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VALIDATED ANALYTICAL METHOD DEVELOPMENT AND CHARACTERIZATION OF FORCED DEGRADATIVE PRODUCTS OF MITAPIVAT AND THEIR TOXICITY PREDICTION BY *IN-SILICO* STUDIES

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Keywords:

RP-HPLC, Mitapivat, Mass characterization, Degradation products, *In-silico* studies

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ABSTRACT: The current study reports to validate a stability indicating RP-HPLC method for estimation of mitapivat in bulk, pharmaceutical formulations and FD characterization by using MS method. The chromatographic separation was accomplished on Waters X-Bridge phenyl column (150× 4.6mm, 3.5µm). With the mobile phase consisting of acetonitrile and 0.1% OPA (80:20v/v) at a flow rate of 1ml/min. The eluents were monitored by UV detector at 234nm. The developed liquid chromatographic method was validated with respect to accuracy, precision, linearity, robustness, range, limit of detection (LOD), limit of quantification (LOQ). The drug was subjected to various stress conditions such as acid, alkali, peroxide, reduction, photolytic, hydrolysis and thermal degradation. Among all the stress condition 5degradation products were obtained they were DP-1, Dp-2, Dp-3, Dp-4, Dp-5. They were subjected to mass characterization. The obtained structures were subjected to *in-silico* studies using Swiss ADME, pk CSM web server, protox2. The SWISS ADME web server and pk CSM web server were used for the prediction of pharmacokinetic properties and toxicity of the drug and its degradation products. The drug and the DP-1 don't show any toxicity. Whereas DP-2, DP-3, DP-4, DP-5 shows immune toxicity. Hence the method was used for impurity profiling for mitapivat.

INTRODUCTION: Mitapivat N-(4-{[4-(Cyclo-propylmethyl) - 1-piperazinyl] carbonyl} phenyl)-8-quinoline sulfonamide. Mitapivat belongs to a group of drugs known as pyruvate kinase activators. In patients with pyruvate kinase deficiency, mitapivat is used to treat hemolytic anemia, a blood condition which happens when red blood cells are destroyed more rapidly than they can be generated within the body.

QUICK RESPONSE CODE

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The FDA has approved it under the Pyrukynd (India) brand in a range of strengths starting at 5 mg [1]. Literature survey revealed that few analytical techniques have been published individually or in combination with other drugs [2-6]. However, no method was reported for estimation of drug by using HPLC method. The development of an established stability indicating RP-HPLC has been attempted. LC-MS/MS method for estimating Mitapivat and FD Characterization; *in-silico* investigations using soft ware's to determine their toxicity [1].

MATERIALS AND METHODS:

Instrument: A Waters Alliance e-2695 type HPLC with a column oven, auto sampler, and degasser was utilized for the analysis. The HPLC system

was linked to the SCIEX QTRAP 5500 mass spectrometer, which features an electro spray ionization interface. The SCIEX program was utilized to the interpretation of the chromatogram's data.

The interface mode of the mass spectrometer has been set to positive ion electrospray ionization. To measure the Mitapivat, a multiple reactions monitoring mode has been implemented. Working Parameters have been set as follows:

- Collision energy: 13V
- Ion spray voltage: 5500V
- Source temperature: 550^{o}C
- Drying gas temperature: 120-250°C
- Collision gas: nitrogen
- Drying gas flow stream: 5 mL/min
- Declustering potential: 40V
- Entrance potential: 10V
- Exit Potential: 7V
- Dwell time: 1sec

Drug Samples: Working standards of Mitapivat were obtained as a gift samples from Shree icon laboratories, Vijayawada, India. Commercially available Mitapivat tablets were purchased from local pharmacy

Determination of Working Wavelength (λmax): The PDA Detector was used to scan the wavelength between 200–400 nm for the highest absorption of the drug solution in the mixture of acetonitrile and 0.1% OPA (80:20) versus acetonitrile and 0.1% OPA (80:20) as a blank. Maximum absorption was demonstrated by the absorption curve at 234 nm. Therefore, the HPLC chromatographic process used 234 nm was the detection wavelength.

Standard Solution Preparation: Weigh and transfer 8 mg of the working standard Mitapivat accurately into a 10 ml dry volumetric flask. Then, add the diluent and sonicate to completely dissolve it. Use the same solvent to bring the volume up to the desired level. (Stock solution) Moreover pipette out 1ml of the above stock solutions into a10ml volumetric flask and diluting to the appropriate mark with diluent (80ppm of Mitapivat).

Sample Solution Preparation: Take 20 commercially available marketed tablets, accurately weighed and further transferred into mortor and pestle. Titurated it to fine powder. And take 50mg equivalent of powder in to a volumetric flask, add diluent and sonicate it up to 30min to dissolve, and centrifuge for 30 min and finally make up to the mark with the same diluent. Then it was filtered through 0.45µ injection filter. Further pipette 0.8 ml of the above stock solutions into a10mlvolumetric flask and dilute up to the mark with diluent. (80ppm of Mitapivat).

The Mitapivat peak was observed at 2.855 min with peak area 3161273, tailing factor 0.90. This trial was optimized.

Preparation of 0.1% OPA Buffer Solution: 1ml of Ortho Phosphoric acid is dissolved in1litre of HPLC grade water and filter through 0.45µ nylon filter.

Preparation of Mobile Phase: The mobile phase was developed by combining 0.1% OPA with 20:80 ACN. A 0.45µ membrane filter was used to filter it out of any impurities that would have affected the final chromatogram.

Chromatographic Condition:

Use Waters Acquity HPLC:

Column: Waters X- Bridge phenyl (150x4.6mm, 3.5µm) Mobile phase ratio: Acetonitrile and 0.1% OPA (80:20)

Detection wavelength: 234nm

Flow rate: 1ml/min

Injection volume: 10µl

Runtime: 5min

Assay Procedure: Measure the area of the Mitapivat peak after injecting 10 uL of the standard into the chromatographic apparatus. Then, use the formulas to get the assay percentage.

Formula for Assay:

%Assay = AT/AS*WS/DS* DT/WT* Average weight /Label claim*P/100*100

Where: AT = average area counts of test (sample) preparation.

AS = average area counts of standard preparation. WS= Weight of working standard taken in mg.

DS = Dilution of working standard in ml.

DT = Dilution of test (sample) in ml.

WT = Weight of test (sample) taken in mg. P = Percentage purity of working standard LC = Label Claim mg/ml.

Method Validation

System Suitability: The theoretical plates for the Mitapivat peak in Standard solution should not be less than 2000. The tailing factor for the peak due to Mitapivat in Standard solution should not be more than 2.0.

Specificity: The ability of an analytical method to quantify an analyte of interest precisely without affect from known and blank contaminants is known as specificity. Blank, standard, sample chromatograms were recorded for this purpose. The fact that the blank chromatogram exhibits no reaction during the drug retention periods indicates that APA response was specific.

Linearity: A series of aliquots were prepared ranging from 20µg/ml to 120µg/ml were prepared using mobile phase from the stock solution. The peak area should be measured after six injections of each concentration into the HPLC apparatus. Graph the relationship between peak area and concentration (on X-axis concentration and on Y-axis Average Peak area) and measure the correlation coefficient.

Range: The interval between the highest and lower levels of analyte (including these levels) that have been proven with precision, accuracy, and linearity is known as the analytical method's range.

Accuracy: For recovery studies 50% 100% 150% solution concentrations were prepared by adding standard solution to pre analysed concentration using standard addition method. The prepared concentrations were then injected into the HPLC system 3 times each and their peak areas and %RSD were calculated.

Precision: The degree of repeatability that an analytical method exhibits under typical operating conditions is known as precision. There are three different types of precision.

1. System precision
2. Method precision
3. Intermediate precision (a. Intra-day precision, b. Inter day precision)

To make sure the analytical system is operating correctly, system precision is verified using a standard chemical substance. The percentage RSD should be calculated in this peak area, where the drug percentage of the six determinations is measured. A homogeneous sample from a single batch should be examined six times for method precision. This shows how a procedure is producing consistent outcomes for a particular batch. This involves six analyses of the sample to determine the percentage RSD. Six solutions of 80 ppm Mitapivat were injected repeatedly to test the instrument's precision.

Robustness: Deliberate changes were made to the Flow rate, Mobile Phase composition, and Temperature Variation as part of the Robustness to assess the effect on the procedure. A range of 0.98 ml/min to 1.02 ml/min was observed for the flow rate. With the use of method flow rate and a variety of flow rates, a standard solution containing 80 ppm of mitapivat was created and examined. It is clear from analyzing the previous information that the approach was greatly impacted by the flow rate change. That means that even with a ±2% deviation from the flow rate, the method remains stable. The change in the ratio of the Organic Phase. Using various kinds of mobile phase ratios, a standard solution containing 80 ppm of mitapivat was produced and analyzed.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The following formula was used to determine the drug performs limit of detection (LOD) and limit of quantification (LOQ) in accordance with international conference harmonization (ICH) standards.

