UPLC - PDA METHOD FOR SIMULTANEOUS DETERMINATION OF GRAMICIDIN, NEOMYCIN AND TRIAMCINOLONE ACETONIDE – AN IN-VITRO STUDY

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Abstract:

Gramicidin, Neomycin, and Triamcinolone are commonly used to treat bacterial infections in the eyes, rheumatoid arthritis, osteoarthritis, and skin disorders. The three components are determined simultaneously using ultra performance liquid chromatography. Waters Aquity ultra performance liquid chromatograph with photo diode array detector was used to develop and validate the method. The chromatographic separation was accomplished with a phenyl column (100 x 2.1mm, 1.7µm) and a mobile phase containing a 30:70 mixture of Methanol and Buffer (1.36g KH_2PO_4 dissolved in 1L distilled water). Before being used for analysis, the mobile phase is filtered through a 0.45µm filter. The auto sampler was kept at room temperature throughout the experiment. The injected sample volume is 5 µl, with a mobile phase flow rate of 0.5 l/min. The eluate was analyzed at 222nm, and the total runtime for analysis is 2.40 minutes. According to ICH guidelines, the research findings for system acceptability, reproducibility, detection limits, quantitation limits, linearity, accuracy, precision, and specificity are all within acceptable bounds.

Key words: Gramicidin, Neomycin, Triamcinolone acetonide, UPLC method, Stress degradation, stability indicating

INTRODUCTION

In the drug development process, high performance liquid chromatography (HPLC) is used for compound separation, identification, and quantification. To improve the speed of analysis, resolution, speed, and sensitivity of an HPLC, a significant advancement is made in instrumentation as well as particle size and column dimension. Waters introduced an instrument called Ultra performance Liquid chromatography (UPLC) in 2004 that uses small porous

particles (sub-2-micron particles) and high pressures of up to 6000 psi to produce better results than conventional HPLC. When compared to HPLC, the instrument also reduces runtime and mobile phase consumption by up to 80%. UPLC is a high-performance version of HPLC that excels in particle chemistry performance, system optimization, detector design, and control(Swartz 2007).

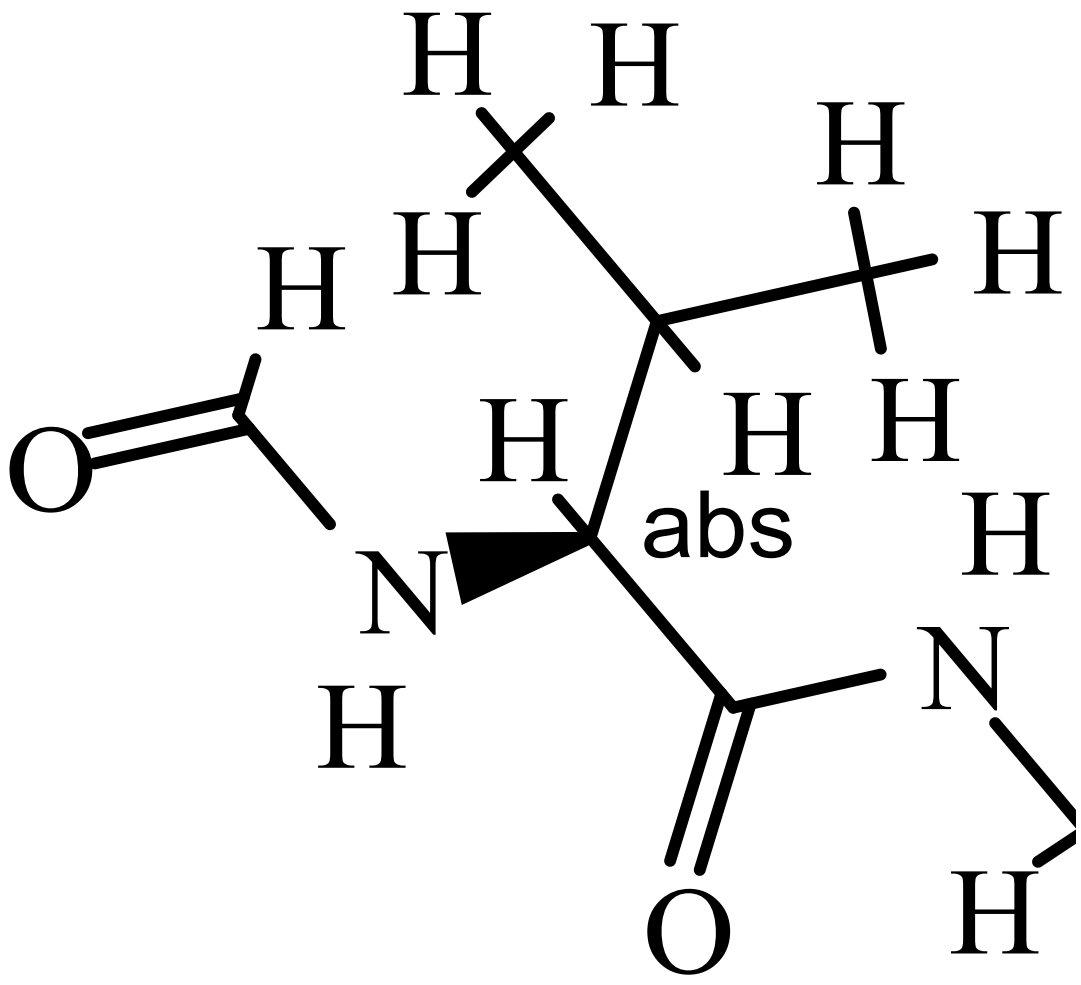
The combination of Gramicidin, Neomycin and Triamcinolone acetonide is used to treat bacterial infections in eyes, rheumatoid arthritis, osteoarthritis, skin disorders and other infections based on patient history.

