanol - water mixture to prepare 50 ppm sample solution. Samples were collected at 30 min, 1hr, 2hr and 4hr time interval respectively.

Oxidative Degradation

Forced degradation in oxidative media was performed by adding 9ml of 15% H₂O₂ to 1 ml above stock solution and mixed it in one set of flasks and these mixtures were heated for up to 4h at 80°C. Then the solution was cooled and diluted with 1:1 ratio of methanol - water mixture to prepare 50 ppm sample solution. Samples were collected at 1hr and 4hr time interval respectively.

Thermal Degradation

For Thermal degradation, solid drugs (20 mg Standard powder) were kept in petri-dish in oven at 100°C for 24 h. Thereafter, 10 mg of drug was transferred to 100 ml volumetric flasks and diluted up to the mark with methanol-water mixture. Again diluted it to prepare 50 µg/ml solution.

Photolytic Degradation

For photo stability, solid drugs (20 mg Standard powder) were kept in Petri-dish indirect sunlight in summer days for 4hr. on a wooden plank. Thereafter, 10 mg of drug was transferred to 100 ml volumetric flasks and diluted up to the mark with methanol-water mixture. Again diluted it to prepare 50 µg/ml solution.

RESULTS AND DISCUSSION

As shown in figure 2

Method validation

System Suitability

The system suitability parameters were evaluated from standard chromatograms obtained by calculating the %RSD of retention times, tailing factor and peak area from 3 replicate injections, It should not be more than 2%. The number of theoretical plates for Natamycin peaks should not be less than 2000. The system suitability parameters are shown in Table 2. These values were well within the limits.

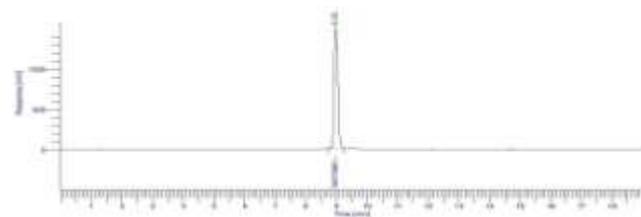


Figure 2: Chromatogram of Natamycin

Table 2: System Suitability data

Retention time	8.96 min
Theoretical Plate	21996.73
Tailing Factor	1.03
LOD	1.825
LOQ	5.308
Peak area	8646012.55
% RSD	0.49

Remarks: **Mean of three determinations

Linearity

The prepared standard solutions of five different concentrations levels ranging from 20 to 100 µg/ml were injected in triplicate into the HPLC column, keeping the injection volume constant (20 µl) and Chromatograms was recorded at 304 nm (Figure 2) and calibration curve was plotted between the mean peak area vs. respective concentration (Table 3 and Figure 3). The regression equation of calibration curves was obtained as $y = 137856x + 304299$ with a correlation coefficient of 0.9983. Where slope (m) is 137856 and intercept (c) is 304299.

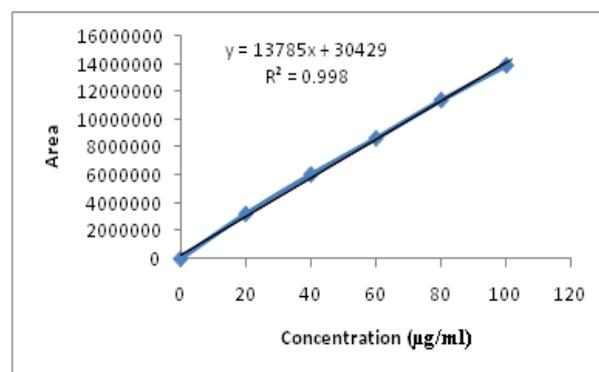


Fig.3: Linearity Plot of Natamycin

Accuracy

The accuracy of the method was carried out by adding known amounts of Natamycin corresponding to three concentration levels; 80%, 100%, and 120% of the label claim in triplicate. The percentage recoveries of Natamycin at each level and each replicate were determined. The recoveries found to be 98.37 to 100.01 for Natamycin (Table 4). It was confirmed from results that the method is highly accurate.

Precision

The precision (intra-day repeatability) was established by analysing three replicates over three concentrations of Natamycin shown in Table 4 and day to day precision (inter-day) was carried out by three concentrations with three replicates. Percentage relative standard deviation (%RSD) is calculated, shown in Table 4 and Table 5 which are within the acceptable criteria of not more than 2.0%.