LOD =3.3 × σ / S LOQ = 10 X σ / S

Mitapivat's LOD was determined to be 0.48µg/mL and its LOQ to be 1.6µg/ml.

Degradation Studies:

Acid Degradation: Pipette 1 ml of above solution into a 10ml volumetric flask and1 ml of 1N HCl was added. Then, the volumetric flask was kept at 60ºC for 1 hour in water bath and then neutralized with 1 N NaOH was added and make up to 10ml with diluents.

Alkali Degradation: Pipette 1ml of above solution into a 10ml volumetric flask and add 1ml of 1NNaOH was added. Then, the volumetric flask was kept at 60ºC for1 hour in water bath and then neutralized with 1N HCl and make up to 10ml with diluent.

Peroxide Degradation: Pipette 1 ml above stock solution was added to a 10 ml volumetric flask, 1 ml of 3 percent w/v hydrogen peroxide was added to the flask and the volume was built up to the mark using diluent. The volumetric flask was then maintained at 60^{o}C for 1 hour in water bath. After that, the volumetric flask was left at room temperature for 15 minutes.

Reduction Degradation: Pipette 1 ml above stock solution was added to a10 ml volumetric flask, 1 ml of 10 percent w/v Sodium bi sulphate was added to the flask and the volume was built up to the mark using diluent. The volumetric flask was then maintained at 60^{o}C for 1 hour in water bath. After that, the volumetric flask was left at room temperature for 15 minutes.

Hydrolysis Degradation: Pipette 1ml of above-stock solution was added to a 10ml volumetric flask, 1ml of HPLC grade water was added to a flask and the volume was built up to the required volume with diluent. The volumetric flask was then maintained at 60^{o}C for1 hour in water bath. After that, the volumetric flask was left at room temperature for 15 minutes.

Photolytic Degradation: Mitapivat sample and control wrapped with aluminum foil was placed in photostability chamber for 3 hours. Subsequently, the material was extracted, diluted using diluents, and introduced into an HPLC for analysis.

Thermal Induced Degradation: A mitapivat sample was collected in a petridish and heated to 105°C in a hot air oven for three hours. Subsequently, the material was extracted, diluted using diluents, and introduced into an HPLC for analysis. A 0.22u syringe was used to filter the fluid through the strained samples.

***In-silico* screening of Forced Degradation Products of Mitapivat [2]:**

Software used in *In-silico* Study of Mitapivat:

1. SwissADME
2. Protox2
3. pKcsm

All the degradation products obtained from the degradation of Mitapivat were screened for toxicity and ADME properties using Swiss ADME, Protox 2, pKcsm[2].

RESULTS AND DISCUSSION:

Collision-Induced Dissociation of Mitapivat:

Mitapivat: Fig 16(B) shows the fragmentation mechanism of Mitapivat and the ESI spectrum showed the most intense [M+H]+ ion of m/z-450.1726. The MS/MS spectrum of Mitapivat displayed abundant product ions at m/z-355.0991 (loss of C6H13N from m/z-450.1726), m/z-228.0569 (loss of C9H7N from m/z 355.0991), m/z-149.0841 (loss of H3NO2S from m/z 228.0569) and m/z-54.0470 (loss of C5H9NO from m/z 149.0841). The MS/MS experiments combined with accurate mass measurements have confirmed the proposed

DP1: The fragmentation mechanism of DP1 has been shown in **Fig. 17(B),** and the most intense [M+H]+ ion of m/z-486.1492 was seen in the ESI spectrum under conditions of acid degradation. Abundant product ions were observed in the DP1 MS/MS spectra at m/z-348.0335 (loss of C8H16N2 from m/z-486.1492), m/z-193.0197 (loss of C7H5ClNO- from m/z 348.0335) and m/z-129.0578 (loss of SO2 from m/z 193.0197). The suggested scheme has been validated by the MS/MS tests in conjunction with precise mass measurements.

DP2: The fragmentation mechanism of DP2 has been shown in **Fig. 18(B)**, and the most intense [M+H]+ ion of m/z-472.1545 was seen in the ESI spectrum under conditions of alkali degradation. Abundant product ions were observed in the DP2 MS/MS spectra at m/z-334.0388 (loss of C8H16N2 from m/z-472.1545), m/z-193.0197 (loss of C7H6NNaO from m/z 334.0388) and m/z-129.0578 (loss of SO2 from m/z 193.0197). The suggested scheme has been validated by the MS/MS tests in conjunction with precise mass measurements.

DP3: The fragmentation mechanism of DP3 has been shown in **Fig. 19(B)** and the most intense [M+H]+ ion of m/z-466.1675 was seen in the ESI spectrum under conditions of peroxide degradation. A lot of product ions were seen in the DP3 MS/MS spectra at m/z-328.0518 (where C8H16N2 was lost from m/z-466.1675), m/z-193.0197 (where C7H7NO2 was lost from m/z 328.0518), and m/z-129.0578 (where SO2 was lost from m/z 193.0197). The suggested scheme has been validated by the MS/MS tests in conjunction with precise mass measurements.

DP4: The fragmentation mechanism of DP4 has been shown in **Fig. 20(B)** and the most intense [M+H]+ ion of m/z-400.2263 was seen in the ESI spectrum under conditions of thermal deterioration. A lot of product ions were seen in the DP4 MS/MS spectra at m/z-262.1106 (where C8H16N2 was lost from m/z-400.2263), m/z-135.0684 (where C9H7N was lost from m/z 262.1106), and m/z-56.0262 (where C5H9N was lost from m/z 135.0684). The suggested scheme has been validated by the MS/MS tests in conjunction with precise mass measurements.

DP5: The fragmentation mechanism of DP5 has been shown in **Fig. 21(B)**, and the most intense [M+H]+ ion of m/z-466.1675 was seen in the ESI spectrum under conditions of hydrolysis degradation.

A lot of product ions were seen in the DP5 MS/MS spectra at m/z-328.0518 (where C8H16N2 was lost from m/z-466.1675), m/z-193.0197 (where C7H7NO2 was lost from m/z 328.0518) and m/z-129.0578 (where SO2 was lost from m/z 193.0197). The suggested scheme has been validated by the MS/MS tests in conjunction with precise mass measurements.

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Parameters	Observation
Instrument used	WatersAlliancee-2695HPLC
Injection volume	10µl
Mobile Phase	ACN and 0.1%OPA(80:20v/v)
Column	Waters X-Bridge Phenyl (150x4.6mm,3.5µm)
Detection Wave Length	234nm
Flow Rate	1 mL/min
Runtime	5min
Temperature	Ambient (25□C)
Mode of separation	Isocraticmode

TABLE 2: SYSTEM SUITABILITY PARAMETERS FOR MITAPIVAT

S. no.	Parameter	Mitapivat
1	Retention time	2.855
2	Plate count	11958
3	Tailing factor	0.90
4	Resolution	----
5	%RSD	0.21

TABLE 3: ASSAY OF MITAPIVAT

Brand	Labelled amount (mg)	Estimated amount (mg)	%purity
Pyrukynd	50	49.75	99.5

TABLE 4: RESULTS OF LINEARITY FOR MITAPIVAT

S. no.	Mitapivat	
	Conc.(µg/ml)	Avg Peak area(n=6)
1	20.00	842684
2	40.00	1569102
3	60.00	2334947
4	80.00	3158539
5	100.00	3934065
6	120.00	4784103
Regression equation	y=39508.05x+4294.14	
Slope	39508.05	
Intercept	4294.14	
R^2	0.99982	

TABLE 5: SYSTEM PRECISION TABLE OF MITAPIVAT

S. no.	Concentration Mitapivat (µg/ml)	Area of Mitapivat
1.	80	3161273
2.	80	3149174
3.	80	3165906
4.	80	3155689
5.	80	3163622
6.	80	3166453
Mean		3160353
S.D		6727.58
%RSD		0.213

TABLE 6: METHOD PRECISION FOR MITAPIVAT

Conc. (µg/ml)	Area for Mitapivat
80	3145206
80	3178521
80	3180740
80	3162963
80	3140687
80	3158842
Average	3161160
Standard Deviation	16535.287
%RSD	0.52

TABLE 7: INTERMEDIATE PRECISION (DAY VARIATION) FOR MITAPIVAT

Days	Injection	Area for Mitapivat
Day 1	1	3156965
	2	3135410
	3	3150137
Day 2	4	3170179
	5	3198746
	6	3138749
Day 3	7	3135411
	8	3170179
	9	3198746
	Average	3158364
	Standard Deviation	23458.795
	%RSD	0.74

