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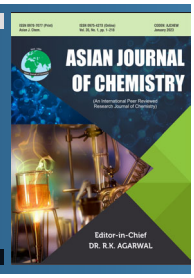


# Liquid Chromatographic-Electrospray Ionization-Mass Spectrometry Method for the Simultaneous Determination of Gramicidin, Neomycin & Triamcinolone Acetonide and Characterization of Novel Forced Degradation Products

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The dosage form iotrim (gramicidin, neomycin and triamcinolone) is a combination of two antibiotics (gramicidin and neomycin) and a steroid (triamcinolone). The antibiotics work by killing the bacteria that cause infections. The steroid blocks the action of chemical messengers (prostaglandins) that make the affected area red, swollen and itchy. Consequently, there was still a need to develop a simple, less time consuming and economical method for the simultaneous determination of gramicidin, neomycin and triamcinolone acetonide. The current work is an effort to develop a fast and reproducible LC-MS technique for the simultaneous estimation of gramicidin, neomycin and triamcinolone acetonide. The objective of the present procedure was to validate and develop a precise and accurate liquid chromatography-mass spectrometry (LC-MS) technique for the simultaneous quantification of gramicidin, neomycin and triamcinolone acetonide. Gramicidin, neomycin and triamcinolone acetonide were monitored on Shimadzu-8045 mass spectrometer equipped with electro spray ionization interface. The retention times of gramicidin, neomycin and triamcinolone acetonide were found at 9.145 min, 7.273 min and 2.435 min, respectively. The limit of detection (LOD) results for gramicidin, neomycin and triamcinolone acetonide were observed to be 0.15, 1.5 and 0.6 µg/mL, respectively while the limit of quantification (LOQ) results were observed to be 0.5, 5, 2 µg/mL concentration, respectively. The linear range for gramicidin, neomycin and triamcinolone acetonide were found in the concentration ranges from 1.25-7.5 µg/mL, 12.5-75 µg/mL and 5-30 µg/mL with regression coefficient of 0.9991, 0.9996, 0.9999, respectively. Accuracy values for gramicidin, neomycin and triamcinolone acetonide were found to be in the range of 98.64%, 99.4%, 99.5% respectively. The % RSD for six replicates in precision was less than 2%. According to ICH Q2(R1) recommendations, this method was successfully tested with LC-MS to confirm the chemical structures of newly produced degradation products of triamcinolone acetonide and neomycin. The developed process was validated efficaciously as per ICH guidelines.

**Keywords: Gramicidin, Neomycin, Triamcinolone acetonide, Electro spray ionization, Limit of detection.**

## INTRODUCTION

The LC-MS is an analytical chemistry procedure that associates liquid chromatography's physical separation skills with mass spectrometry's mass analysis capabilities. It's been used in drug development for a long time including, metabolite identification, metabolic stability testing, *in vivo* drug testing, glycoprotein mapping, impurity detection, natural product dereplication, peptide mapping and bio-affinity screening. For the study ingredient difference phenotypic cloning, LC-MS evaluates the contents and classes of distinct groups of grown plant cells and selects the couple of assemblies with the greatest difference in ingredient content. In the areas of genomics, lipidomics, metabolomics and proteomics, among other things, the use of LC-MS has progressed into a prevailing two-dimensional (2D) hyphenated technology for the study of proteins, nucleic acids, amino acids, lipids, carbohydrates and/or in the main classification [1].

Gramicidin is effective against Gram-positive organisms, with the exception of Gram-positive *Bacilli*, as well as some Gram-negative species, such as *Neisseria*. Gramicidin is primarily used topically, either alone or in conjunction with other antibiotics [2]. It has a molecular formula of $C_{99}H_{140}N_{20}O_{17}$ and a molecular weight of 1882.332 g/mol. Neomycin kills a varied range of bacteria, including Gram-positive and Gram-negative bacteria, as well as acid-fast bacteria. Streptomycin-sensitive

and streptomycin-resistant bacteria, including *M. tuberculosis* var. hominis, are both susceptible to it. It doesn't work against fungus. Neomycin is bacteriostatic as well as highly bactericidal and prevents the establishment of resistant bacterium strains among the sensitive types [3]. Triamcinolone acetonide (TA), a synthetic glucocorticoid corticosteroid, has a strong anti-inflammatory effect, while also having a lengthy ocular distribution due to its lipophilic nature and poor aqueous solubility increased amounts of tight-junction proteins are thought to be a mechanism of action for triamcinolone acetonide, which reduces artery leakage and angiostatic effects by inhibiting and down regulating vascular endothelial growth factor [4].

Literature survey for gramicidin revealed that only HPLC [2,5,6] is reported. For neomycin, HPLC [5,7-11], LC-MS [12-16], high-performance anion-exchange chromatography (HPAEC) [17] and capillary electrophoresis (CE) [18] are few analytical techniques. Only two analytical procedures for triamcinolone acetonide analysis *viz*. HPLC [19-22] and LC-MS [23-26] are reported.

To date, the stability and forced degradation studies for the simultaneous estimation of gramicidin, neomycin and triamcinolone acetonide have not been performed sufficiently. Moreover, broader research on drug quantification and degradation rates under various stress conditions is needed. The above information will be valuable for understanding the chemical stability of gramicidin, neomycin, triamcinolone acetonide and developing a suitable formulation and screening for appropriate storage conditions. To the best of our knowledge, no research presents a fully validated stability-indicating LC-MS method for simultaneous estimation of gramicidin, neomycin and triamcinolone acetonide in the presence of all known degradation products. Consequently, there was still a need to develop a simple, less time consuming and economical method for the simultaneous determination of gramicidin, neomycin, triamcinolone acetonide and characterization of forced degradation products. Therefore, the current work was an effort to develop a fast and reproducible LC-MS technique for the simultaneous estimation of gramicidin, neomycin, triamcinolone acetonide by following ICH method validation guidelines.

## EXPERIMENTAL

Merck provided all of the HPLC grade solvents. Filters were employed to filter all of the solvents and solutions used. Gramicidin, neomycin and triamcinolone acetonide drug samples were obtained from Shree Icon Labs as gift samples. Instruments utilized in this work were: liquid chromatography (Waters, Alliance HPLC e-2695 system) coupled to mass spectroscopy (Shimadzu-8045) instrument and chemical glassware. The Waters, Alliance HPLC e-2695 system coupled to Shimadzu-8045 mass spectrometer equipped with electrospray ionization interface was used to execute the process. Separation of gramicidin, neomycin and triamcinolone acetonide were successfully achieved by the Agilent eclipse XDB (150 × 4.6 mm, 3.5 µ) column and gradient mobile phase composition of solution A- acetonitrile: 0.1% formic acid (60:40) and solution B- methanol, using 1 mL/min flow rate and 10 µL of injection volume. The temperature sustained in the auto sampler and column was ambient.

HPLC was coupled to a mass spectrophotometer in the forced degradation investigation. The typical operational basis settings for mass spectrometer scan of gramicidin, neomycin and triamcinolone acetonide on positive electrospray ionization mode were enhanced. The ion spray voltage was set at 5500 V, collision energy at 15 V, declustering potential at 40 V and ultrapure nitrogen gas was used as collision gas.