Gramicidin isa mixture of six antibiotics obtained from bacillus brevis. It is active against most gram – positive bacteria like bacillus subtillis, staphylococcus aureus and against selective gram- negative bacteria like Neisseria. It is used in the treatment of dermatological and ophthalmic infections individually or in combination with other antibiotics. It has a molecular formula of $C_{99}H_{140}N_{20}O_{17}$ and having molecular mass 1882.3 g/mol(Orwa 2001).

Neomycin is an aminoglycoside antibiotic. It is used to treat skin infections caused by bacteria. It is active against staphylococci and gram-negative bacilli, either alone or in combination with other anti-biotics, anti-fungal and corticosteroids. The molecular formula of neomycin is $C_{23}H_{46}N_6O_{13}$ with a molecular weight of 614.644 g/mol (Blanchard et al. 2016).

Neomycin Triamcinolone Acetonide

Triamcinolone acetonide is a synthetic glucocorticosteroid used topically to treat various skin conditions like eczema, dermatitis, allergies rashes etc. it works by activating natural substances in the skin to reduce swelling, redness and itching. The molecular formula of Triamcinolone acetonide is $C_{24}H_{31}FO_6$ and its molecular mass is 434.5g (Yilmaz, Cordero-Coma, and Federici 2011).



Gramicidin

The survey of literature reports few methods for the determination of Gramicidin using HPLC(Orwa 2001; Adams et al. 1997; Salom et al. 1997) and HPLC - MS(Gaddey and Sundararajan 2023). HPLC(Adams et al. 1997; Permata et al. 2019; M. Sathwik 2018; B. Balaswami 2018), LC - MS(Hamidi et al. 2020; Zu et al. 2018; Stypulkowska et al. 2013; Mascher, Unger, and Mascher 2007), High Performance anion – exchange

chromatography(Hanko and Rohrer 2007), Capillary electrophoresis(Huidobro, Garcia, and Barbas 2009) and HPLC - MS(Gaddey and Sundararajan 2023) were used in the determination Neomycin. HPLC(van Heugten et al. 2018; Vieira Mde, Singh, and Derendorf 2010; Sudsakorn, Kaplan, and Williams 2006), LC – MS (Gaddey and Sundararajan 2023; Sun et al. 2018; Liu et al. 2015; Cesar et al. 2011) were the methods reported for the determination of Triamcinolone acetonide.

EXPERIMENTAL

The Gramicidin, Neomycin and triamcinolone acetonide pharmaceutical standards were procured from Aurobindo Pharma limited. All chemicals were procured from Merck, Mumbai. Milli – Q Plus water purification system was used to prepare high pure water. The equipment used is Waters Aquity Ultra Performance Liquid Chromatograph with Photo Diode Array detector, binary solvent manager and auto sampler system. Empower – 2 software is used to monitor and process the signal. The sample prepared is sonicated by using Cyient make before analysis. Gramicidin, Neomycin and Triamcinolone acetonide were separated by using phenyl column (100 x 2.1mm, 1.7µm) using a mobile phase containing 30:70 mixture of Methanol and Buffer (1.36g of KH_2PO_4 dissolved in 1L distilled water). The mobile phase is filtered using 0.45µm filter before used for analysis. Ambient temperature was maintained in the auto sampler throughout the analysis. The volume of the sample injected is 5 µl, with a flow rate of mobile phase is 0.5 µl / min. The analysis of the eluate was carried at 222nm and the total runtime for analysis is 2.40 min.

