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Forced Degradation Analysis Of Spiramycin And Characterization Of Degradation Product By Rp-Hplc And Lc-Ms/Ms

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Abstract:

The objective of present work was to develop and validate forced degradation analysis and characterization of degradation products of sixteen membered lactone ring macrolide antibiotic Spiramycin under hydrolytic, oxidative, thermal and photolytic stress degradation condition as per the International Conference on Harmonization (ICH) guidelines. A simple, sensitive and accurate RP-HPLC method was developed by using C_{18} Water X Bridge® column (250 x 4.6 mm, 5 µm) using isocratic elution programme. The drug was found to be linear at the concentration range of 1-100 µg/ml, with a regression coefficient (r^2= 0.999). Validation of developed method was performed as per ICH Q2A R1 guideline and results al all parameters were within the acceptable limit. Drug was found to degraded in hydrolytic degradation condition and was stable at oxidative, thermal and photolytic condition. A complete mass fragmentation route was established with the help of LC-MS/MS method, and data obtained from mass spectrum were employed to characterize the degradation products and structure was elucidated.

Keywords: Forced degradation, Spiramycin, RP-HPLC, LC-MS/MS, degradation products.

Introduction:

Degradation of drug substance and drug products at a condition more severe than the accelerated condition (40^0C ± 5^0C, 75 ± 5% RH) are known as forced degradation or stress degradation. Forced degradation studies plays a key role in development of stability indicating methods. A specific stability indicating approach is developed to determine the active substance in presence of debasement products, diluent and other additives anticipate in pharmaceutical formulation. In order to measure active substance as well as degradation products in presence of diluent and additives selective stability indicating method was developed [1]. Regulatory guidelines such as ICH includes [ICH Q1A (R2), ICH Q1B, ICH Q2A(R1), ICH Q3BR2, ICH Q6A], WHO, EMA, FDA 21 CFR section 211 are needed for forced degradation studies, identification of degraded products and development and validation of stability indicating approach [2-9]. There is no such information provided by these guidelines regarding the specific practical approach for forced degradation studies.

Spiramycin, an antibiotic and antiparasitic agent approved by British Pharmacopoeia Commission produced from Streptomyces ambofaciens. It is commonly known as Rovamycin in a European countries. Spiramycin is 16-membered lactone ring macrolide antibiotics used to treat infection

caused by gram +ve and gram -ve bacteria such as Streptococci, Pneumococci, Leginella spp., Mycoplasma and Toxoplasma gonidia. Its mechanism of action was to inhibit protein synthesis in 50s subunit of bacterial ribosomes causing polypeptide chain to dissociate from ribosome [10]. Chemically it is known as 2-[(4R,5S,6S,7R,9R,10R,11E,13E,16R)-6-[(2S,3R,4R,5S,6R0-5-[(2S,4R,5S,6S)-4,5-dihydroxy-4,6-dimethyloxan-2-yl]oxy-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-10-[(2R,5S,6R)-5-(dimethylamino)-6-methyloxan-2-yl]oxy-4-hydroxy-5-methoxy-9,16-dimethyl-2-oxo-1-oxacyclohexadeca-11,13-dien-7-yl]acetaldehyde as shown in Fig 1, having a chemical formula $C_{43}H_{74}N_2O_{14}$ and molecular weight of 843.02 g/mol [11]. As per the review of literature various methods such as, separation and determination of components of spiramycin in bulk powders and in pharmaceutical preparations by high-performance liquid chromatography [12], solid phase extraction and HPLC determination of spiramycin in plasma and vitreous concentrations [13], determination of spiramycin and neospiramycin in plasma and milk of lactating cows by reversed-phase high-performance liquid chromatography [14], a new reversed phase HPLC method for the determination of spiramycins I, II and III [15], development and validation of an reversed-phase liquid chromatographic method for analysis of spiramycin and related substances [16], characterization of impurities in spiramycin by liquid chromatography/ion trap mass spectrometry [17], improved liquid chromatographic method for quality control of spiramycin using superficially porous particles [18], efficient and simple HPLC method for spiramycin determination in urine samples and in pharmaceutical tablets [19]. From that we came to know that no stability indicating RP-HPLC method and characterization of degradations products by LC-MS/MS was available for spiramycin. Hence, a rapid, sensitive, accurate, precise and robust RP-HPLC method has been developed for the forced degradation analysis of spiramycin and characterization of degradation product by LC-MS/MS.