### Preparation of standard solution

**Preparation of solution A:** Triamcinolone acetonide (5 mg) was added to 7 mL of dilutant in a 10 mL dry volumetric flask and sonicated for 30 min. Then, diluents were used to bring the solution up to the final volume.

**Preparation of solution B:** Gramicidin (5 mg) was added to 7 mL of dilutant in a 10 mL dry volumetric flask and sonicated for 30 min. Then, diluents were used to bring the solution up to the final volume.

**Preparation of standard stock solution:** Neomycin (5 mg) was transferred to a 10 mL clean dry volumetric flask and then added 4 mL of solution A and 1 mL of solution B. The solution was sonicated for 30 min and then diluted to the desired volume with diluents.

**Preparation of standard solution:** 1 mL of the above-prepared stock solution was pipetted out and diluted to the final volume in a 10 mL volumetric flask. This was the standard solution.

**Preparation of sample solution:** Transferred ointment (iotrim, 1000 mg) in a 10 mL dry volumetric flask, a sample solution containing triamcinolone acetonide, neomycin and gramicidin was prepared followed by the addition of 7 mL of diluents and the mixture was sonicated for 30 min. The whole solution was centrifuged for 15 min before diluting to the final volume. A 10 mL volumetric flask was filled with 2 mL of stock solution, which was then diluent filled to the final volume.

### Method validation

**System suitability test:** Six replicates of gramicidin, neomycin sulphate and triamcinolone acetonide working standards samples were injected to assess system suitability and metrics such as tailing factor (K), relative retention time, plate number (N), resolution and peak asymmetry of samples were investigated.

**Linearity:** Six different concentrations of gramicidin, neomycin and triamcinolone acetonide in the range of 1.25-7.5 µg/mL, 12.5-75 µg/mL and 5-30 µg/mL, respectively were prepared. Each solution was injected into the instrument and the peaks were recorded. The regression coefficient was then calculated by plotting a curve between the average peak area and the concentration.

**Accuracy:** Recovery studies have been used to verify the method's accuracy at 80%, 100% and 120% level. A known amount of gramicidin, neomycin sulphate and triamcinolone acetonide spiked discretely to pre-analyzed samples of the stated levels. The percent recovery of each level was calculated after each spiked level was administered into the LC system.

**Method precision:** Method precision was executed by spiking the sample with gramicidin, neomycin and triamcinolone acetonide at 100% of the quantified limit concerning the sample concentration in six preparations. Six homogenous replicates were injected. Then, the amount of gramicidin, neomycin and triamcinolone acetonide was calculated to determine the percent relative standard deviation.

**Intermediate precision:** In six preparations, the intermediate precision was achieved by spiking the sample with gramicidin, neomycin sulphate and triamcinolone acetonide at 100% of the prescribed limit in terms of sample concentration. The intermediate precision investigation was conducted on various days with various analysts.

**Sensitivity (LOD and LOQ):** The LOD and LOQ were evaluated by the signal-to-noise ratio of three and ten times, respectively.

**Robustness:** Small adjustments in the chromatographic settings such as flow (-) (0.9 mL/min), flow (+) (1.1 mL/min), organic phase (-) (55B:45A), organic phase (+) (65B:35A) were used to test the method's robustness.

**Specificity:** Two separate samples were injected and compared to placebo controls. Any interference peaks in the LC chromatograms for the drug matrix (combination of the medicine and placebos) were evaluated.

### Forced degradation studies

**Peroxide degradation:** Sample (1000 mg) placed in a 10 mL volumetric flask was mixed with 7 mL of diluents and sonicated for 15 min to dissolve the contents. Then, 1 mL of 30% $H_2O_2$ was added and the flask was heated for 30 min and air-conditioned to room temperature. After cooling, the sample was diluted to volume with diluent and mixed. Further 2 mL was pipetted out and made up with diluent. The above solution was injected after 24 h. The chromatogram was taken to determine the sample's stability.

**Reduction degradation:** Sample (1000 mg) placed in a 10 mL volumetric flask was mixed with 7 mL of diluents and sonicated for 15 min. Then, 1 mL of 30% sodium bisulphate was added and the flask was heated for 30 min and air-conditioned to room temperature. After cooling, the sample was diluted to volume with diluent and mixed. Further 2 mL was pipetted out and made up with diluent. The prepared solution was injected after 24 h. The chromatogram was taken to determine the sample's stability.

**Acid degradation:** Sample (1000 mg) placed in a 10 mL volumetric flask was mixed with 7 mL of diluents and sonicated for 15 min. Then, 1 mL of 1 N HCl was added, heated for 30 min and cooled to room temperature. After cooling, 1 mL of NaOH was added to neutralize the solution and sample was diluted with diluent and mixed. A further 2 mL was pipetted and diluted. The aforesaid solution was injected after 24 h. The chromatogram was taken to determine the sample's stability.

**Base degradation:** Sample (1000 mg) placed in a 10 mL volumetric flask was mixed with 7 mL of diluents and sonicated for 15 min. Then, 1 mL of 1 N NaOH was added and the flask was heated for 30 min and cooled to room temperature. After cooling, 1 mL of HCl was added to neutralize the solution and sample was diluted with diluent and mixed. Further 2 mL was pipetted out and made up with diluent. The final solution was injected after 24 h. The chromatogram was taken to determine the sample's stability.

**Hydrolytic degradation:** Sample (1000 mg) placed in a 10 mL volumetric flask was mixed with 7 mL of diluents and sonicated for 15 min. Then, 1 mL of HPLC grade water was added and the flask was heated for 30 min and cooled to room temperature. After cooling, the sample was diluted to volume with diluent and mixed. Further 2 mL was pipetted out and made up with diluent. The prepared solution was injected after 24 h. The chromatogram was taken to determine the sample's stability.

**Thermal degradation:** Sample (1000 mg) was exposed at 105 ºC for 6 h and then placed into a 10 mL volumetric flask. To this sample, 7 mL of diluents were added and then sonicated. The sample was diluted to volume with diluent and mixed. Further 2 mL was pipetted and made up with diluent. The prepared solution was injected after 24 h. The chromatogram was taken to determine the sample's stability.

**Photostability degradation:** Sample (1000 mg) was exposed to sunlight for 6 h and then transferred into a 10 mL volumetric flask. To this sample, 7 mL of diluents were added and sonicated. The sample was diluted to volume with diluent and mixed. Further 2 mL was pipetted out and made up the final volume with diluent. The prepared solution was injected after 24 h. The chromatogram was taken to determine the photostability of the sample.