Table 3: Linearity Data of Natamycin

Concentration µg/ml	Area 1	Area 2	Area 3	Mean
0	0	0	0	0
20	3483440.42	3095140.13	3080320.21	3219633.587
40	5954665.14	6348651.91	5842436.39	6048584.48
60	8810413.12	8596269.39	8531355.14	8646012.55
80	11200369.88	11564803.59	11405988.79	11390387.42
100	13886126.41	13532826.15	14214561.76	13877838.11

Table 4: Accuracy-Recovery Study of Natamycin by Standard-Addition Method

Sr. no.	Initial Conc. µg/ml	Amt. added µg/ml	Area	Amt. found µg/ml	Amt. Recover µg/ml	% Recovery	Mean %	%RSD
1	60	0	8646012.57	60.51	60.51	99.16	99.34	0.38
2			8594322.45	60.14	60.14	99.77		
3			8650482.25	60.54	60.54	99.10		
1	60	48	15146012.57	107.66	47.66	99.29	99.02	0.24
2			15122457.25	107.49	47.49	98.94		
3			15115478.24	107.44	47.44	98.83		
1	60	60	16712025.14	119.02	59.02	98.37	99.01	0.89
2			16847644.9	120.00	60.00	100.01		
3			16735472.2	119.19	59.19	98.65		
1	60	72	18399245.88	131.26	71.26	98.97	99.31	0.53
2			18406481.61	131.31	71.31	99.04		
3			18492457.2	131.94	71.94	99.91		

Table 5: Precision of Natamycin (Intraday)

Sr.	Conc. (µg/ml)	Area1	Area2	Area3	Mean	SD	RSD%
1	40	6325342.22	6222331.21	6245889.21	6264520.88	53973.80	0.86
2	60	8622567.21	8557381.32	8656814.31	8612254.28	50512.34	0.59
3	80	11604955.83	11416234.59	11493641.89	11504944.10	94866.92	0.82

Table 6: Precision of Natamycin (Interday)

Sr.	Conc. (µg/ml)	Area1	Area2	Area3	Mean	SD	RSD%
1	40	6325462.22	6222181.21	6325742.22	6291128.55	59710.31	0.96
2	60	8622687.21	8557231.32	8622967.21	8600961.91	37872.06	0.44
3	80	11605075.83	11416084.59	11605355.83	11542172.08	109195.07	0.95

LOD and LOQ

The LOQ was found to be 1.2 µg/ml and the LOD was found to be 3.66 µg/ml. Lower values of these parameter indicates more sensitivity of the method.

Robustness

The results obtained from assay of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions. The value of assay determined for the same sample under original conditions and robustness conditions indicates that the developed method was robust for effect of changed wavelength (± 2 nm) and impact of flow rate (± 0.2), that evaluation were based on the system suitability parameters such as retention time, tailing factor and theoretical plates. Results were shown in Table 7. **Assay for Natamycin Ophthalmic Suspension.**

Assay procedure was repeated six times for the analysis of homogenous sample of marketed formulation and results are compare with that of the standard. The % Assay was calculated as 98.38 % which was with in the acceptable range of 98.0 to 102 %.

Forced Degradation Study

Natamycin standard were found to be less stable under oxidative, alkaline, acidic and photolytic conditions. A chromatogram of acid hydrolysis performed at 80 °C for 4 hr showed two major degradant having Rt 8.40 min and 13.37 (Figure 4). A chromatogram of basic hydrolysis performed at 80 °C for 4 hr showed two major degradant having Rt of 6.97 min and 7.57 respectively (Figure 5). A chromatogram of oxidised degradation performed at 80 °C for 4 hr showed major degradant having Rt of 6.67 min (Figure 6). A chromatogram of photolytic degradation performed to sunlight at normal temperature for 4 hr showed three major degradant having Rt of 4.57, 6.39 and 10.80 min respectively (Figure 7). A slight

decomposition was seen on exposure of Natamycin drug solution to thermal degradation, 5.13 % of the drug was decomposed (Figure 8). The free alcoholic-OH, amino NH₂ and carboxyl -COOH group present in its chemical structure might be responsible for the reported degradation. The drug has undergone photolysis (29.41) oxidative (24.13 %), alkaline (9.18 %) acidic (8.62%) degradation in 4 hr. (Table 8)

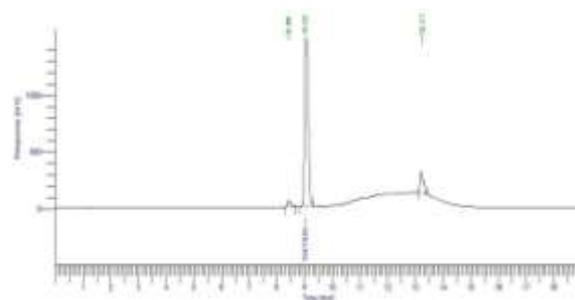


Fig.4: Chromatogram of 0.01 N HCl treated Natamycin at 80 °C temperature for 4 h.