Materials and Methods:

Chemicals:

The working standard of Spiramycin was obtained from Indian Pharmacopoeia Commission, New Delhi. Chemicals (dipotassium hydrogen orthophosphate, ammonium acetate, sodium hydroxide, HCl, H_2O_2) and solvents (acetonitrile, acetic acid, formic acid) were of HPLC and LC-MS/MS grade purchase from SD Fine chemicals. Solvent water was obtained from Milli Q RO system.

Instrumentation and Chromatographic conditions:

Shimadzu single pan digital balance, Shimadzu (UV-1700) Spectrophotometer, Shimadzu Prominence HPLC system LC-20 AT-VP solvent delivery system (pump) Hamilton injector with 20 µl loop SPD 20A UV detector Lab solution CS software for data management and Shimadzu LC-MS/MS 8030 system equipped with electrospray ionization (ESI) interface, LC-20AD pump, SPD-M20 PDA detector, CTO-20AC column oven, CBM-20 alite controller and SIL-20AC auto sampler and Lab Solution data station were used. The chromatographic conditions consist of Water X Bridge®C_{18} (250 x 4.6 mm, 5 µm) column at ambient temperature was selected, and dipotassium hydrogen orthophosphate and acetonitrile were used as mobile phase at the ratio of (40:60 % v/v) and set at a flow rate of 0.8 ml/min. 10 µl of injection volume was employed and detected at 231 nm.

Stress studies:

Forced degradation studies were carried out as per ICH guideline Q1A (R2). The drug was exposed to different stress conditions like acidic hydrolysis, basic hydrolysis, neutral, oxidation, thermal and photolysis. The evaluation was accomplished by HPLC with UV detector and LC-MS/MS technique were employed for

characterization of debasement products produced during forced degradation. Hydrolytic degradation of Spiramycin was accomplished separately under acidic, alkaline, neutral condition by treating of 1mg/ml solution of drug at room temperature for 24 hrs using (0.1M HCl), (0.1 M NaOH) and water. Spiramycin solution, 1ml of 1mg/ml was treated with 1ml of 0.3% H_2O_2 at room temperature for oxidation at 24 hrs. Thermal and photolytic degradation was accomplished on dry sample. In case of dry heat degradation, the drug powdered was heated in an oven at 75^0C for a period of 24 hrs. For photolytic degradation it was carried out under sunlight for a period of 24 hrs. Drug was stressed to natural sunlight along with control samples. After completion of degradation studies, the samples were diluted with mobile phase to get the desired concentration and injected into HPLC system for analysis.

Preparation of stock solution and working standard solution

Preparation of Stock solution:

A standard solution of 1 mg/ml of Spiramycin was prepared by dissolving 10 mg of Spiramycin in 10 ml of Acetonitrile, labeled and stored the solution in a refrigerator below 8°C.

Preparation of working standard solution:

1 ml from 1 mg/ml solution of Spiramycin was diluted to 10 ml in a standard flask with acetonitrile to achieve a concentration of 100 µg/ml solution for HPLC. From the above solution, different working standard solutions were prepared, as per respective concentrations of solutions need by diluting with the acetonitrile.

Preparation of Mobile phase:

8.70 g of dipotassium hydrogen orthophosphate was accurately weighed and transferred in a 500 ml volumetric flask and dissolved in 250 ml water, adjusted the pH 6 with 0.1 M HCl solution and made up the volume up to mark and filtered through membrane filter having 0.45 micro pore size.

UV for selection of wavelength

From the stock solution 10 µg/ml of solution was prepared with acetonitrile and scanned over the range of 200-400 nm and spectrum was recorded. It was observed that in 231 nm maximum absorbance was obtained. Hence, 231 nm was the λ_{max} of spiramycin and it was preferred as suitable wavelength for detection. The UV-spectrum of spiramycin was shown in Fig 2.