TABLE 8: ACCURACY RESULTS OF MITAPIVAT

Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	%Recovery	Mean %Recovery
50%	1586487	4.0	4.02	100.5	100.3
	1574096	4.0	3.98	99.5	
	1593432	4.0	4.03	100.8	
100%	3165234	8.0	8.01	100.1	99.6
	3130265	8.0	7.92	99.0	
	3148796	8.0	7.97	99.6	
150%	4722130	12.0	11.95	99.6	99.7
	4698521	12.0	11.89	99.1	
	4758103	12.0	12.04	100.3	

TABLE 9: ROBUSTNESS RESULTS OF MITAPIVAT

Parameter	Mitapivat				
	Condition	Retention time (min)	Peak area	Tailing	Plate count
Flow rate Change (mL/min)	Less flow (0.98ml)	2.892	3111575	0.98	12053
	Actual flow (1ml)	2.855	3161273	0.90	11958
	More flow (1.02ml)	2.785	3170798	0.86	11871
Organic Phase change	Less Org (78.4:21.6)	2.910	3127258	0.95	12097
	Actual (80:20)	2.847	3149174	0.92	11962
	More Org (81.6:18.4)	2.736	3196341	0.83	11824

TABLE 10: SENSITIVITY PARAMETERS (LOD & LOQ)

Name of drug	LOD (µg/ml)	s/n	LOQ (µg/ml)	S/n
Mitapivat	0.48	3	1.6	10

TABLE 11: FORCED DEGRADATION RESULTS OF MITAPIVAT

Stress condition	% assay after degradation	Purity Angle	Purity Threshold	%Degradation
Control	100	7.395	11.028	0
Acid	87.9	7.387	11.022	12.1
Alkali	89.3	7.380	11.035	10.7
Peroxide	85.6	7.372	11.036	14.4
Reduction	98.0	7.345	11.058	2.0
Thermal	87.3	7.303	11.052	12.7
Photolytic	96.1	7.362	11.019	3.9
Hydrolysis	89.0	7.374	11.023	11.0

TABLE 12: LC-MS/MS DATA OF MITPIVAT AND ITS DEGRADATION PRODUCTS AND SOME MAJOR FRAGMENTS

	Molecular Formula	Calculated Mass	Observed Mass	Error	Major Fragment Ions
Mitapivat	$C_{24}H_{26}N_4O_3S$	450.1726	450.1734	1.777096	55.0475,150.0849, 229.0575 and356.0997
DP1	$C_{24}H_{27}ClN_4O_3S$	486.1492	486.1501	1.851284	130.0585,194.0208 and 349.0347

DP2	$C_{24}H_{25}N_4NaO_3S$	472.1545	472.1553	1.694361	130.0586,194.0205 and 335.0397
DP3	$C_{24}H_{26}N_4O_4S$	466.1675	466.1683	1.716121	130.0581,194.0208 and 329.0524
DP4	$C_{25}H_{28}N_4O$	400.2263	400.2270	1.749010	57.0272,136.0691 and 263.1111
DP5	$C_{24}H_{26}N_4O_4S$	466.1675	466.1682	1.501606	130.0582,194.0205 and 329.0526

TABLE 13: ADME PREDICTION (SWISS ADME)

Parameters	Mitapivat	DP-1	DP-2	DP-3	DP-4	DP-5
Solubility	Soluble	Moderate	Moderate	Moderate	Moderate	Moderate
GI absorption	High	High	High	High	High	High
BBB Permeation	No	No	No	No	Yes	No
Skin permeation	-7.79	-7.08cm/s	-7.12cm/s	-7.43cm/s	-6.13cm/s	-7.06cm/s
P-gpsubstrate	Yes	Yes	Yes	Yes	Yes	Yes
CYP1A2inhibitor	No	No	No	NO	Yes	No
CYP2C19inhibitor	Yes	Yes	Yes	No	No	Yes
CYP2C9 inhibitor	Yes	No	Yes	Yes	No	No
CYP2D6 inhibitor	Yes	Yes	Yes	No	Yes	Yes
CYP3A4 inhibitor	Yes	No	Yes	Yes	Yes	Yes

TABLE 14: PROTOX2 PREDICTION

Parameters	Mitapivat	DP-1	DP-2	DP-3	DP-4	DP-5
Predicted LD50	3160mg/ kg	3160mg/ kg	3160mg/kg	3160mg/kg	1000mg/kg	3160mg/kg
Toxicity class	4	5	5	5	4	5
Toxicity	-	-	Immunotoxicity	Immunotoxicity	Immunotoxicity	Immunotoxicity
Probability	-	-	0.94	0.98	0.81	0.92

TABLE 15: PKCSM PREDICTION

Fraction unbound, BBB permeability	-1.112	-0.366	0.831	-0.698	0.336	-0.95
CNS permeability	-2.541	-3.383	-2.692	-2.095	-1676	-2.57
METABOLISM	No Yes	No Yes No	No Yes No	No Yes No	Yes Yes Yes	No Yes No
CYP2D6 substrate, CYP3A4 substrate, CYP2D6 inhibitor CYP3A4 inhibitor	No Yes	No	No	Yes	Yes	Yes
EXCRETION Total clearance	0.599	0.88	1.864	0.487	1.131	0.532
RenalOCT2 inhibitor	No	Yes	Yes	Yes	Yes	Yes
TOXICITI	No 0.232	No	No 0.154	No	No	No 0.064
AMES		-0.328		-0.123	-0.108	
Max tolerated dose(human)	No Yes	No Yes	No Yes 2.936	No Yes 2.798	No Yes 2.880	No Yes
hERG1inhibitor, hERG2 inhibitor	2.665	2.746				2.505
Oralratacute toxicity (LD 50) Oral rat			1.689	1.818	2.004	
chronic toxicity	0.945	2.494				2.04
Hepatotoxicity	Yes	Yes	Yes	Yes	Yes	Yes