PREPARATION OF STOCK SOLUTIONS

The stock solutions of Gramicidin, Neomycin and Triamcinolone acetonide were prepared by weighing 25mg, 250mg and 100mg of compounds, carefully transferring them into volumetric flask of 100 ml capacity. This is then dissolved and diluted with diluent and sonicated to give 25 mg/l, 250 mg/l and 100mg/l respectively. The diluent used is 30:70 mixture of Methanol and Buffer.

PREPARATION OF WORKING SOLUTIONS

The working solutions were prepared by taking 5 ml of the standard solution in a clean and dry volumetric flask of 50ml capacity such that 1ml of working solution contains 2.5mg, 25mg and 10mg of Gramicidin, Neomycin and Triamcinolone acetonide respectively.

METHOD VALIDATION

System suitability:

Gramicidin, Neomycin and Triamcinolone acetonide were taken in six replicates and injected into the system. System suitability is assessed by calculating the tailing factor, retention time, plate number and Peak asymmetry.

Linearity:

The linearity of the method is validated by taking six different concentrations of Gramicidin (6.25 µg/ml – 37.5 µg/ml), Neomycin (62.5 µg/ml - 375 µg/ml) and Triamcinolone acetonide (25 µg/ml – 300 µg/ml) respectively. Linearity plot is plotted by taking concentration on x – axis and average peak area count on y – axis. Regression coefficient is also calculated for the above plot.

Accuracy:

The accuracy of an analytical procedure expresses the degree of agreement between the value accepted as a standard true value and the value discovered. The accuracy of the method is assessed by carrying the recovery studies at spiked levels of 50, 100 and 150 percent of Gramicidin (12.5, 25 and 37.5 mg / ml), Neomycin (125, 250 and 375 mg / ml) and Triamcinolone acetonide (50, 100 and 150 mg/ml). the percent recovery at each level was collected and mean standard deviation is calculated.

Method Precision:

It is the level of agreement among individual test results when the procedure is repeated on multiple samples. In six injections, gramicidin, Neomycin, and Triamcinolone acetonide were spiked at 100 percent of the quantified limit for the sample concentration. Their amounts were calculated and percent RSD is determined.

Intermediate precision:

It is the measure of precision under defined set of conditions. 1000 mg each of Gramicidin, Neomycin and Triamcinolone acetonide were spiked and the intermediate precision is calculated by various analysts in various time intervals.

Limit of Detection and Limit of Quantification:

Limit of detection is the smallest concentration that can be determined by the analytical procedure with accuracy and precision. Limit of quantification is the minimum concentration at which the analyte can be quantitively determined. This is evaluated by the signal to noise ratio of three to ten times.

Robustness:

Robustness of the method determines the capacity of the method to remain unaffected by variations in method parameters.

Specificity:

Specificity is the ability to assess the analyte clearly in the presence of components that are expected to be present. The method is evaluated for any interferences in the drug matrix.

Forced Degradation studies:

Acid degradation

5 ml of the sample stock solution was taken in a 50 ml volumetric flask. This is combined with 0.1N HCl and left for 15 minutes. The solution is neutralized with 1 ml of NaOH before being diluted and analyzed. Steps are repeated with 1N HCl.

Alkali degradation

Alkali degradation is tested using 0.1N NaOH and 1N NaOH. 5 ml of stock solution filled in a 50 ml volumetric flask. The mixture is then left fr 15 minutes. Then 0.1N HCl and 1N HCl are added to titrate (trial 1 and trial 2). After dilution, it is analyzed.

Peroxide degradation

In two tests, peroxide degradation is carried out using 10% and 30% H2O2. A 50 ml volumetric flask is filled with 5 ml of the sample stock solution. To this 1 ml of H2 O2 is then added, set aside for 15 minutes., diluted with the appropriate amount of diluent and then examined.

Reduction degradation

Reduction degradation tests were performed with 10% and 30% sodium bisulphate. In a 50 ml volumetric flask, 5ml of sample stock solution is added. After 15 minutes, add 1 ml of sodium bisulphate solution. This is diluted and evaluated.

Thermal degradation

5 m l of sample stock solution is taken into two 50 ml volumetric flasks. This is exposed at 105°C for 3 hours and another one for 6 hours respectively. These were diluted with the diluent and then analyzed.

Hydrolysis degradation

5 ml of sample stock solution is put into a 50 ml volumetric flask. 1 ml of HPLC grade water is added. After 15 minutes, it is diluted and tested. Similarly, the sample is examined after 15 minutes by adding 3 ml of HPLC grade water.

Photolytic degradation

Two 50 ml volumetric flasks are taken. To this 5 ml of stock solution is added. These flasks were exposed to sunlight for 3 hours and 6 hours respectively. These were diluted and tested.