## RESULTS AND DISCUSSION

**Method optimization:** Using trial and error procedures, the optimal LC-MS method was developed. Agilent eclipse XDB (150 × 4.6 mm, 3.5 µ) column and a gradient mobile phase composition of acetonitrile: 0.1% formic acid (60:40) and methanol using 1 mL/min flow rate and 10 µL of injection volume developed an efficient approach after multiple trials. The temperature in the column and auto sampler was kept constant at room temperature. The gramicidin, neomycin and triamcinolone acetonide in the optimized chromatogram were eluted at 9.145, 7.273 and 2.435 min, respectively (Fig. 1). By fine tuning the variables that contribute to system performance, a strategy with high sensitivity and selectivity was developed.

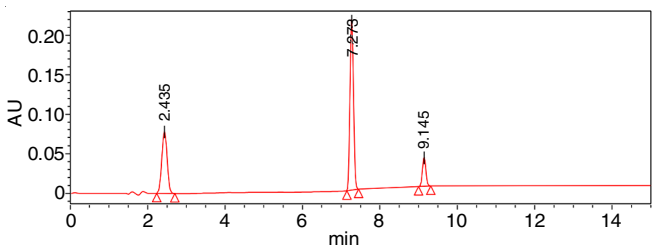


Fig. 1. Optimized chromatogram of HPLC of gramicidin, neomycin and triamcinolone acetonide

**Method validation:** The devised approach was validated bestowing to the ICH Q2 requirements. The projected method was successfully validated according to ICH principles and

the findings were within acceptable limits. Hence, this was employed for the determination of gramicidin, neomycin and triamcinolone acetonide.

**System suitability:** The system suitability characteristics were investigated and used to determine the best settings. The retention period, tailing factor (T) and theoretical plate number (N) were all studied for this purpose. The total number of theoretical plates was over 2000, which is enough for the system suitability test. According to the standards, the tailing factor was within the specified limitations. These findings demonstrated that the proposed strategy can produce data of acceptable quality. All the system suitable parameters were agreed and were within the limits. The results are tabulated in Table-1.

**Linearity:** The linearity of the proposed approach was tested in the ranges of 5-30 µg/mL, 12.5-75 µg/mL and 1.25-7.5 µg/mL for triamcinolone acetonide, neomycin and gramicidin, respectively. The $R^2$ values of the calibration curves for triamcinolone acetonide, neomycin and gramicidin were found to be 0.9999, 0.9996 and 0.9991, respectively. The correlation coefficient was more than 0.98, which was within the allowed ranges (NLT 0.99) (Fig. 2). As a result, the findings revealed that the peak area and analyte concentration showed a strong correlation (Table-2).

**Accuracy:** Gramicidin, neomycin and triamcinolone acetonide were recovered at 98.64%, 99.4% and 99.5%, respectively, from the varying quantities of spiked sample solutions (Table-3). The recovery rate was determined to be between 98% and 102%. This implies that the proposed approach was extremely accurate and the results were within the ICH guidelines' acceptable ranges.

TABLE-1
SYSTEM SUITABILITY RESULTS

| Triamcinolone acetonide | | | Neomycin | | | Gramicidin | | |
|---|---|---|---|---|---|---|---|---|
| RT (min) | USP plate count | Tailing | RT (min) | USP plate count | Tailing | RT (min) | USP plate count | Tailing |
| 2.435 | 5388 | 1.03 | 7.273 | 27001 | 0.89 | 9.145 | 44429 | 0.56 |
| 2.428 | 5452 | 0.86 | 7.281 | 27618 | 0.92 | 9.137 | 44562 | 0.54 |
| 2.431 | 5468 | 0.85 | 7.274 | 27621 | 0.97 | 9.145 | 44685 | 0.58 |
| 2.425 | 5487 | 0.89 | 7.270 | 27558 | 0.92 | 9.144 | 44736 | 0.51 |
| 2.432 | 5896 | 0.82 | 7.264 | 27365 | 0.95 | 9.138 | 44682 | 0.58 |
| 2.437 | 5877 | 0.86 | 7.261 | 27485 | 0.95 | 9.145 | 45687 | 0.59 |

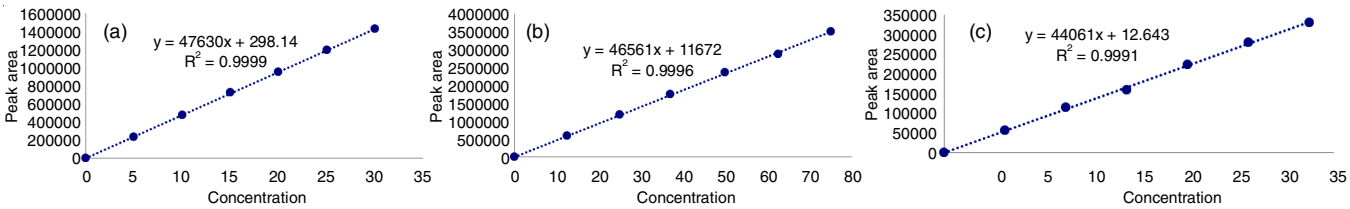


Fig. 2. Linearity plot of triamcinolone acetonide (a), neomycin (b) and gramicidin (c)

TABLE-2
LINEARITY RESULTS OF TRIAMCINOLONE ACETONIDE, NEOMYCIN AND GRAMICIDIN

| Parameters | Triamcinolone acetonide | Neomycin | Gramicidin |
|---|---|---|---|
| Linearity (µg/mL) | 5-30 | 12.5-75 | 1.25-7.5 |
| Regression equation | y = 47630x + 298.14 | y = 46561x + 11672 | y = 44061x + 12.643 |
| Slope | 47630 | 46561 | 44061 |
| Intercept | 298.14 | 11672 | 12.643 |
| Correlation coefficient ($R^2$) | 0.9999 | 0.9996 | 0.9991 |

TABLE-3
RECOVERY RESULTS OF GRAMICIDIN, NEOMYCIN AND TRIAMCINOLONE ACETONIDE

| Recovery level (%) | Gramicidin | | | | Neomycin | | | | Triamcinolone acetonide | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | A | B | C | D | A | B | C | D |
| 80 | 0.45 | 0.448 | 99.6 | 98.64 | 4 | 4.037 | 100.9 | 99.4 | 1.6 | 1.582 | 98.9 | 99.5 |
| | 0.45 | 0.444 | 98.7 | | 4 | 3.979 | 99.5 | | 1.6 | 1.621 | 101.3 | |
| | 0.45 | 0.446 | 99.1 | | 4 | 3.995 | 99.9 | | 1.6 | 1.596 | 99.8 | |
| 100 | 0.50 | 0.494 | 98.8 | 98.64 | 5 | 4.917 | 98.3 | 99.4 | 2.0 | 1.991 | 99.6 | 99.5 |
| | 0.50 | 0.493 | 98.6 | | 5 | 4.945 | 98.9 | | 2.0 | 1.971 | 98.6 | |
| | 0.50 | 0.491 | 98.2 | | 5 | 4.907 | 98.1 | | 2.0 | 1.989 | 99.5 | |
| 120 | 0.55 | 0.540 | 98.2 | 98.64 | 6 | 5.932 | 98.9 | 99.4 | 2.4 | 2.370 | 98.8 | 99.5 |
| | 0.55 | 0.540 | 98.2 | | 6 | 5.987 | 99.8 | | 2.4 | 2.402 | 100.1 | |
| | 0.55 | 0.541 | 98.4 | | 6 | 6.029 | 100.5 | | 2.4 | 2.381 | 99.2 | |

A = Amount spiked (mg); B = Amount recovered (mg); C = Recovery (%); D = Mean % recovery

**Precision:** The percent relative standard deviation values for method precision results of gramicidin, neomycin and triamcinolone acetonide were found to be 0.684%, 0.0442% and 0.018%, respectively (Table-4). The intermediate precision results of gramicidin, neomycin and triamcinolone acetonide were found to be 0.3969%, 0.0702% and 0.0604%, respectively (Table-5). The results were well under the accepted ± 2% limit.