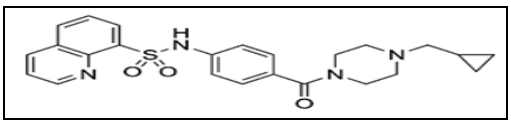

FIG. 1: MOLECULAR STRUCTURE OF MITAPIVAT

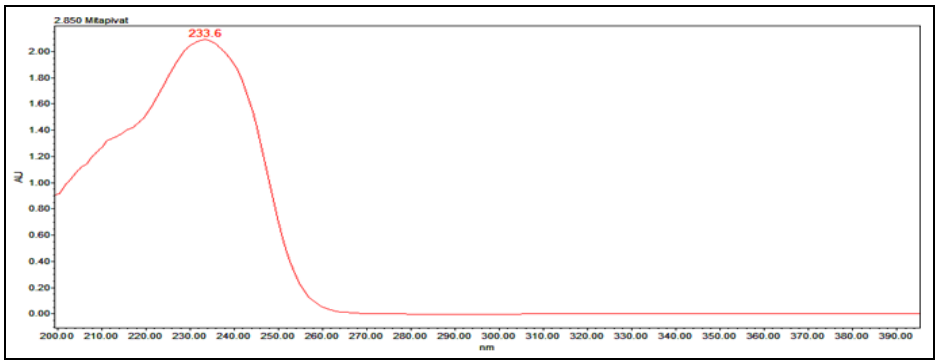

FIG. 2: PDA- SPECTRUM OFMITAPIVAT

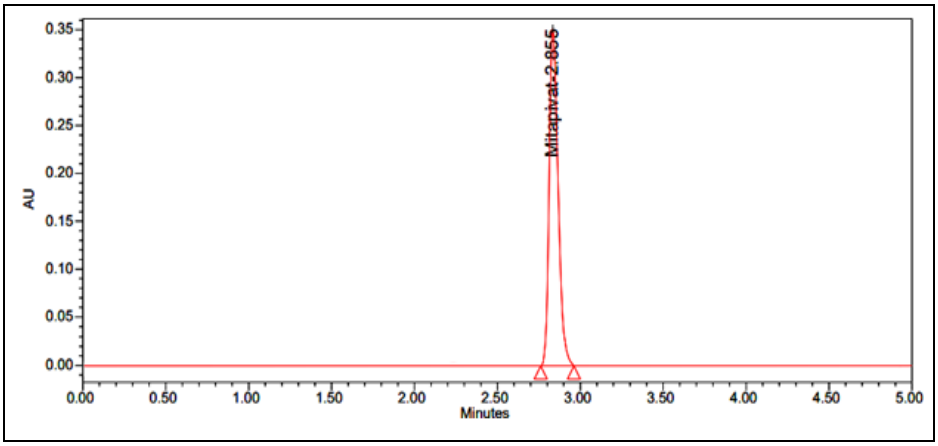

FIG. 3: OPTIMIZED CHROMATOGRAM

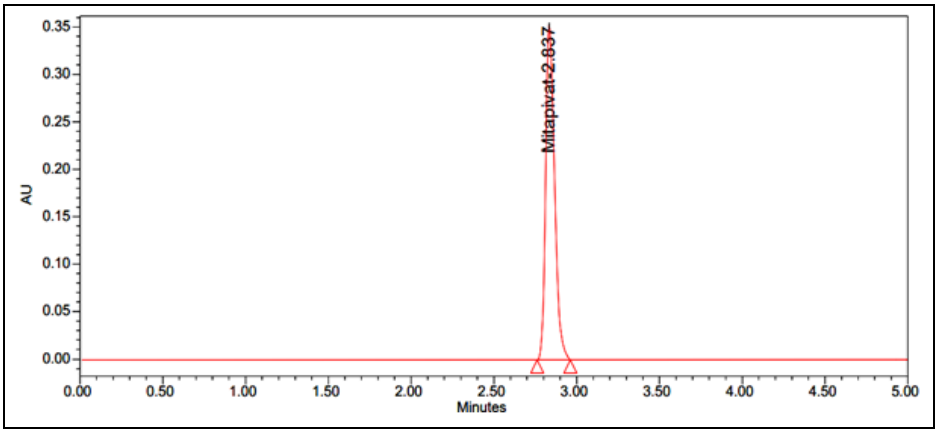

FIG. 4: CHROMATOGRAM OF FORMULATION

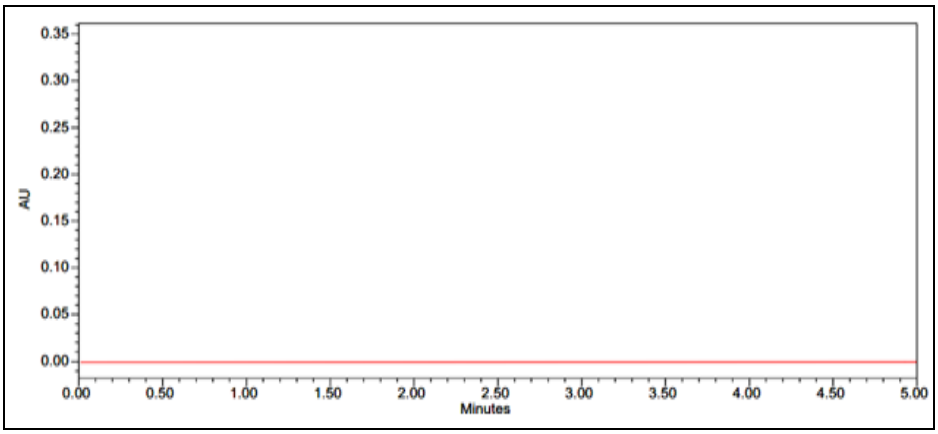

FIG. 5: CHROMATOGRAM OF BLANK

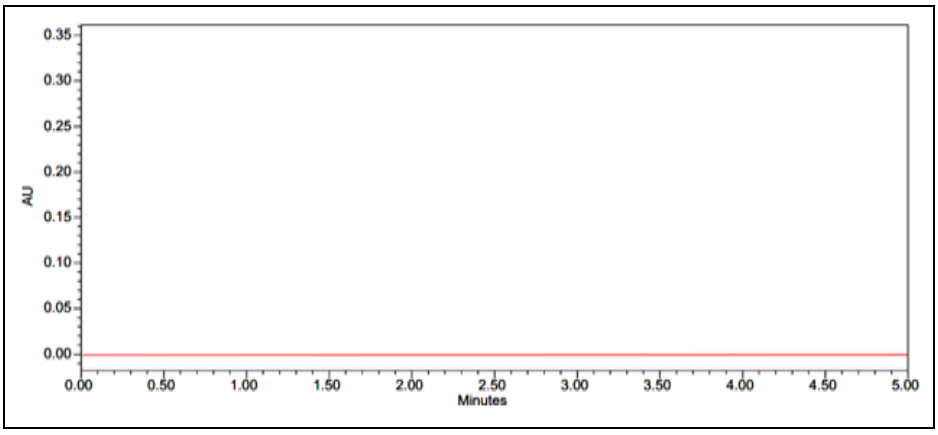

FIG. 6: CHROMATOGRAM OF PLACEBO

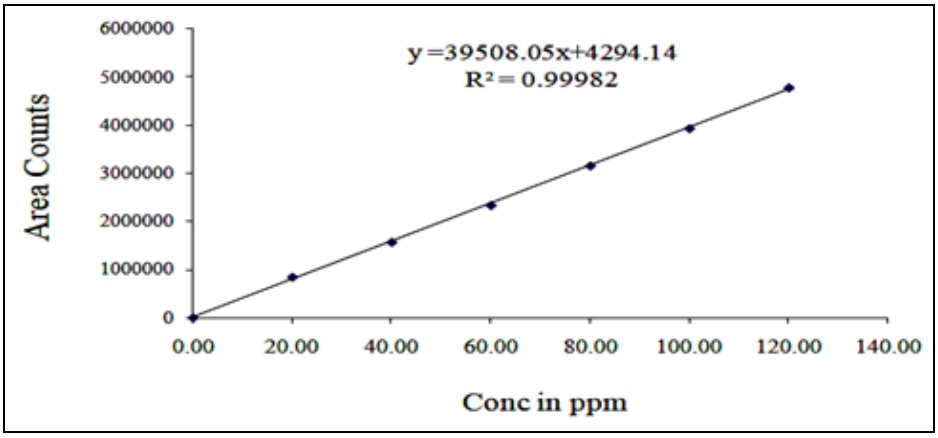

FIG. 7: CALIBRATION CURVE FOR MITAPIVAT AT 259NM

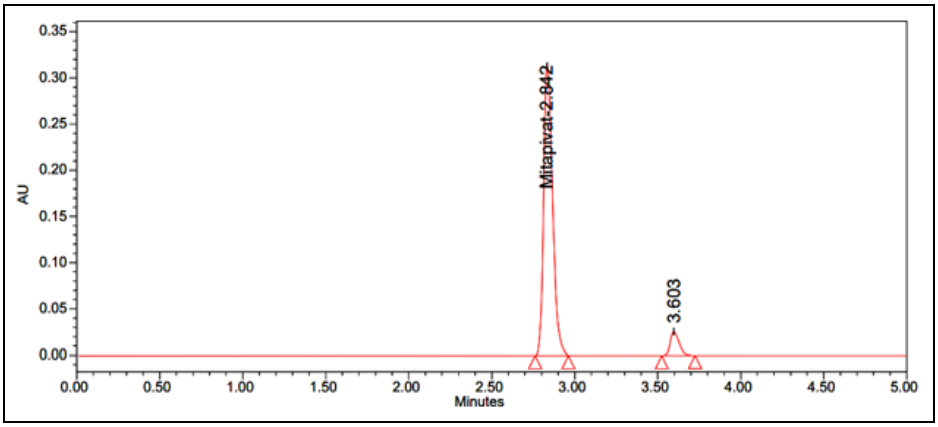

FIG. 8(A): CHROMATOGRAM OF ACID DEGRADATION

Degradation Studies:

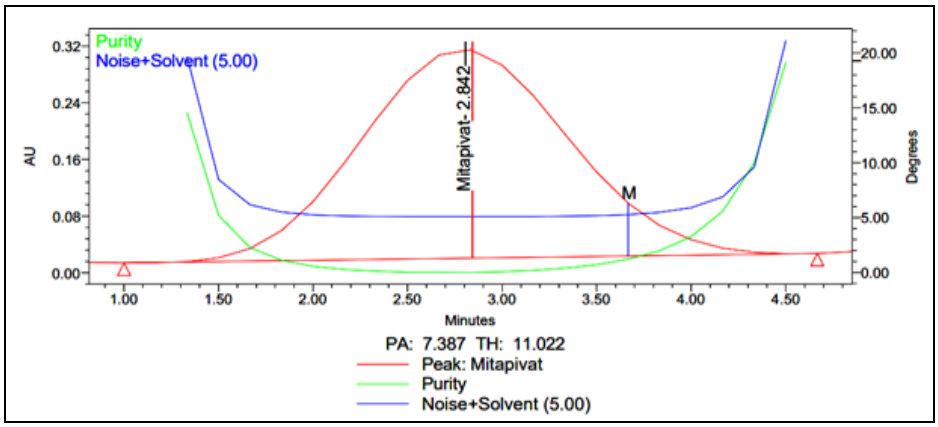

FIG. 8(B): PURITY PLOT OF ACID DEGRADATION

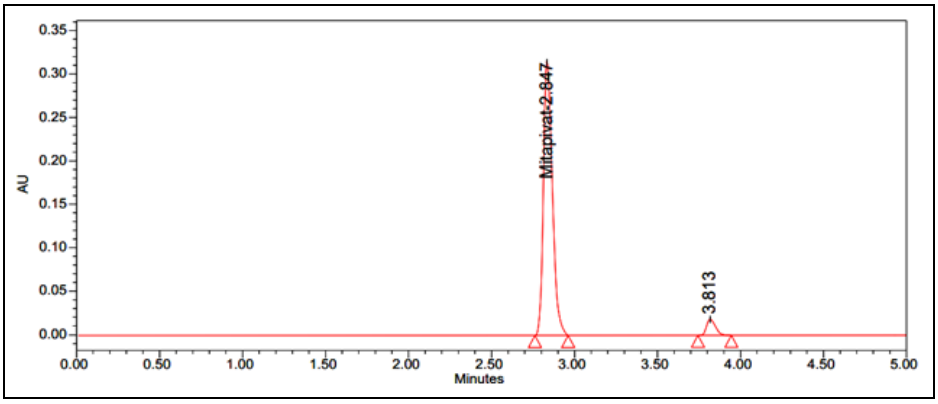

FIG. 9 (A): CHROMATOGRAM OF ALKALI DEGRADATION

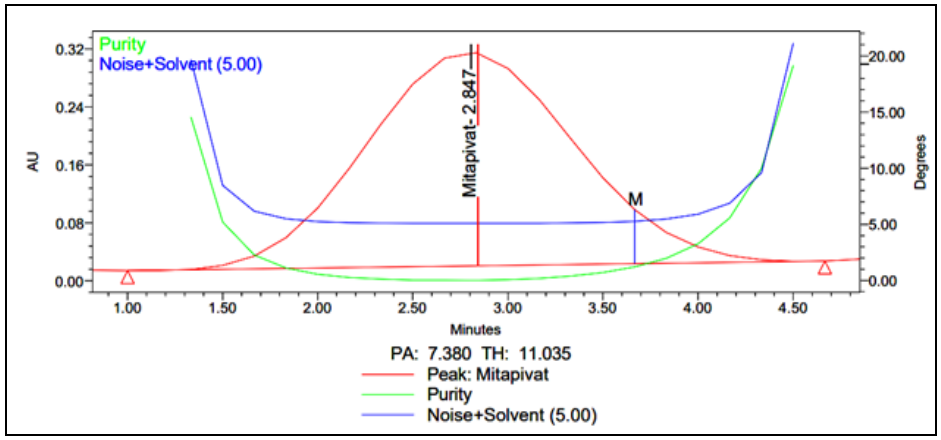

FIG. 9(B): PURITY PLOT OF ALKALI DEGRADATION

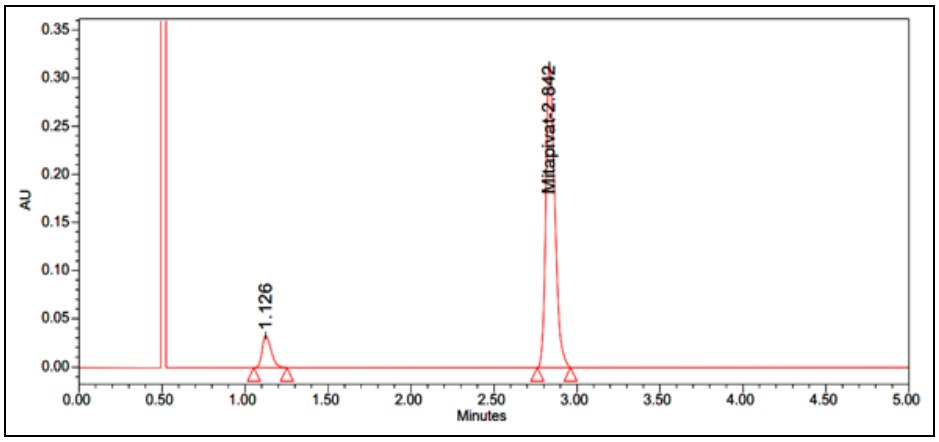

FIG. 10(A): CHROMATOGRAM OF PEROXIDE DEGRADATION

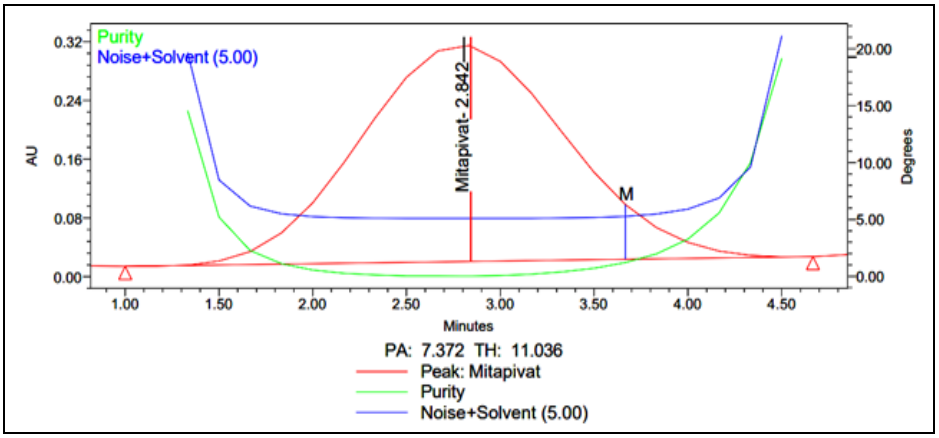

FIG. 10(B): PURITY PLOT OF PEROXIDE DEGRADATION

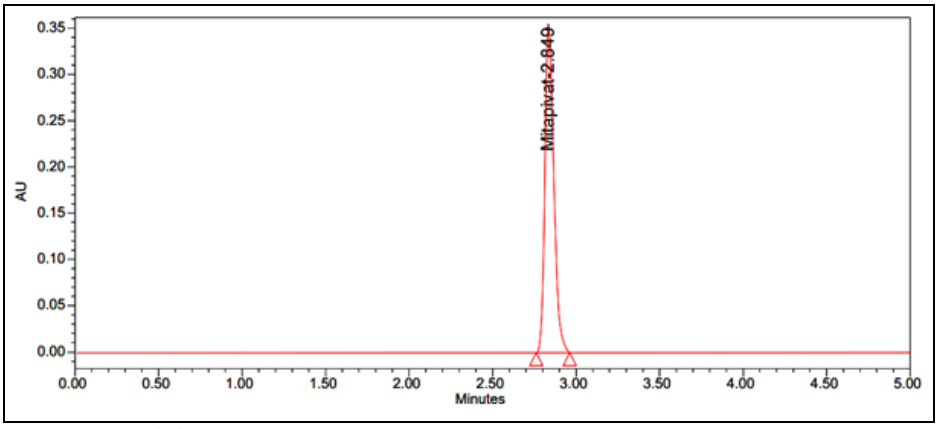

FIG. 11(A): CHROMATOGRAM OF REDUCTION DEGRADATION

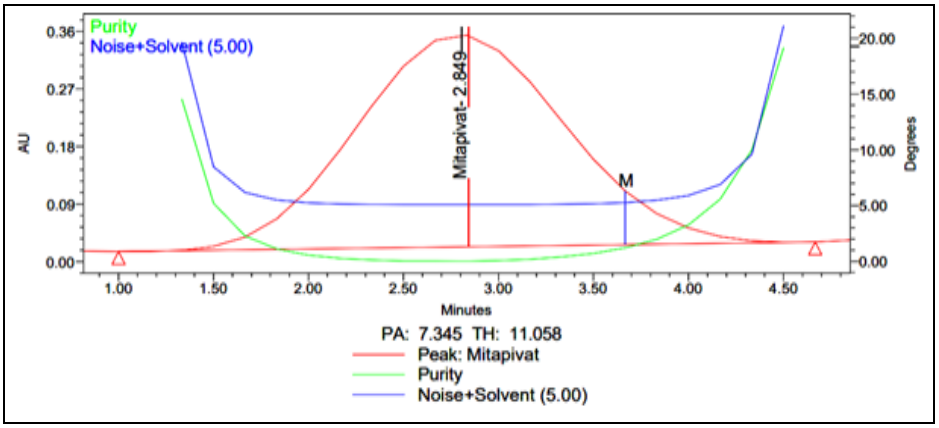