RESULTS AND DISCUSSIONS

Method Optimization

The chromatographic conditions were established after testing with various mobile phase ratios in isocratic mode and different stationary phases. The compositional changes in the mobile phase enable an improvement in resolution and tolerable retention times. Based on the composition of the drug matrix, phenyl column (100mm x 2.1mm, 1.7µm) is used as stationary phase and 30:70 mixture of methanol and KH_2PO_4 is used as mobile phase. At 222nm, the mobile phase flow rate was optimized to be 0.5 ml/min. the retention times for Gramicidin, Neomycin and Triamcinolone acetonide were determined to be 1.835, 1.330 and 0.924 minutes. Under these optimized conditions, the tailing factors were found to be 1.02, 0.65 and 0.86 respectively. Indicating the effective elution, the theoretical plates were found to be 12567, 9687 and 5897

respectively. For six replicates, the percent RSD values were found to be 0.353, 0.67 and 0.08 respectively. The proposed method is precise and has been validated in accordance with ICH guidelines.

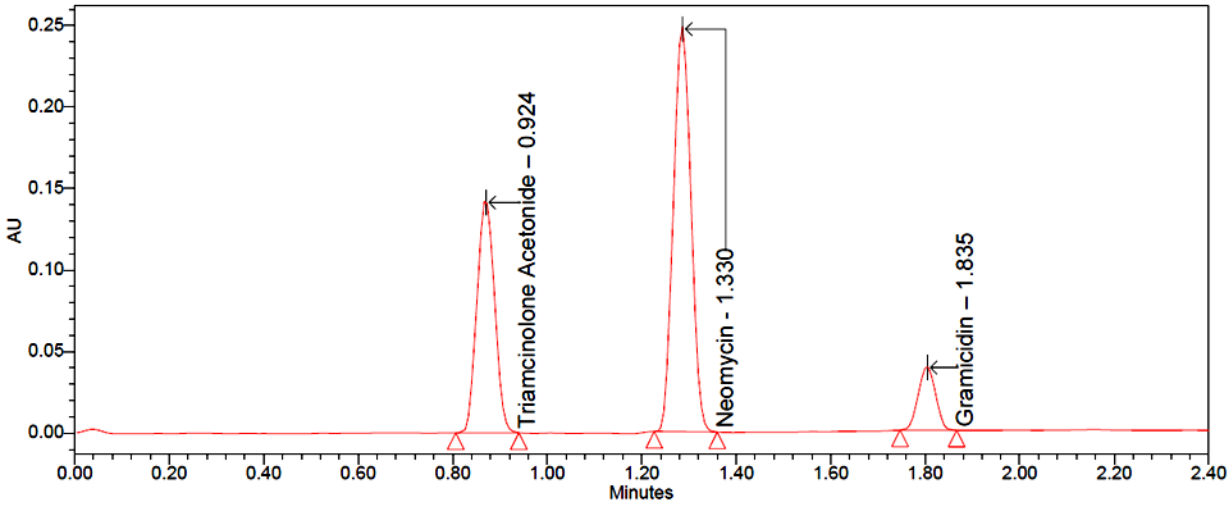


Figure 1 – Optimized chromatogram of UPLC of Gramicidin, Neomycin and Triamcinolone acetonide.

Method Validation

The proposed methodology was validated in accordance with ICH principles and tested in accordance with ICH Q2 requirements; the results were within acceptable limits. As a result, the method is used to determine Gramicidin, Neomycin, and Triamcinolone Acetonide.

System suitability

It is a crucial component of method validation, examining the performance and relationship of tools, software, and analysis. By injecting 25 µg/ml, 250 µg/ml and 100 µg/ml of Gramicidin, Neomycin and Triamcinolone acetonide, in six replicates, the method's suitability is examined. The results are tabulated below.

Table – 1 – System Suitability Parameters

Parameter	Acceptance Requirement	Name of the API		
		Gramicidin	Neomycin	Triacinolone acetonide

Plate count*	</2000	12567	9687	5897
Tailing*	>/ 2.0	1.02	0.65	0.86
Resolution*	</ 2.0	7.33	5.81	-
RSD in percent	>/ 2.0	0.385 %	0.671 %	0.082 %
Retention Time	</ 2.0	1.835	1.330	0.924

Linearity

The linearity of the process determines the ranges of the procedure within which the observations are obtained. Linearity is determined by plotting the concentration-peak area calibration curve. The plot shows that Gramicidin, Neomycin, and Triamcinolone acetonide were linear in concentrations ranging from 0 g/ml to 37.5 g/ml, 375 g/ml, and 150 g/ml, respectively. The findings are summarized.