TABLE-4
METHOD PRECISION RESULTS

| S. No. | Area of triamcinolone acetonide | Area of neomycin | Area of gramicidin |
|---|---|---|---|
| 1 | 951347 | 2368865 | 227896 |
| 2 | 951856 | 2367749 | 226985 |
| 3 | 951768 | 2389659 | 227443 |
| 4 | 951596 | 2388831 | 225598 |
| 5 | 951544 | 2387451 | 225637 |
| 6 | 951642 | 2388631 | 223698 |
| Mean | 951625.5 | 2381864.33 | 226209.5 |
| S.D. | 178.20 | 10531.05 | 1547.677 |
| %RSD | 0.0187 | 0.4421 | 0.6841 |

TABLE-5
INTERMEDIATE PRECISION RESULTS

| S. No. | Area of triamcinolone acetonide | Area of neomycin | Area of gramicidin |
|---|---|---|---|
| 1 | 951854 | 2377524 | 225638 |
| 2 | 951576 | 2377452 | 224755 |
| 3 | 951442 | 2378647 | 225578 |
| 4 | 951351 | 2374521 | 223578 |
| 5 | 952758 | 2375721 | 224557 |
| 6 | 952451 | 2374812 | 223664 |
| Mean | 951905.33 | 2376446.16 | 224628.33 |
| S.D. | 575.82 | 1668.51 | 891.69 |
| %RSD | 0.0604 | 0.0702 | 0.3969 |

**Sensitivity:** The LOD results of gramicidin, neomycin and triamcinolone acetonide were observed to be 0.15, 1.5 and 0.6 µg/mL, respectively, whereas LOQs values of gramicidin, neomycin and triamcinolone acetonide were 0.5, 5 and 2 µg/mL, respectively, indicating that the method had good sensitivity.

**Robustness:** The percent RSD of gramicidin, neomycin and triamcinolone acetonide was less than 2.0 in all of the deliberately changed chromatographic settings. The system suitability parameters were not significantly changed and all of them were passed. The results are shown Table-6.

**Specificity:** The specificity of the method was assessed in terms of interference caused by the presence of any other placebos. The LC-MS chromatograms recorded for blank and placebo (Fig. 3) exhibited nearly no intrusive peaks within the retention time ranges. Thus, the LC-MS method in this work was specific and selective.

TABLE-6
RESULTS OF ROBUSTNESS

| Condition | %RSD of triamcinolone acetonide | %RSD of neomycin | %RSD of gramicidin |
|---|---|---|---|
| Flow rate (+) 0.9 mL/min | 0.12 | 0.05 | 0.34 |
| Flow rate (-) 1.1 mL/min | 0.01 | 0.04 | 0.06 |
| Organic phase (+) 55B:45A | 0.11 | 0.03 | 0.09 |
| Organic phase (-) 65B:35A | 0.07 | 0.03 | 0.32 |

**Degradation studies:** Different types of stressed conditions have been undertaken according to ICH recommendations. Few degradation products were observed during the study. The tailing factor, plate count, % RSD, % degradation were in the limits for forced degradation studies (Table-7).

**Forced degradation studies of triamcinolone acetonide**

**DP1:** The fragmentation mechanism of DP1 was depicted in Fig. 4 and the ESI spectrum revealed the most intense [M+H] ion of *m/z* -468.17, which was detected under acid degeneration conditions. The MS spectrum of DP1 revealed numerous product ions at *m/z*-420.19 (loss of $CH_3Cl$), *m/z*-362.19 (loss of $C_2H_6O_2$) and *m/z*-278.17 (loss of $C_4H_8O_2$). **DP2:** The ESI spectra showed the highest [M+H] ion of *m/z* -450.21 observed under alkali degeneration conditions and Fig. 5 exhibited the fragmentation mechanism of **DP2**. Multiple product ions at *m/z*-362.19 (loss of $C_3H_8O_3$) and *m/z*-278.17 (loss of $C_4H_8O_2$) were found in the MS spectra of DP2. **DP3:** DP3 fragmentation mechanism is depicted in Fig. 6 and the ESI spectrum revealed the most intense [M+H] ion of *m/z* -392.16 seen under peroxide degeneration conditions. The MS spectrum of DP3 revealed several product ions at *m/z*-278.17 (loss of $C_4H_6O_4$) and *m/z*-118.03 (loss of $C_{17}H_{23}FO_2$). **DP4:** The most intense [M+H] ion of *m/z* -438.24 found under the reduction degeneration circumstances was visible in the ESI spectrum and Fig. 7 displayed the DP4 fragmentation mechanism. The MS spectra of **DP4** at *m/z*-352.24 (loss of $C_3H_8O_3$) and *m/z*-266.17 revealed several product ions (loss of $C_5H_{12}O$).

**Forced degradation studies of neomycin**

**DP5:** The fragmentation mechanism of **DP5** was depicted in Fig. 8 and the ESI spectrum revealed the most intense [M+H] ion of *m/z* -648.27, which was detected under acid degeneration conditions. The MS spectra of **DP5** revealed several product