Validation of developed method:

The validation of developed method was performed as per ICH guideline Q2A (R1). The linearity test samples for Spiramycin assay were prepared from working standard solution with the help of mobile phase at seven different concentrations level from 1-100 µg/ml. The analysis was carried out in triplicate by injecting 20 µl of each concentration solution. To obtain the calibration graph the peak areas were plotted against corresponding concentration and the linear regression r^2 value was calculated. Specificity of the method was determined by injecting the blank sample and peak was recorded. The inter-day and intraday precision were determined at 80%, 100% and 120% of drug solution in triplicate in same day and on different consecutive three days. Accuracy of the developed method was determined by the analysis of known concentration of drugs, and different concentration spiked with sample in triplicate and the percentage recovery was determined. LOD and LOQ was determined based on signal to noise ration i.e., 3:1 and 10:1.

Assay of Tablets

Twenty tablets were weighed accurately, powdered and a weight of the powder equivalent to one tablet of spiramycin (3 M.I.U) was transferred to a 100 ml volumetric flask, dissolved the contents with acetonitrile and

filtered. The filtered solution was made up the volume with acetonitrile to obtain a concentration of 10 mg/ml of spiramycin. The above solution was further diluted with acetonitrile to achieve a concentration of 50 µg/ml

Characterization of degradation products

The degradation products were characterized by using LC-MS/MS. Based on m/z value obtained from positive ESI mode, the structure of the degradation products was predicted and degradation pathways was established. The expected structure and molecular weights of degradation products were determined.

Results and Discussion

Development and optimization of chromatographic condition

Many trials have been performed by using various mobile phase, flow rate and stationary phase. During the optimization of process, preliminary examination was performed using C_{18} column using different mobile phase such as acetonitrile: water in the ratio of (20:80, 50:50, 40:60, 30:70), water: methanol in the ratio of (50:50, 60:40, 70:30, 80:20), 0.1% formic acid: acetonitrile in the ratio of (50:50, 60:40, 70:30, 80:20) at different flow rate 0.5 ml/min, 0.8 ml/min and 1ml/min. Under such conditions peak symmetry and resolution were not satisfactory hence, phosphate buffer pH 6: acetonitrile (40:60% v/v) can be used as mobile phase at the flow rate of 0.8 ml/min. The optimized chromatogram was shown in Fig 3.

Validation

Specificity

The developed method was found to be specific as no peak was eluted at the retention of drug at 1.95 when blank sample was injected under optimized conditions.

Linearity

The drug response was found to be linear at the seven different concentrations ranged from 1-100 µg/ml and the regression equation was found to be Y= 56662x + 69377 with corelation coefficient of 0.999. Standard deviation of calibration curve was found within the limits. The graph of calibration curve was shown in Fig 4, and values were provided in Table I.

Accuracy and precision

Accuracy of the method was evaluated by conducting recovery studies in triplicate at three concentration level. The % recovery of the accuracy was found to be 98.6% which is within the limits. The data obtained from precision studies was provided in Table III.

Limit of Detection and Limit of Quantification (LOD & LOQ)

The LOD and LOQ were evaluated based on the standard deviation of response and signal to noise ratio. LOD and LOQ were found to be 0.110 µg/ml and 0.330 µg/ml respectively.

Robustness

The robustness of the assay method was performed by small change in chromatographic conditions which include wavelength ±2 nm, mobile phase ratio ±2 v/v and flow rate ±0.2 ml. The robustness of the developed method was evaluated using six replicate of same concentration and %RSD was found to be less than 2.

System suitability

The system suitability of the developed method was accomplished to determine parameters such as theoretical plates (N), asymmetry factor (As) and tailing factor (T) as per ICH Q2A R1 guideline. The result was obtained within the limits and provided in Table V.

Degradation studies on Spiramycin

The degradation studies on Spiramycin were accomplished under various stress conditions determined by HPLC. The typical chromatograms of separation of degraded

products and Spirmycin were shown in Fig 5. The concentration of the drug remained and % of the drug degraded were provided in Table II.

Hydrolysis degradation

The drug showed degradation of 13.31% in 0.1 M HCl, 27.34 % in 0.1 M NaOH and 7.43 % in neutral condition at room temperature for a period of 24 hrs. During hydrolytic condition spiramycin was found to be unstable.

Oxidation degradation

The drug was stressed under 0.3% H_2O_2 at room temperature for a period of 24 hrs and was found to be stable at oxidative condition (Fig 5).

Thermal degradation

The chromatogram indicates that spiramycin was not degraded after 24 hours when exposed to heat and was stable at thermal condition (Fig 5).