Table – 2 - LINEARITY RESULTS

Linearity	**Gramicidin**		**Neomycin**		**Triamcinolone acetonide**	
	Amount*	**Peak area**	**Amount***	**Peak Area**	**Amount***	**Peak area**
Linearity - 1	0	0	0	0	0	0
Linearity – 2	6.25	59337	62.5	431715	25	246894
Linearity – 3	12.5	132045	125.0	833987	50	463878
Linearity – 4	18.75	190437	162.5	1077973	75	708996
Linearity – 5	25.00	242224	250.0	1614341	100	926706
Linearity – 6	31.25	305632	312.5	2087487	125	1175456
Linearity - 7	37.50	365632	375.0	2432107	150	1391457
Regression Equation	Y = 9712.37 X + 2936.89		Y = 6506.11 X + 14427.05		Y = 9277.6 X + 6092.11	
Slope	9712.37		6506.11		9277.6	
Intercept	2936.89		14427.05		6092.11	
CC	0.9993		0.9996		0.9998	

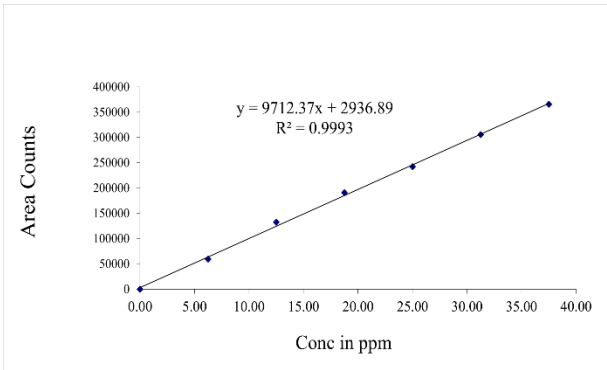


Fig. 2. Linearity – Gramicidin

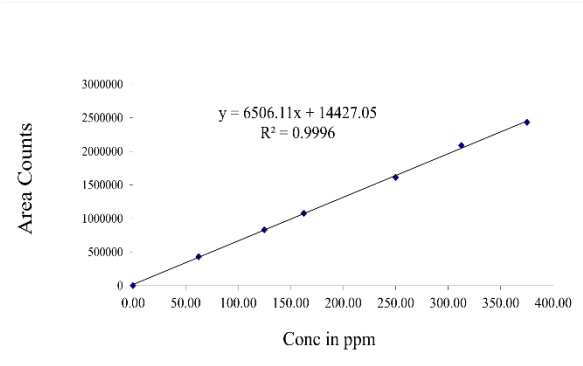


Fig. 2. Linearity – Neomycin

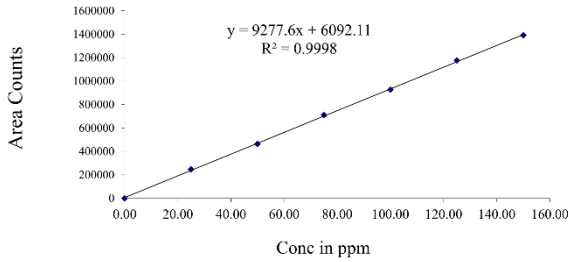


Fig. 2. Linearity – Triamcinolone acetonide

Accuracy

The accuracy of a procedure determines its appropriateness. Three recovery trials were conducted. (50, 100 and 150 percent levels). APIs containing 12.5, 25.0, and 37.5 g/ml Gramicidin, 125, 250, and 375 g/ml Neomycin, and 50, 100, and 150 g/ml Triamcinolone acetonide were prepared. At each spike stage, the test solution was given three times. The RSD values were discovered to be < ± 2 percent, while the recovery results were close to 100%. The recovery data were correct, and the percent recovery, mean, and relative standard deviations were calculated. The following table summarizes the findings.

Table – 3 - RESULTS FOR ACCURACY

S. No	% Level	%Gramicidin Recovered	%Neomycin Recovered	%Triamcinolone acetonide Recovered

1	50	99.9	100.5	100.6
2	100	98.9	99.3	100.1
3	150	99.4	100.7	99.7
Mean		99.4	100.2	100.1
SD		0.5	0.757	0.451
Percent RSD		0.5	0.76	0.45

Precision:

Method Precision (Intraday Precision)

It's the proximity of homogeneous mixture sample readings. The procedure's precision is determined by measuring six injections 25 µg / ml of Gramicidin, 250 µg / ml of Neomycin and 100 µg / ml of Triamcinolone acetonide. The results for method precision were tabulated as follows in table 4.