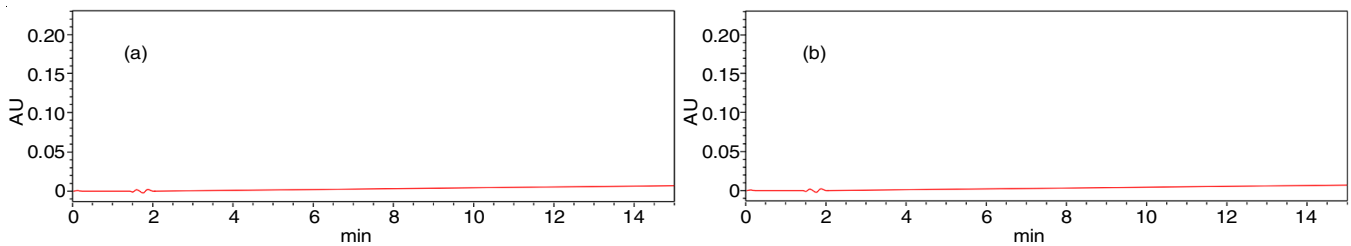


Fig. 3. (a) Blank and (b) Placebo chromatogram of HPLC

TABLE-7
RESULTS OF FORCED DEGRADATION STUDY FOR GRAMICIDIN, NEOMYCIN AND TRIAMCINOLONE ACETONIDE

| Time (h) | Acid | | Alkali | | Peroxide | | Reduction | | Thermal | | Photo stability | | Hydrolysis | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R (%) | D (%) | R (%) | D (%) | R (%) | D (%) | R (%) | D (%) | R (%) | D (%) | R (%) | D (%) | R (%) | D (%) |
| Gramicidin | | | | | | | | | | | | | | |
| Initial | 99.7 | 0.2 | 99.2 | 0.7 | 99 | 0.9 | 99.1 | 0.8 | 99.3 | 0.6 | 99.4 | 0.5 | 99.8 | 0.1 |
| 6 h | 99.1 | 0.8 | 98.5 | 1.4 | 98.2 | 1.7 | 98.7 | 1.2 | 98.4 | 1.5 | 98.9 | 1 | 99.2 | 0.7 |
| 12 h | 98.3 | 1.6 | 97.9 | 2 | 97.1 | 2.8 | 98.3 | 1.6 | 98.1 | 1.8 | 98.6 | 1.3 | 98.6 | 1.3 |
| 18 h | 97.7 | 2.2 | 97.3 | 2.6 | 96.5 | 3.4 | 97.8 | 2.1 | 96.9 | 3 | 98.6 | 1.3 | 97.6 | 2.3 |
| 24 h | 96.5 | 3.4 | 96.2 | 3.7 | 95.7 | 4.2 | 97 | 2.9 | 96.7 | 3.2 | 97.9 | 2 | 97.2 | 2.7 |
| Neomycin | | | | | | | | | | | | | | |
| Initial | 99 | 1 | 98.9 | 1.1 | 98.4 | 1.6 | 99.5 | 0.5 | 98.8 | 1.2 | 99.1 | 0.9 | 99.2 | 0.8 |
| 6 h | 95.9 | 4.1 | 95.2 | 4.8 | 94.3 | 5.7 | 98.6 | 1.4 | 93.2 | 6.8 | 98.2 | 1.8 | 98.1 | 1.9 |
| 12 h | 91.8 | 8.2 | 91.8 | 8.2 | 91.6 | 8.4 | 97.3 | 2.7 | 91.7 | 8.3 | 97.5 | 2.5 | 97.2 | 2.8 |
| 18 h | 88.3 | 11.7 | 88.8 | 11.2 | 87.9 | 12.1 | 96.6 | 3.4 | 88.5 | 11.5 | 96 | 4 | 96.3 | 3.7 |
| 24 h | 85.2 | 14.8 | 84.1 | 15.9 | 83.1 | 16.9 | 94.4 | 5.6 | 86.4 | 13.6 | 95.3 | 4.7 | 95 | 5 |
| Triamcinolone acetonide | | | | | | | | | | | | | | |
| Initial | 99.4 | 0.6 | 99.3 | 0.7 | 98.9 | 1.1 | 99.6 | 0.4 | 99.5 | 0.5 | 99.6 | 0.4 | 99.8 | 0.2 |
| 6 h | 96.2 | 3.8 | 96.3 | 3.7 | 95.7 | 4.3 | 96.9 | 3.1 | 98.3 | 1.7 | 98.8 | 1.2 | 98.8 | 1.2 |
| 12 h | 91 | 9 | 91.4 | 8.6 | 90.5 | 9.5 | 93.5 | 6.5 | 97.8 | 2.2 | 97.4 | 2.6 | 98.4 | 1.6 |
| 18 h | 86.6 | 13.4 | 86.9 | 13.1 | 86 | 14 | 89.5 | 10.5 | 97.3 | 2.7 | 96.9 | 3.1 | 98 | 2 |
| 24 h | 83.2 | 16.8 | 84.5 | 15.5 | 83 | 17 | 86.7 | 13.3 | 95.8 | 4.2 | 96.5 | 3.5 | 97 | 3 |

R = Recovered (%); D = Degraded (%)