Table 7: Robustness data for Natamycin

Parameters	Variation	Rt (min)	Tailing factor	Plate Count
Wavelength	302 nm	8.96	1.04	22157.91
	304 nm	8.99	1.10	21922.43
	306 nm	8.90	1.03	21558.19
Flow Rate	0.8 ml/min	9.24	1.21	29256.67
	1 ml/min	8.93	1.04	21596.59
	1.2 ml/min	8.71	1.29	18526.73

Table 8: Assay of 5 % w/v Natamycin Ophthalmic Formulation

	Area	Mean	% Assay
Sample	1	7389564.23	98.38 %
	2	7299447.11	
	3	7312685.19	
	4	7328699.48	
	5	7279826.14	
Std. 50 ppm	6	7299895.83	7412025.263
	1	7411248.89	
	2	7431268.17	
	3	7393558.73	

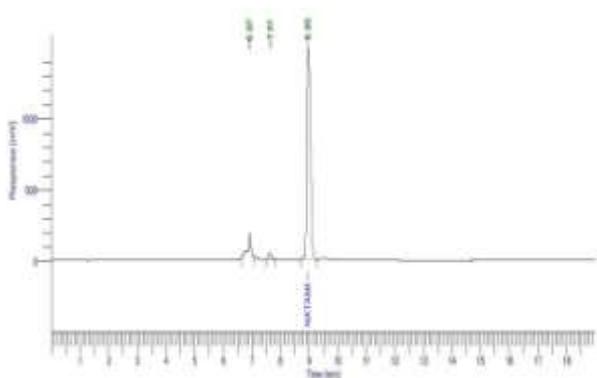


Fig.5: Chromatogram of 0.01 N NaOH treated Natamycin at 80 °C temperature for 4h.

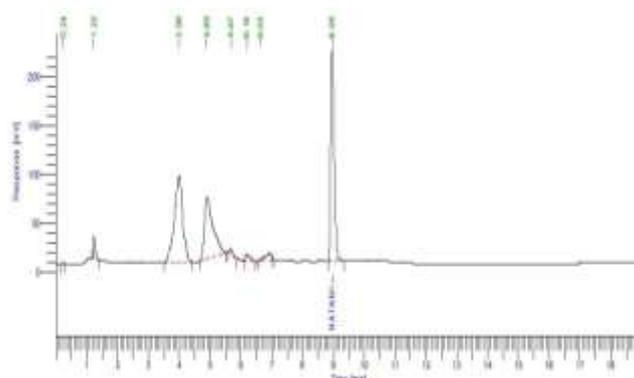


Fig.7: Chromatogram of Natamycin treated Photolysis for 4 hr.

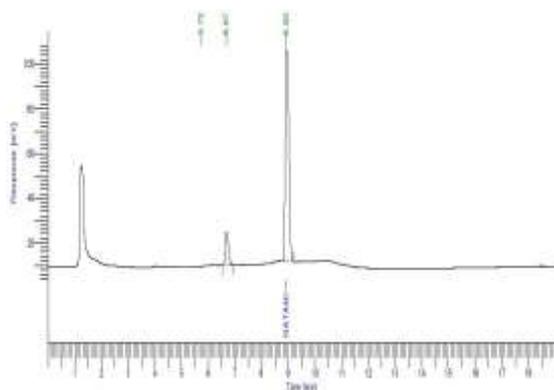
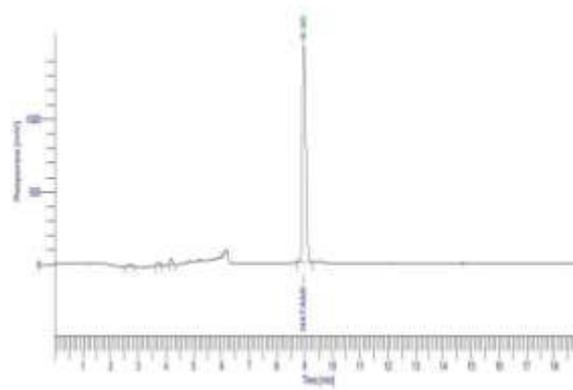
Fig. 6: Chromatogram of 0.1 N H₂O₂ treated Natamycin at 80 °C temperature for 4 h.

Fig.8: Chromatogram of Natamycin at Thermal condition for 24 h.

Table 9: Force Degradation Data for Natamycin at Different Condition

Degradation Condition	Time	% Degradation
Base, 0.01 N NaOH	30 min	5.51
	1 hr.	6.98
	2 hr.	8.36
	4 hr.	9.18
Acid, 0.01 N HCl	30 min	3.89
	1 hr.	5.03
	2 hr.	6.28
	4 hr.	8.62
Oxidative, 15% H ₂ O ₂	1 hr.	15.19
	4 hr.	24.13
Thermal Degradation Photolysis	24 hr.	5.13
	4 hr.	29.41

CONCLUSION

The validated RP-HPLC Method employed here proved to be simple, precise, specific, robust, accurate and stability indicating. The developed method was found to be sensitive and selective for analysis of Natamycin in 5% w/v Ophthalmic Solution without any interference from the excipients. The stability-indicating method determined the API peak and also the peaks of degradation products formed under variability of conditions. After exposure of Natamycin to stress conditions, it was established that the drug is susceptible to acid, base hydrolysis; oxidation, and photolytic degradation with maximum degradation observed in photolysis followed by oxidative degradation. Therefore this method can be employed for monitoring the stability of Natamycin drug substance commercially.

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