Photolytic degradation

The chromatogram indicates that 7.98% of spiramycin was degraded after 24 hours. The drug was slightly degraded under sunlight due to the presence of chromophore (Fig 5).

Estimation of drugs in formulation

Estimation of Spiramycin in dosage forms by HPLC method was carried out using above optimized chromatographic conditions. The sample solution was prepared and the chromatogram was recorded. The typical chromatogram of the sample solution is given in Fig 6. The percentage of the drug found in formulation, standard deviation was calculated and presented in Table IV. The result of analysis indicated that the amount of drug was in good agreement with the label claim of the formulation.

Characterization of degradation samples by LC-MS/MS

The LC-MS/MS analysis of the spiramycin drug was performed based on the conditions mentioned earlier and spectrum showed a molecular ion peak [M+H] at 843 and along with two major fragment ions at m/z of 912 and 776. The most abundant peaks 912 and 776 were formed with the additional product and fragmented peak respectively as shown in Fig 7-9. Mass spectra of spiramycin and its degradants were analysed in positive Electron spray ionization mode. The output of the mass spectrometer was validated by injecting the standard solution of drugs.

Conclusion

A rapid, specific, sensitive and robust stability indicating RP-HPLC method was developed for stress degradation analysis of spiramycin using C_{18} column. The developed method was validated as per ICH Q2A R1 guideline. The LC-MS/MS study was performed for characterization of degradation product. The m/z values obtained from mass studies helps to predict the structure of degradation products and establishment of degradation pathway. They are thus suitable for the estimation of drug in raw materials under stress conditions. The newly developed analytical methods can be used in the fields of research institutions, academic institutes, quality control department in industries, approved testing laboratories, biopharmaceutics & bioequivalence studies and clinical & pharmacokinetic studies after suitable modification

Conflict of Interest

The author declares that they have no conflict of interest.

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Table I: Linearity and Range

S. No.	Spiramycin Concentration (µg/ml)	Peak area for Spiramycin
1	1	105892
2	20	1280242
3	30	1820063
4	40	2244541
5	50	2879931
6	75	4272221
7	100	5787847

Table II**:** Summary of forced degradation studies of Spiramycin

Stress conditions	Drug recovered (%)	Drug decomposed (%)
Standard drug	100	-
Acidic hydrolysis	86.69	13.31
Alkaline hydrolysis	72.66	27.34
Neutral	92.57	7.43
Oxidative degradation	100	-
Thermal degradation	100	-
Photolytic degradation	92.02	7.98

Table III: Accuracy and Precision studies of Spiramycin

Sample (µg/ml)	Amount found (µg/ml) ± SD*	Intra day		Inter day	
		Accuracy	Precision (% RSD) **	Accuracy	Precision (% RSD) **
50	49.34±0.28	98.68	0.78	98.62	1.38

*SD: Standard Deviation

**RSD: Relative Standard Deviation.

Table IV: Assay and recovery studies for Spiramycin formulation

Formulation	Label claim	Amount taken for assay(µg/ml)	Amount found ±SD (N=6)	Found mg/tab	Recovery %
S_1	3 M I U	50	49.34 ± 0.29	2.96 MIU	98.6

S_1: Spiramycin Tablet from market

Table V: System suitability studies

S. No.	Parameters	Values obtained for Spiramycin
1	Theoretical Plate (N)	2560
2	Linearity range (µg/ml)	1-100
2	Regression equation	Y=56662x+69377
3	Correlation coefficient	0.999
4	Asymmetry factor	1
5	Tailing Factor	1.09
6	LOD (µg/ml)	0.110
7	LOQ (µg/ml)	0.330