Table – 4 – Results of Method precision (intraday precision)

S. No	Gramicidin			Neomycin			Triamcinolone Acetonide		
	Amount*	Peak Area	Assay %	Amount*	Peak Area	Assay %	Amount*	Peak Area	Assay %
1	25	242319	98.2	250	1619274	99.8	100	927679	100.2
2		243247	98.6		1609553	99.2		927432	100.2
3		243054	98.5		1594189	98.3		923387	99.7
4		245138	99.4		1599510	98.6		920485	99.4
5		242055	98.1		1594115	98.2		922066	99.6
6		247359	100.3		1613067	99.4		921679	99.5
Mean		98.9			98.9			99.8	
SD		0.846			0.646			0.35	
RSD		0.86			0.65			0.35	

%									

* - Amount in µg / ml

Intermediate precision (inter day Precision)

On separate days, various researchers examined six replicates of the sample solution with various tools. Peak zones have been defined to evaluate RSD% averages. The results are displayed in the table below.

Table – 5 – Results for inter day precision

S. No	Gramicidin			Neomycin			Triamcinolone acetonide		
	Amount*	**Peak Area**	**Assay %**	**Amount***	**Peak Area**	**Assay %**	**Amount***	**Peak Area**	**Assay %**
1	**25**	**243430**	**98.6**	**250**	1621234	100.2	**100**	924786	99.9
2		**245320**	**99.4**		1611328	99.6		922367	99.6
3		**243461**	**98.6**		1620384	100.1		921542	99.5
4		**244312**	**98.9**		1613784	99.7		925143	99.9
5		**245761**	**99.5**		1614128	99.8		926357	100.0
6		**246124**	**99.7**		1619354	100.1		921687	99.5
Mean		**99.1**			99.9			**99.7**	
SD		**0.479**			0.248			**0.225**	
RSD %		**0.48**			0.25			**0.23**	

* - Amount in µg / ml

Sensitivity

Calibration curves were used to determine the detection and quantitation limits. The detection and quantitation limits of API were established using UPLC by injecting lower and higher

amounts of the standard solution. The detection limits and quantitation limits of Gramicidin, Neomycin and Triamcinolone acetonide are 0.033, 0.325 and 0.13 µg / ml and 0.108, 1.075 and 0.430 µg / ml respectively.

Table- 6 – Detection and Quantitation limits

Gramicidin				Neomycin				Triamcinolone acetonide			
Detection Limit		Quantitation Limit		Detection Limit		Quantitation Limit		Detection Limit		Quantitation Limit	
Amount *	s/n	Amount *	s/n	Amount *	s/n	Amount *	s/n	Amount *	s/n	Amount *	s/n
25	3	25	10	250	3	250	10	100	3	100	10