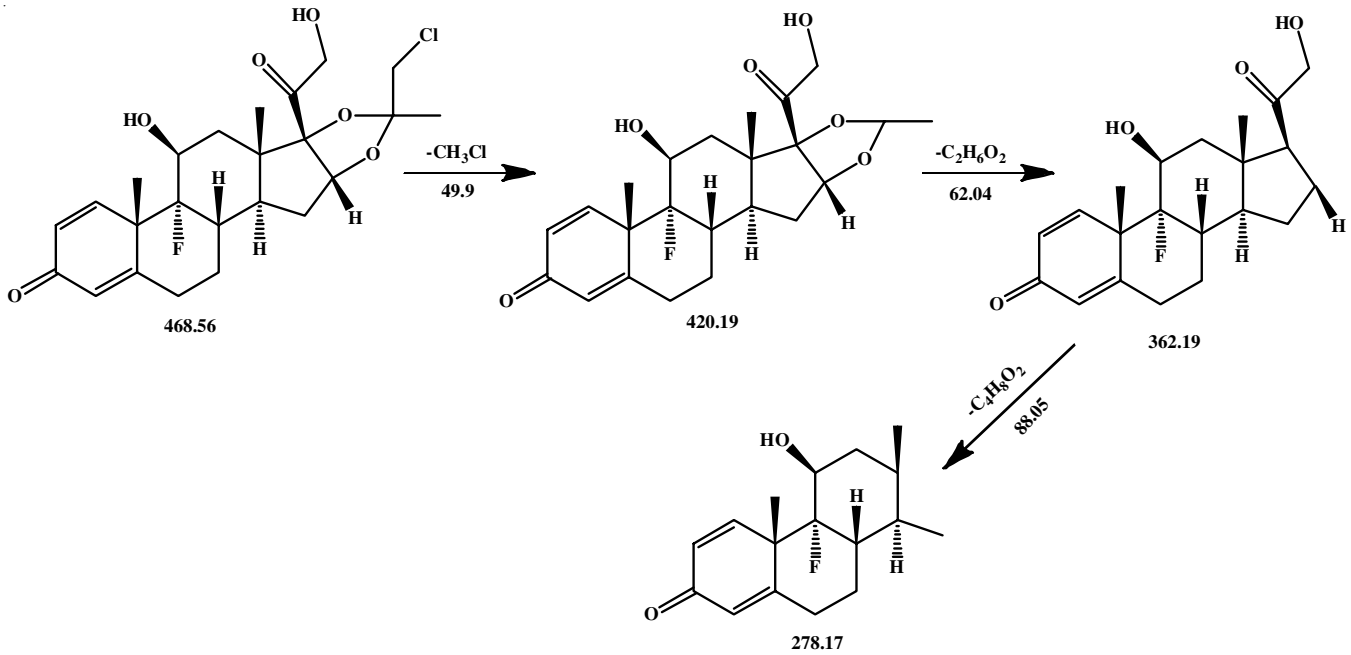


Fig. 4. Degradation products of triamcinolone acetonide acid impurity-**DP1**

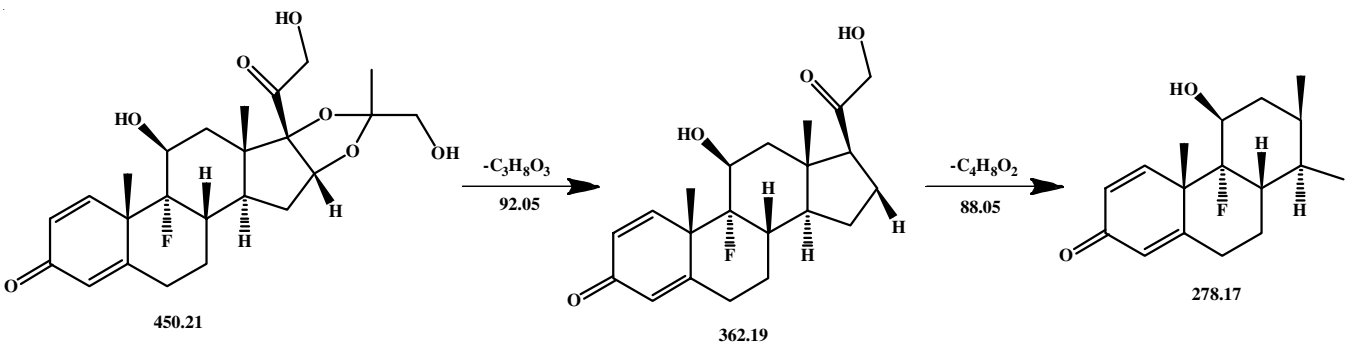


Fig. 5. Degradation products of triamcinolone acetonide alkali impurity-**DP2**

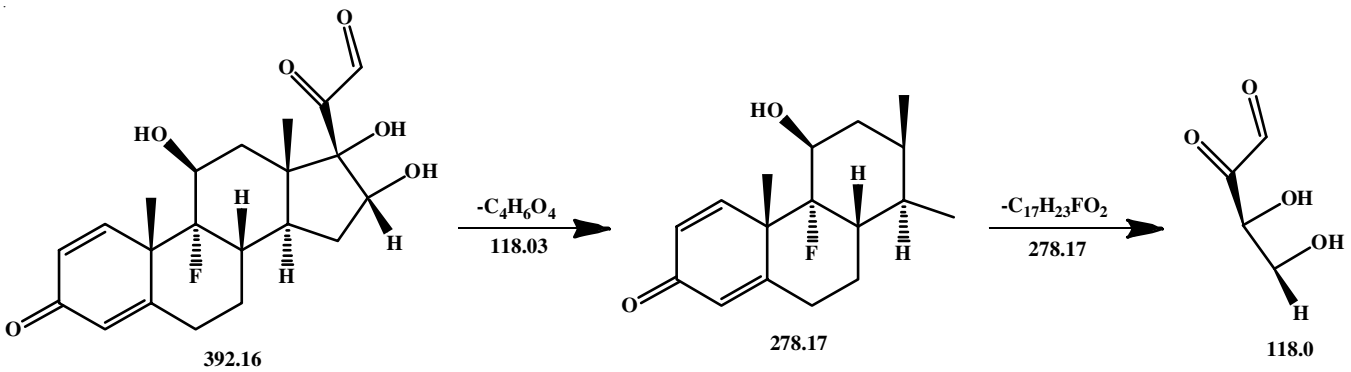


Fig. 6. Degradation products of triamcinolone acetonide peroxide impurity-**DP3**

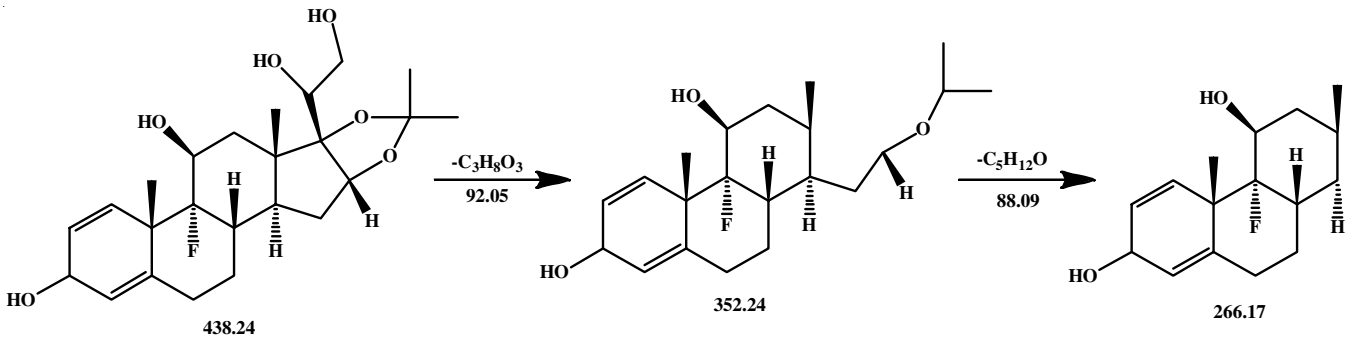


Fig. 7. Degradation products of triamcinolone acetonide reduction impurity-**DP4**