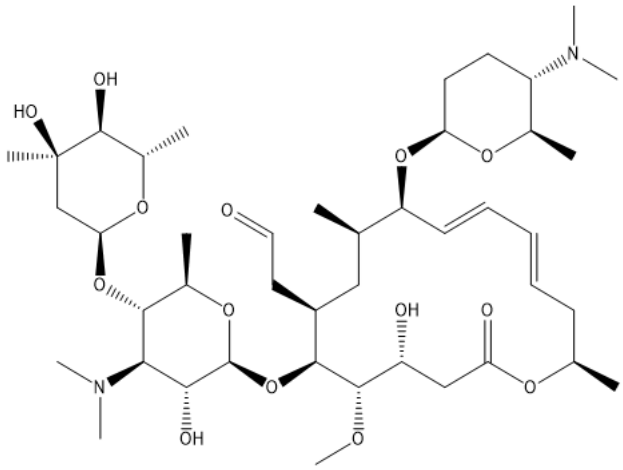

Figure 1. Structure of Spiramycin

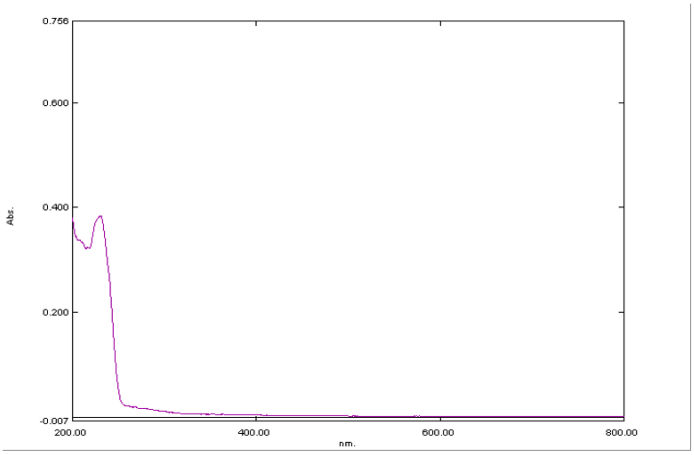

Figure 2. U V spectra of Spiramycin

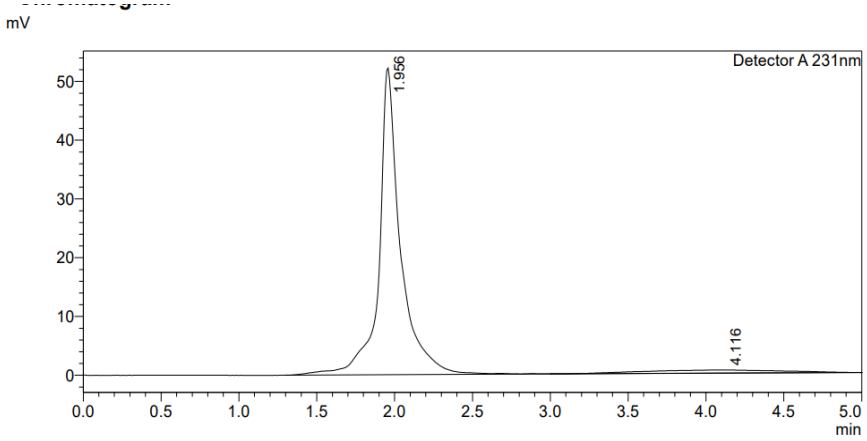

Figure 3. Typical HPLC chromatogram of Spiramycin standard

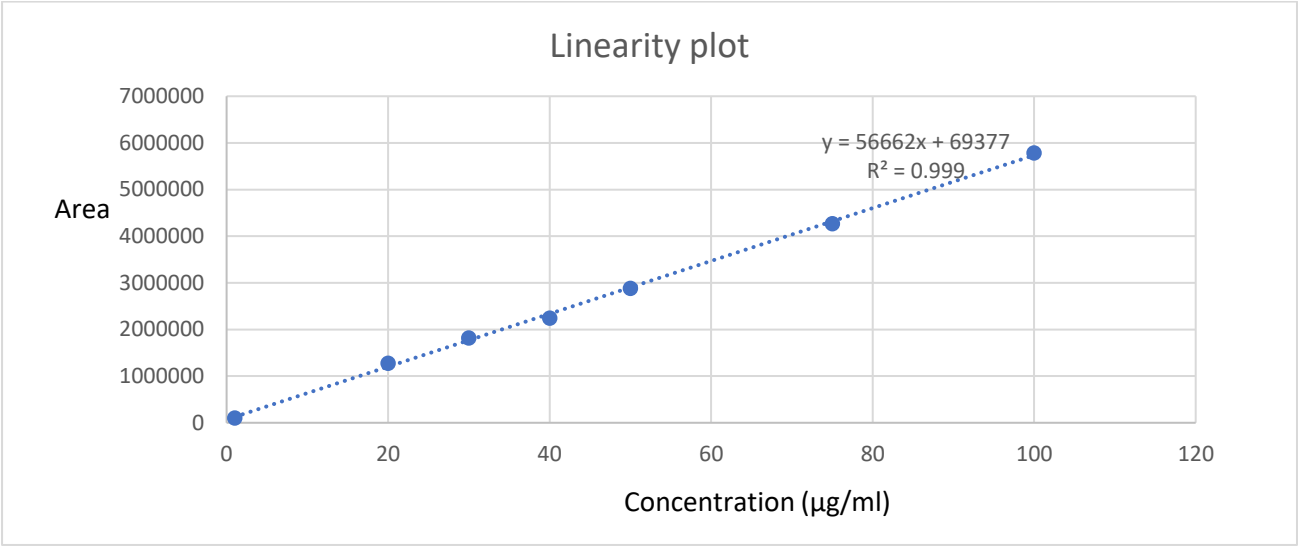