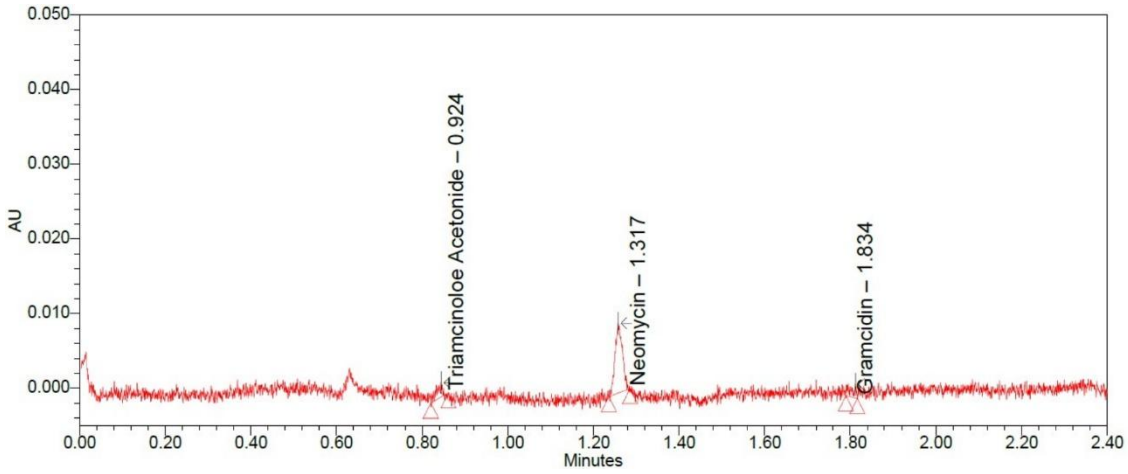


Fig. 3: Chromatogram for Detection limit

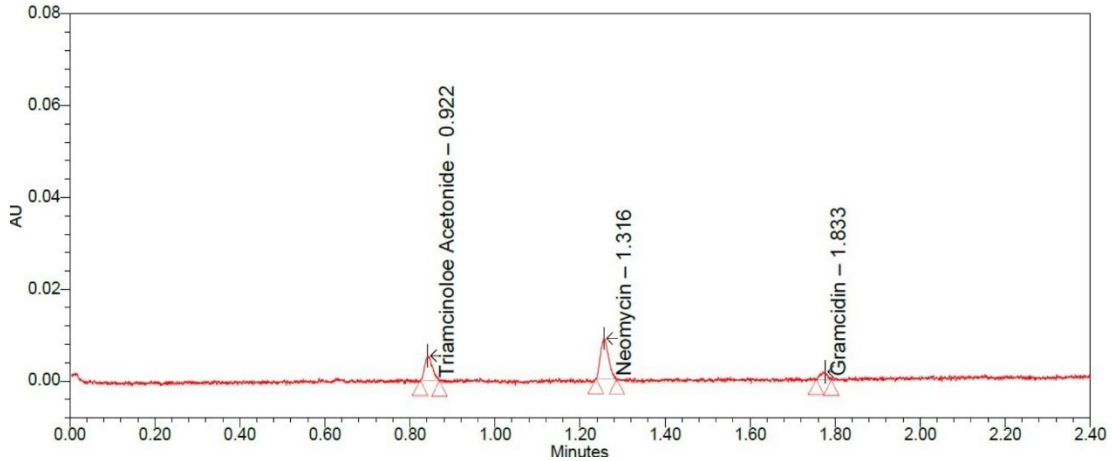


Fig. 3: Chromatogram for Quantitation limit

Robustness

The experiment was designed to assess the robustness of changing flow rate and organic content in mobile phase.

Table – 7: Robustness

Specification Name	Percent RSD		
	Gramicidin	**Neomycin**	**Triamcinolone acetonide**
Rate of Flow (0.8 ml/min)	0.9	0.21	0.21
Rate of Flow (1.2 ml/min)	0.51	1.28	0.15
Org Plus (66:34)	0.99	1.22	0.32
Org Minus (54:46)	0.5	0.99	0.3

Specificity

The specificity of the method was assessed in terms of interference caused by the presence of other placebos. Within the retention time images, the UPLC chromatograms for blank and placebo showed almost no intrusive peaks. As a result, the UPLC method employed in this study was precise and selective.