FIG. 11(B): PURITY PLOT OF REDUCTION DEGRADATION

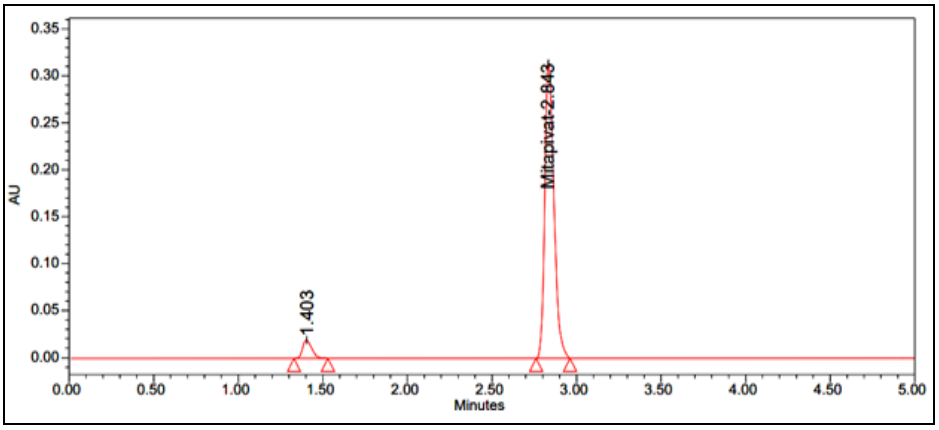

FIG. 12(A): CHROMATOGRAM OF HYDROLYSIS DEGRADATION

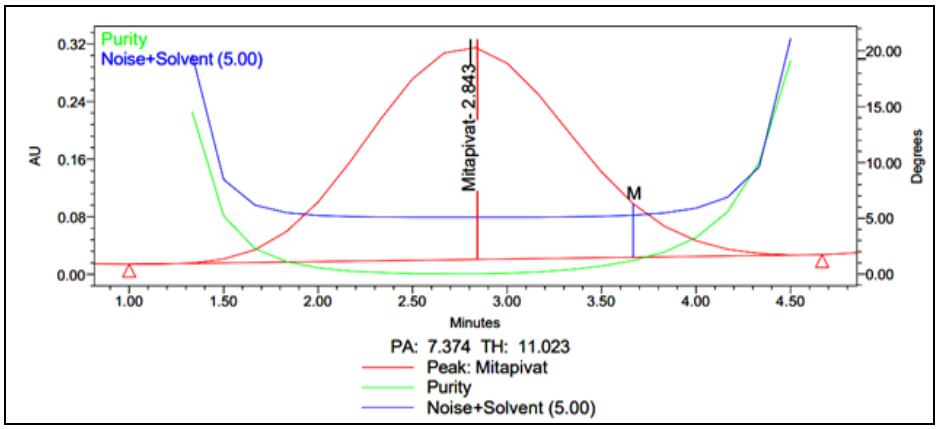

FIG. 12(B): PURITY PLOT OF HYDROLYSIS DEGRADATION

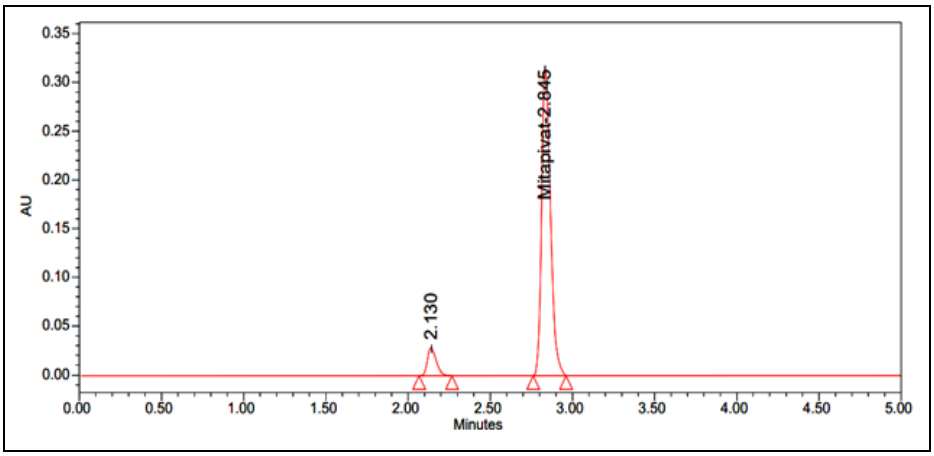

FIG. 13(A): CHROMATOGRAM OF THERMAL DEGRADATION

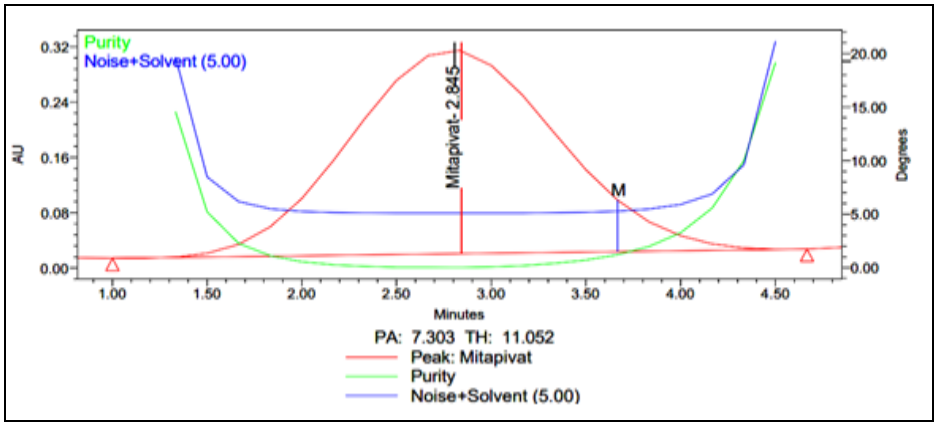

FIG. 13(B): PURITY PLOT OF THERMAL DEGRADATION

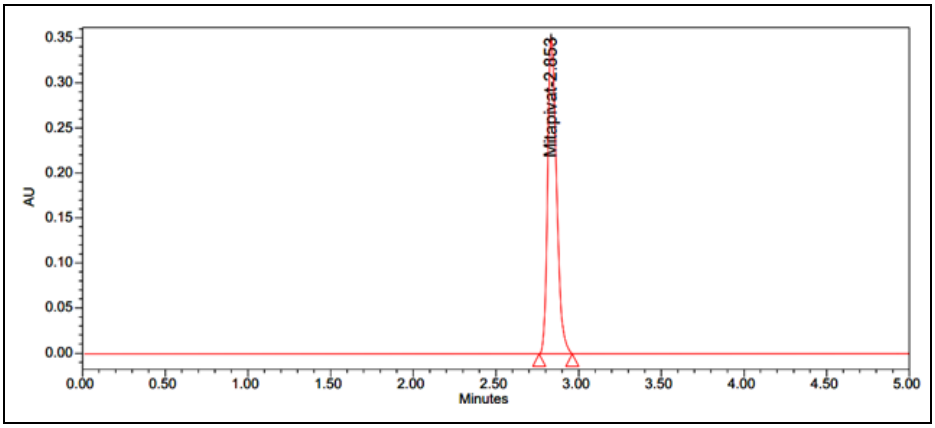

FIG. 14(A): CHROMATOGRAM OF PHOTOLYTIC DEGRADATION

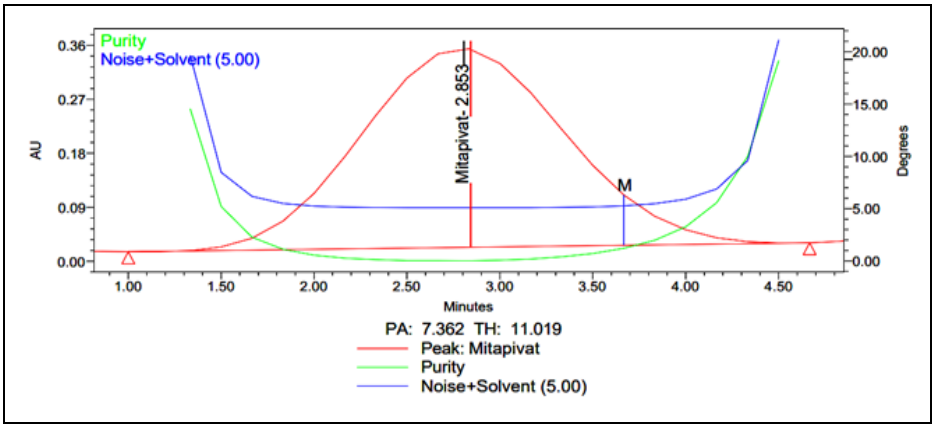

FIG. 14 (B): PURITY PLOT OF PHOTOLYTIC DEGRADATION