Figure 4. Calibration curve of Spiramycin

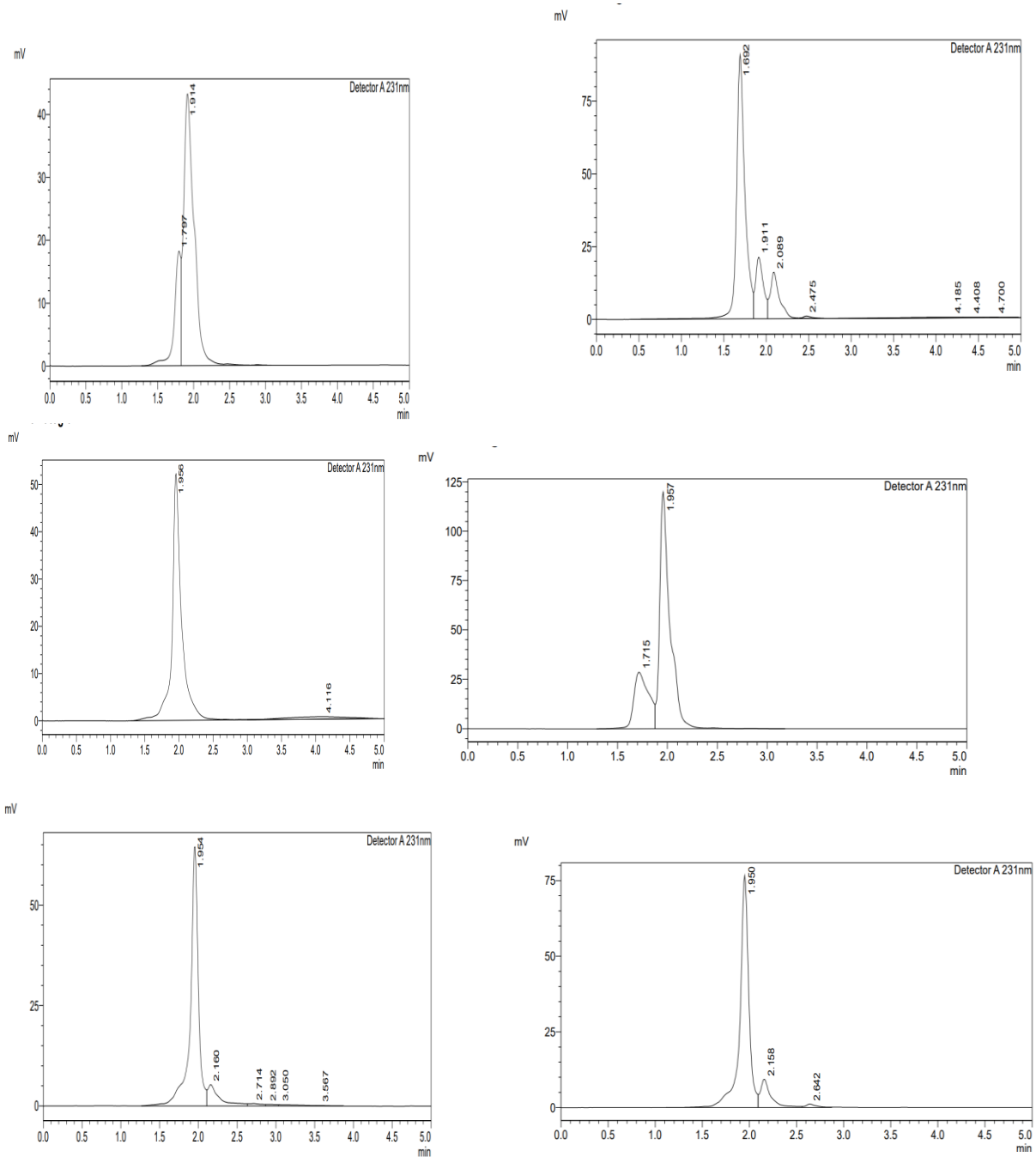

Figure 5. A typical HPLC chromatograms of A. acid degradation, B. base degradation, C. neutral degradation, D. oxidative degradation, E. thermal degradation and
F. photolytic degradation.