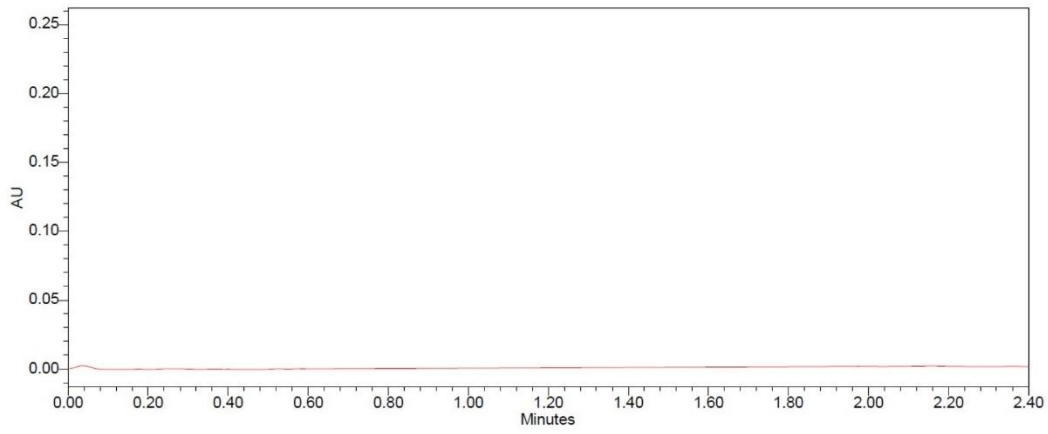


Fig. 4: Chromatogram of Blank

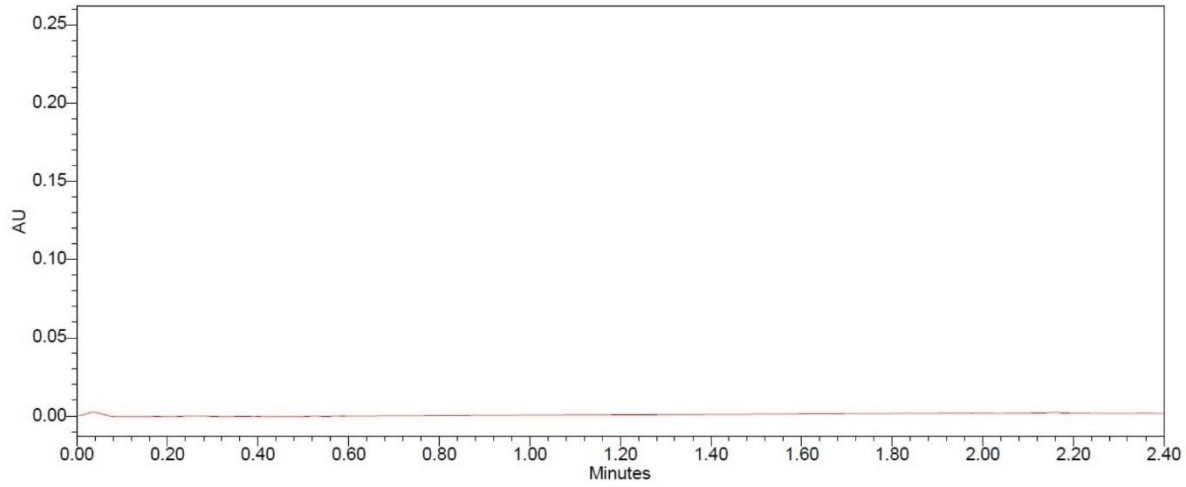


Fig. 4: Chromatogram of Placebo

Stability

At room temperature and 2°C - 8°C, the stability of the standard was investigated. The results are given below.

Table - 8: Stability studies at room temperature

S. No	Stability	Gramicidin		Neomycin		Triamcinolone acetonide	
	Room Temperature	% Assay	% Dev	% Assay	% Dev	% Assay	% Dev
1	**Initial**	99.9	0.00	100.0	0.00	100	0.00
2	**6 hrs**	98.5	- 1.40	99.6	-0.40	99.5	-0.50
3	**12 hrs**	98.2	- 1.70	99.1	-0.90	99.1	-0.90
4	**18 hrs**	97.3	- 2.60	98.9	-1.10	98.6	-1.40
5	**24 hrs**	97.1	- 2.80	98.4	-1.60	98.3	-1.70

Table - 9: Stability at 2°C - 8°C

S. No	Stability	Gramicidin		Neomycin		Triamcinolone acetonide	
	2°C - 8°C	% Assay	% Dev	% Assay	% Dev	% Assay	% Dev
1	Initial	99.9	0.00	100.0	0.00	100.0	0.00
2	6 hrs	98.8	-1.10	99.5	-0.50	99.4	-0.60
3	12 hrs	98.6	-1.30	99.2	-0.80	99.3	-0.70
4	18 hrs	98.0	-1.90	98.1	-1.90	98.8	-1.20
5	24 hrs	96.3	-3.60	97.8	-2.20	98.4	-1.60

Forced degradation studies:

ICH guidelines were followed for the various types of stressed conditions. During the study, only a few degradation products were formed.

Table - 10: Forced degradation studies

Degradation Condition		No. of deg. products	Gramicidin		Neomycin		Triamcinolone acetonide	
			% Assay	% Deg.	% Assay	% Deg.	% Assay	% Deg.
Control		0	99.8	0.2	99.9	0.1	99.9	0.1
Acid degradation (HCl)	0.1N	0	96.2	3.6	97.2	2.7	97.5	2.4
	1N	2	95.1	4.7	86.8	13.1	86.5	13.4
Alkali degradation (NaOH)	0.1N	0	96.7	3.1	96.3	3.6	97.8	2.1
	1N	2	94.2	5.6	87.3	12.6	87.1	12.8
Peroxide degradation (H_2O_2)	10%	1	95.7	4.1	95.7	4.2	96.7	3.2
	30%	3	95.6	4.2	84.4	15.5	85.5	14.4
Reduction degradation (Na_2SO_4)	10%	0	96.4	3.4	96.8	3.1	96.9	3.0
	30%	2	96.2	3.6	89.5	10.4	88.0	11.9

Thermal degradation (at 105°C)	**3 hrs**	0	97.2	2.6	97.0	2.9	96.3	3.6
	6 hrs	0	96.6	3.2	96.6	3.3	96.5	3.4
Hydrolysis degradation (adding H_2O)	**1 ml**	0	97.8	2.0	97.7	2.2	97.9	2.0
	3 ml	0	97.3	2.5	94.7	5.2	97.1	2.8
Photo degradation (Sun light)	**3 hrs**	0	98.9	0.9	98.3	1.6	95.8	4.1
	6 hrs	0	97.1	2.7	95.6	4.3	96.4	3.5

Conclusion:

A precise and sensitive UPLC method for the simultaneous determination of Gramicidin, Neomycin, and triamcinolone acetonide was proposed. The method proposed is highly selective, specific, and sensitive. A few degradation products were detected during the forced degradation of the combination, and all other method validation parameters were evaluated in accordance with ICH guidelines.

Conflict of Interests:

The authors declare that is no conflict of interest regarding the publication of this article.

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