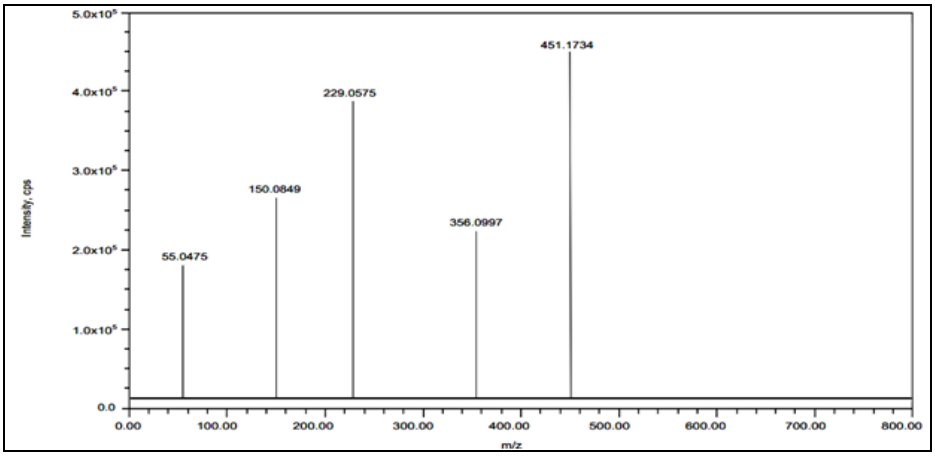

FIG. 15(A): MASS SPECTRA OF MITAPIVAT

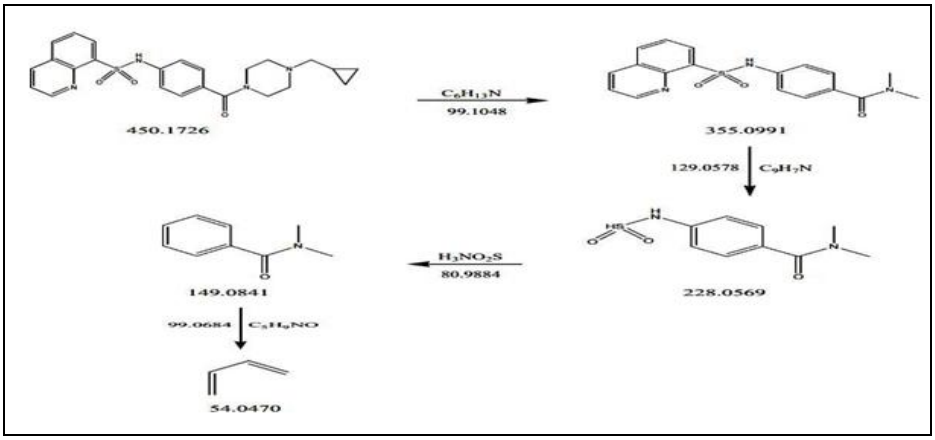

FIG. 15(B): FRAGMENTATION PATHWAY OF MITAPIVAT

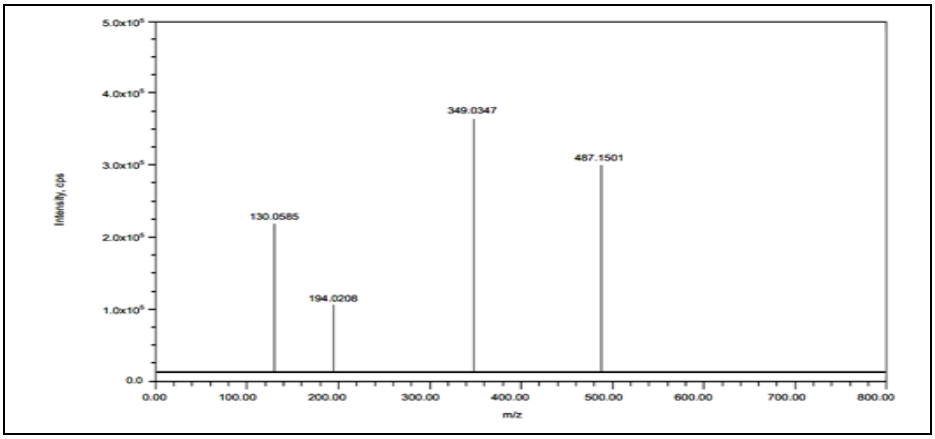

FIG. 16 (A): MASS SPECTRA OF ACID IMPURITY (DP-1)

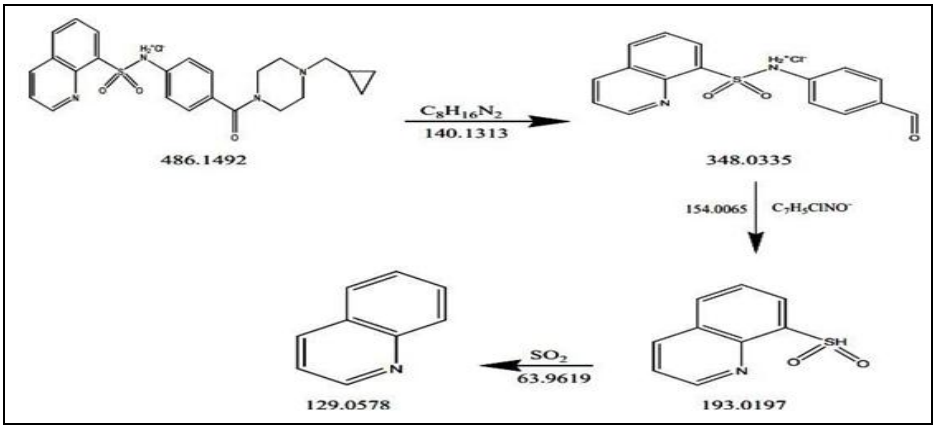

FIG. 16(B): FRAGMENTATION PATH WAY OF ACID IMPURITY (DP-1)

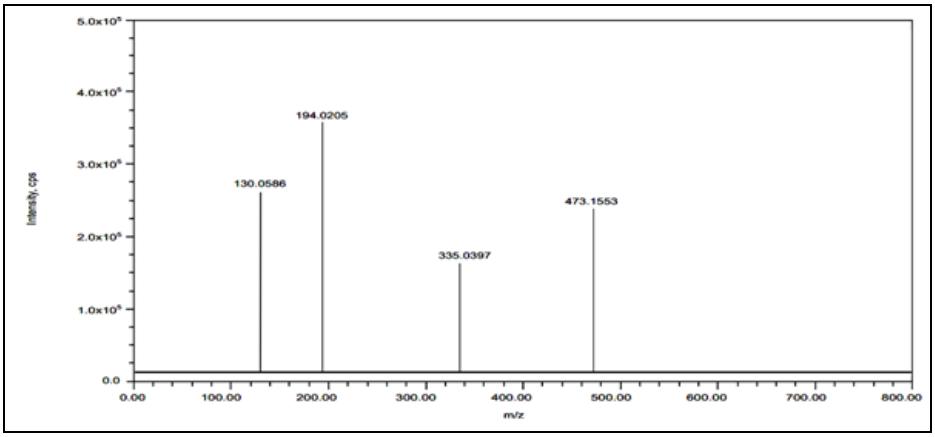

FIG. 17 (A): MASS SPECTRA OF ALKALI IMPURITY (DP-2)

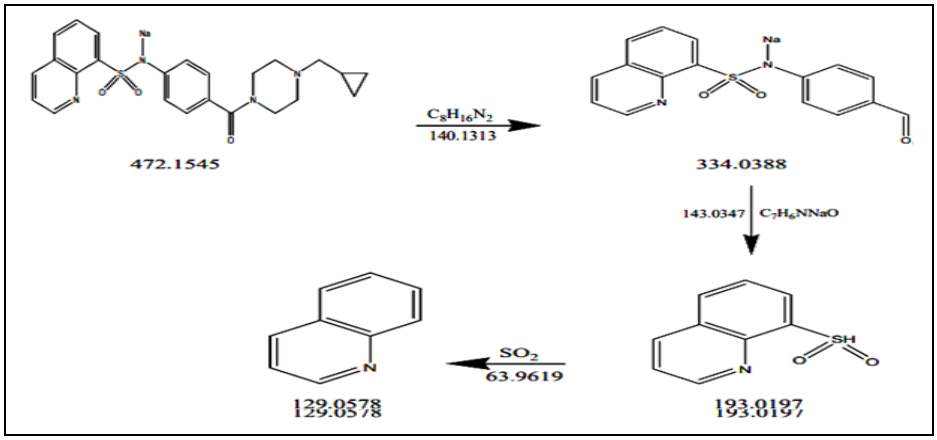

FIG. 17(B): FRAGMENTATION PATHWAY OF ALKALI IMPURITY (DP-2)