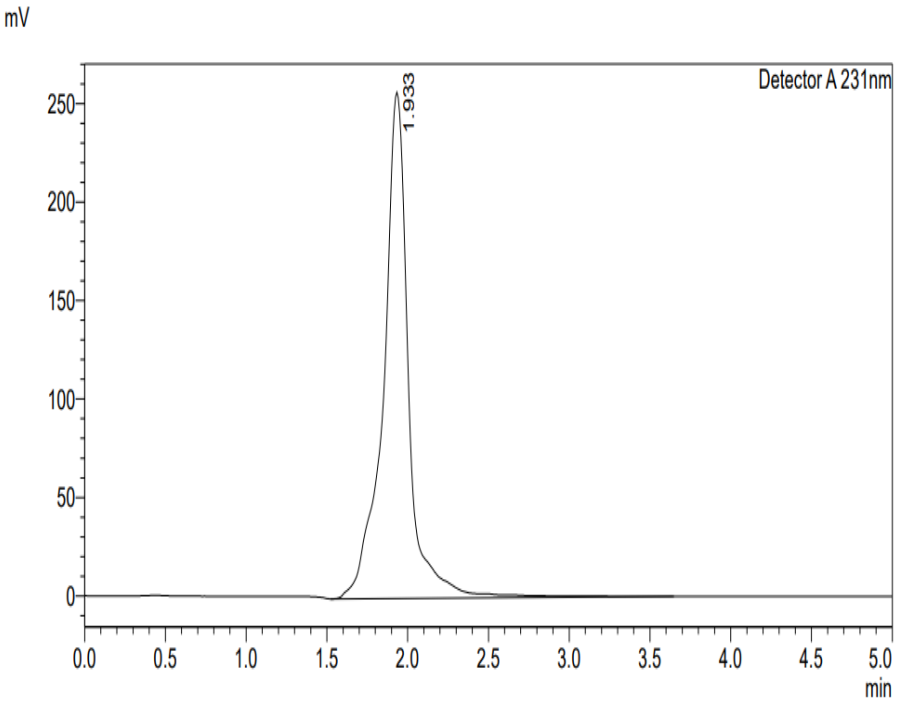

Figure 6. Typical HPLC chromatogram of Spiramycin Tablet formulation

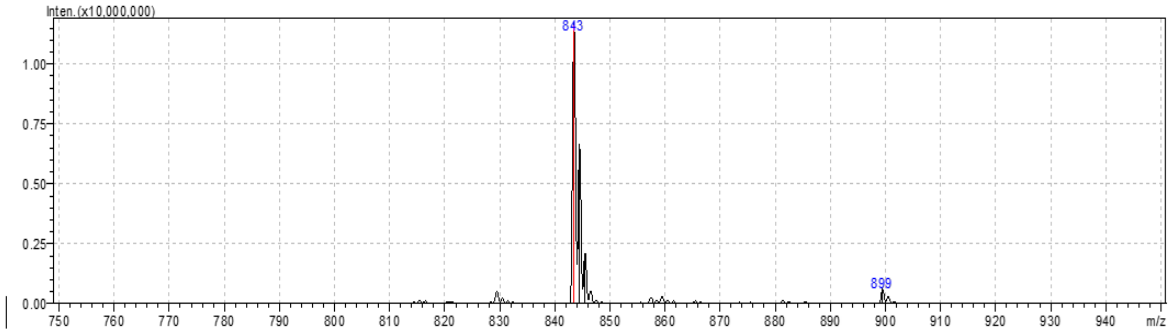

Figure 7. LC-MS/MS spectra of Spiramycin in ESI positive mode