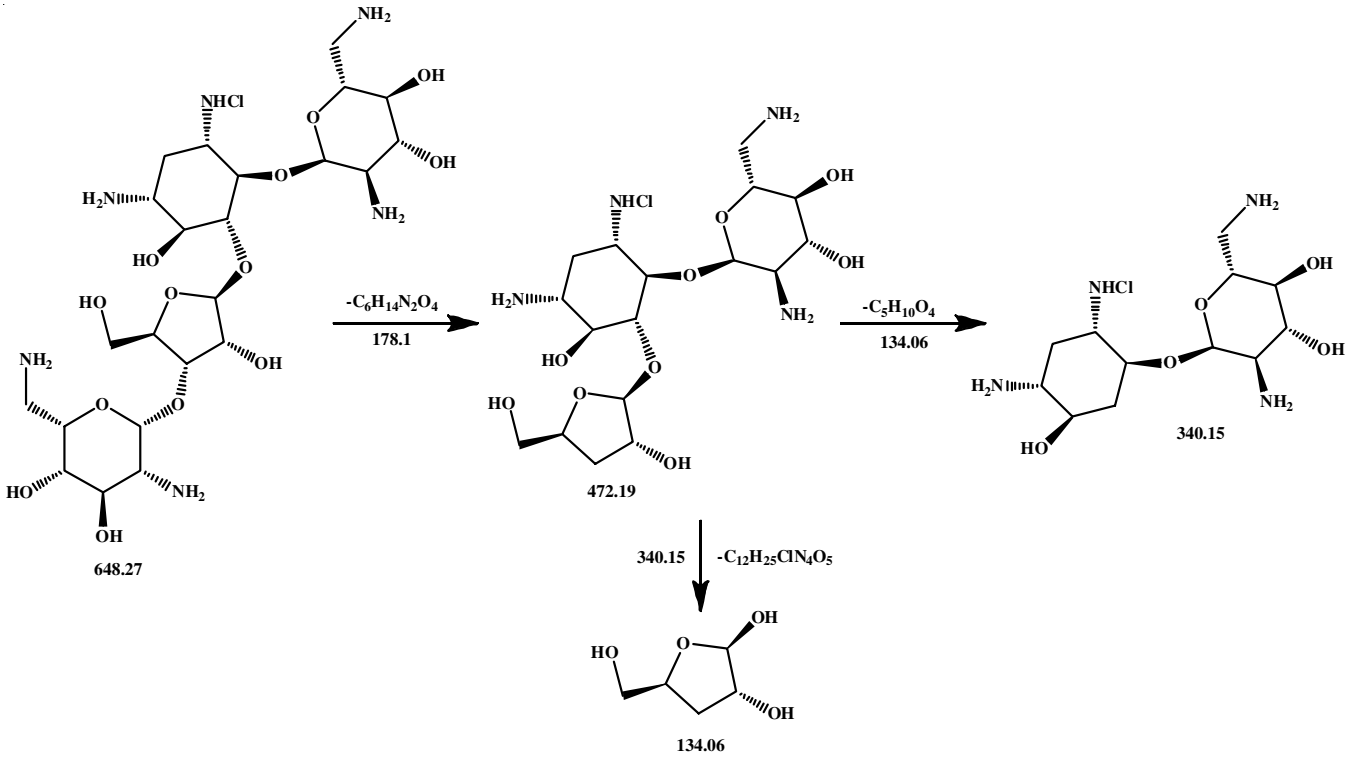


Fig. 8. Degradation products of neomycin acid impurity-**DP5**

ions at *m/z*-472.19 (loss of $C_6H_{14}N_2O_4$), *m/z*-340.15 (loss of $C_5H_{10}O_4$) and *m/z*-134.06 (loss of $C_5H_{10}O_4$) (loss of $C_{12}H_{25}ClN_4O_5$). **DP6:** The most powerful [M+H] ion, identified under alkali degeneration conditions, was visible in the ESI spectrum at *m/z* -630.31 and the **DP6** fragmentation mechanism was shown in Fig. 9. The MS spectra of **DP6** revealed the several product ions at *m/z*-454.23 (loss of $C_6H_{14}N_2O_4$) and *m/z*-306.19 (loss of $C_5H_{10}O_5$). **DP7:** The strongest [M+H] ion, identified during peroxide degeneration, was displayed in the ESI spectrum at *m/z* -644.29. The fragmentation mechanism of DP7 is shown