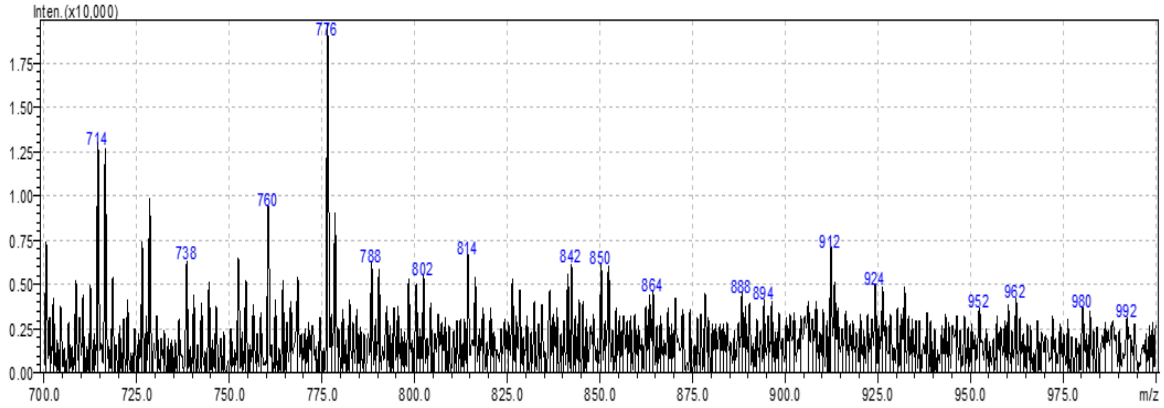

Figure 8. LC-MS/MS spectrum of DP-I

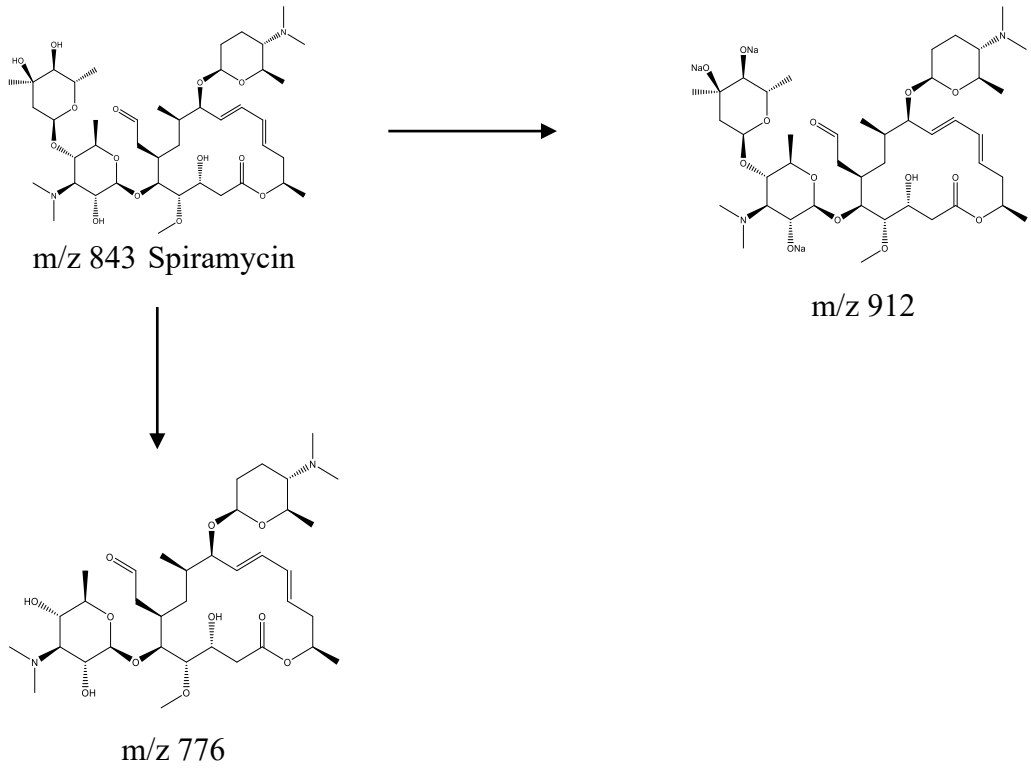

Figure 9. Postulated basic degradation mechanism of Spiramycin