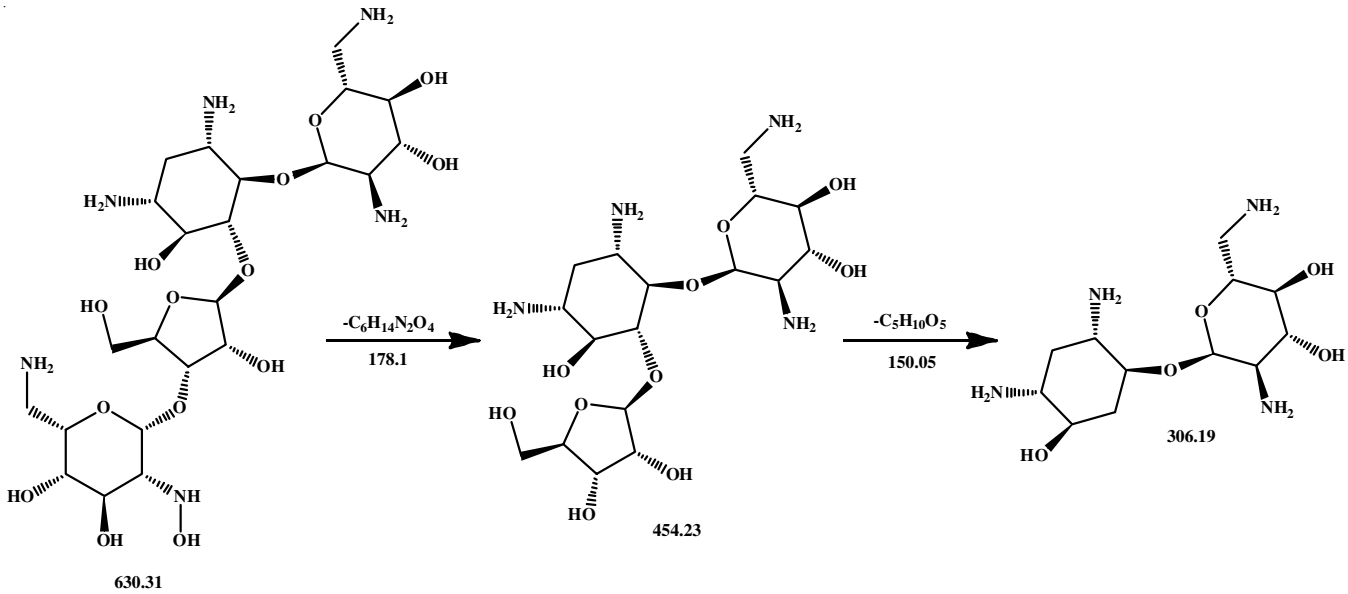


Fig. 9. Degradation products of neomycin alkali impurity-**DP6**

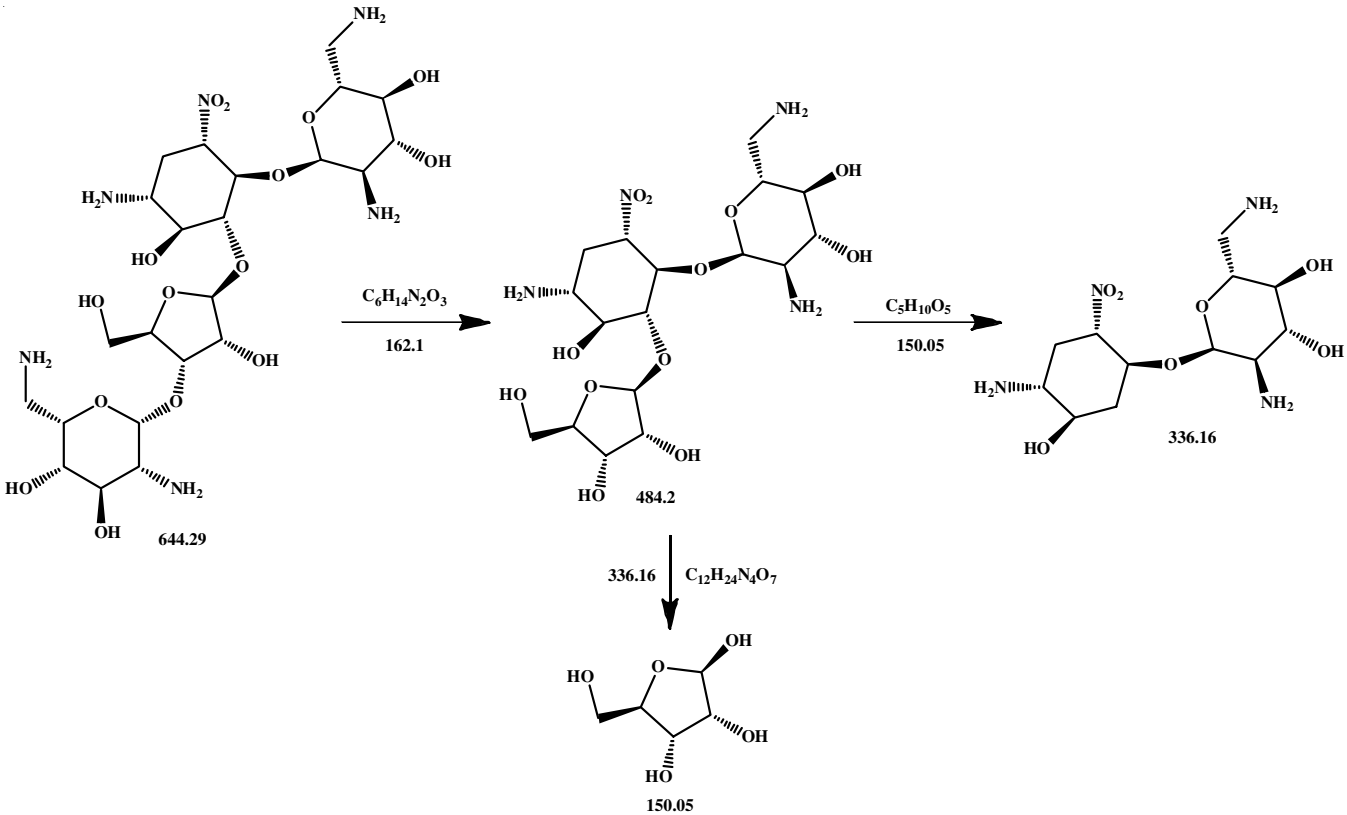


Fig. 10. Degradation products of neomycin peroxide impurity-**DP7**

in Fig. 10. The MS spectra of **DP7** revealed the presence of several product ions at *m/z*-484.2 (loss of $C_6H_{14}N_2O_3$), *m/z*-336.16 (loss of $C_5H_{10}O_5$) and *m/z*-150.05 (loss of $C_{12}H_{24}N_4O_7$). **DP8:** The strongest [M+H] ion, identified as a result of thermal degeneration, was discernible in the ESI spectrum at *m/z* -596.3 and Fig. 11 showed the **DP8** fragmentation mechanism. Multiple product ions at *m/z*-420.22 (loss of $C_6H_{14}N_2O_4$), *m/z*-288.18 (loss of $C_5H_{10}O_4$) and *m/z*-134.06 (loss of $C_{12}H_{24}N_4O_4$) were discovered in the MS spectra of **DP8**.

## Conclusion

For the simultaneous assessment of gramicidin, neomycin and triamcinolone acetonide pure and pharmaceutical dose form, a precise and sensitive LC-MS approach was proposed. The proposed approach was highly sensitive, precise and specific. Few degradation products were observed during the forced degradation studies. The tailing factor, plate count, % RSD and % degraded were in the limits for forced degradation studies. This demonstrates that the procedure was precise and

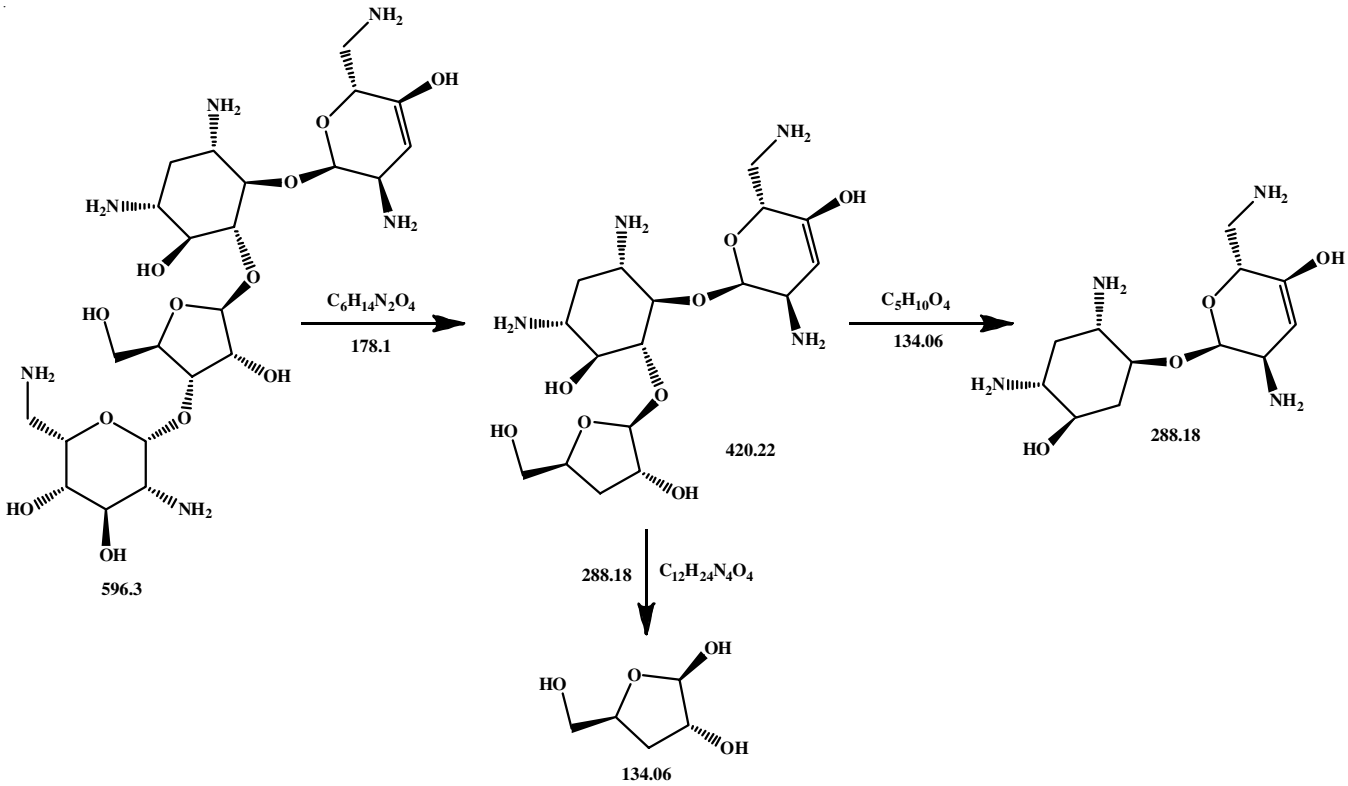


Fig. 11. Degradation products of neomycin thermal impurity-**DP8**

stable. Also, there were no degradation products found in the triamcinolone acetonide. As a result, this approach will be used to identify and quantify gramicidin, neomycin and triamcinolone acetonide in the process chemistry and quality control departments.

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## CONFLICT OF INTEREST

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

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