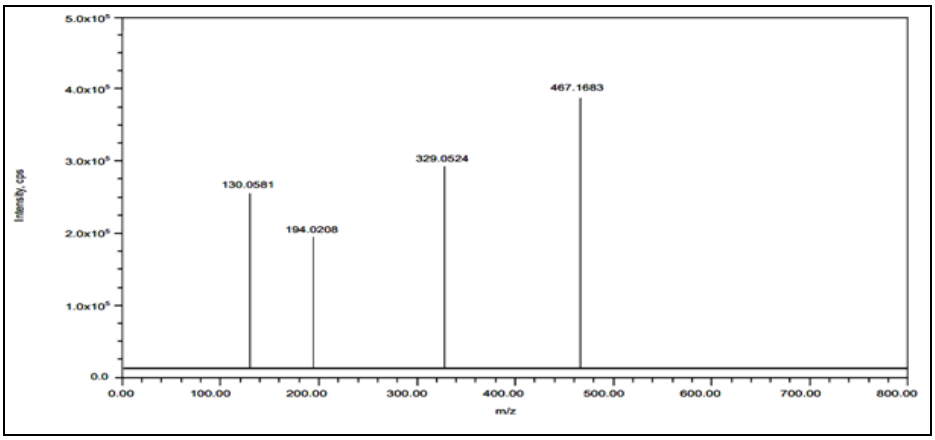

FIG. 18 (A): MASS SPECTRA OF PEROXIDE IMPURITY (DP-3)

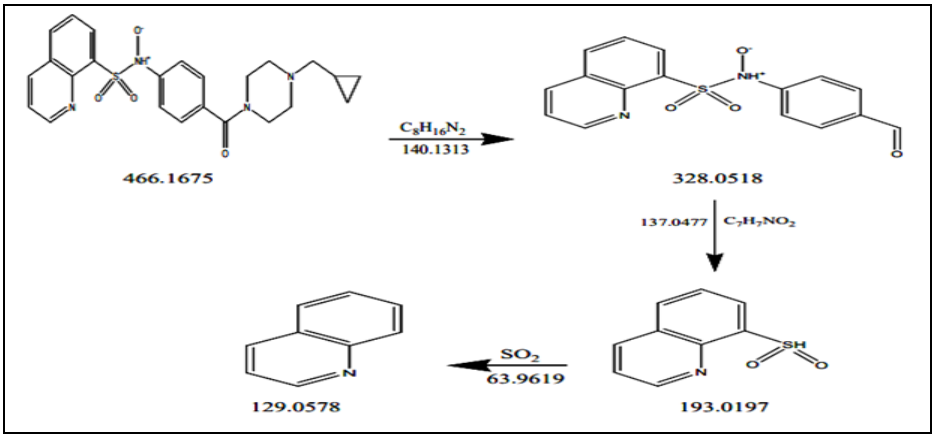

FIG. 18(B): FRAGMENTATION PATHWAY OF PEROXIDE IMPURITY (DP-3)

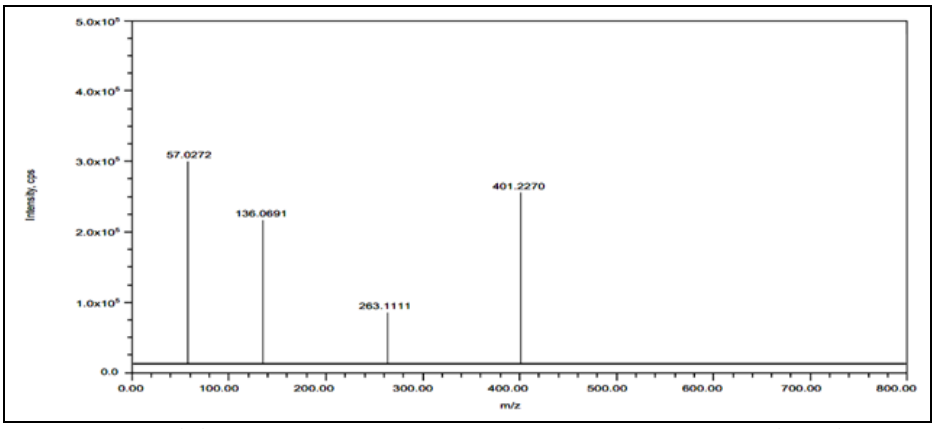

FIG. 19(A): MASS SPECTRA OF THERMAL IMPURITY (DP-4)

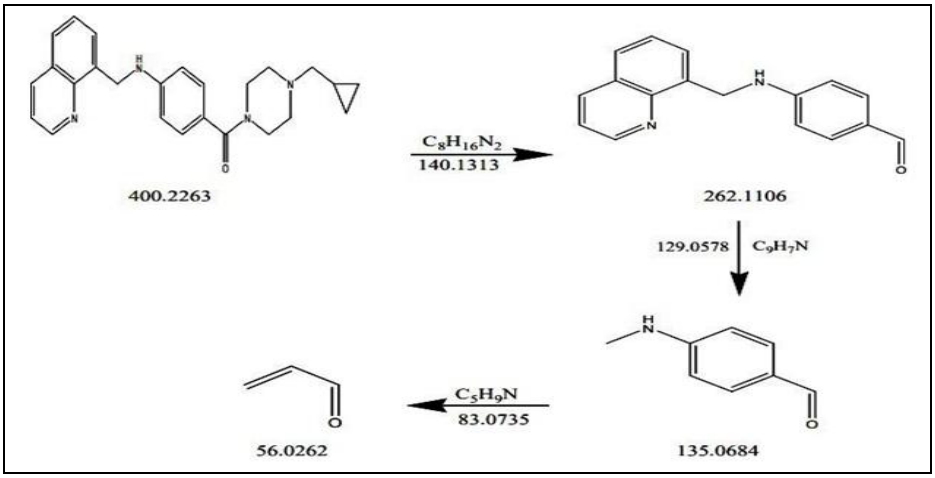

FIG. 19(B): FRAGMENTATION PATHWAY OF THERMAL IMPURITY (DP-4)

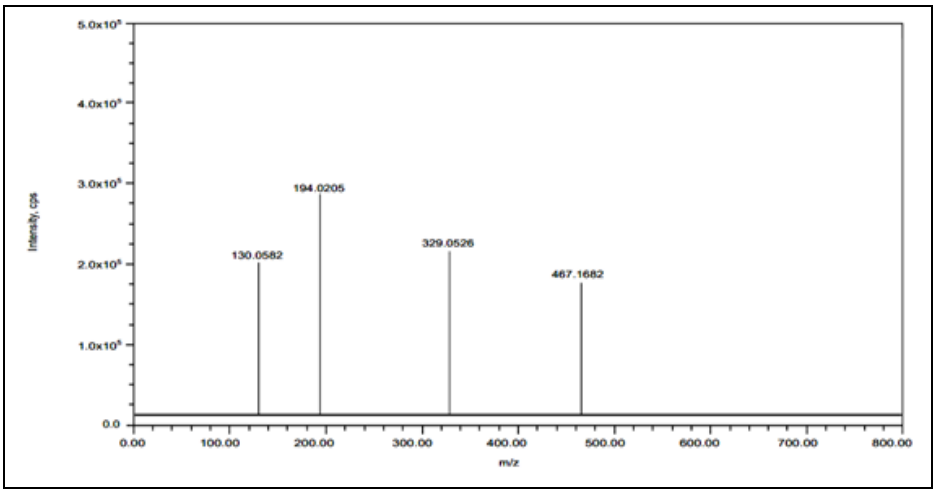

FIG. 20(A): MASS SPECTRA OF HYDROLYSIS IMPURITY (DP-5)

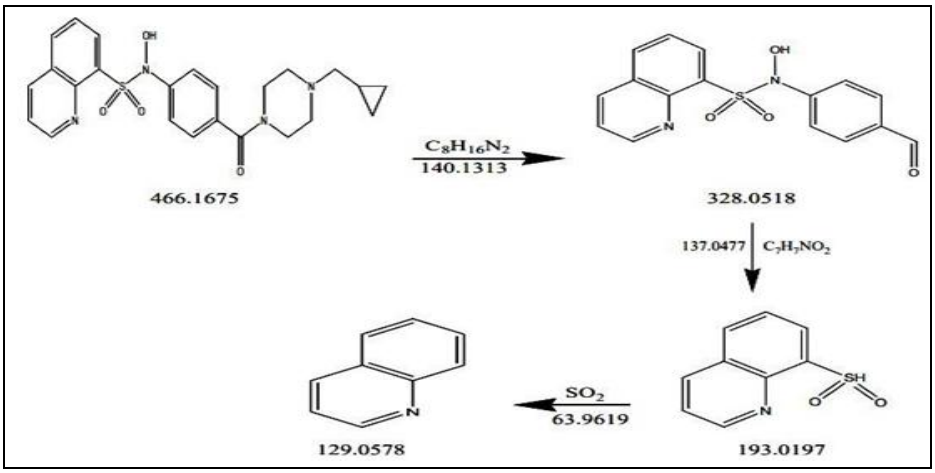

FIG. 20(B): FRAGMENTATION PATH WAY OF HYDROLYSIS IMPURITY (DP-5)

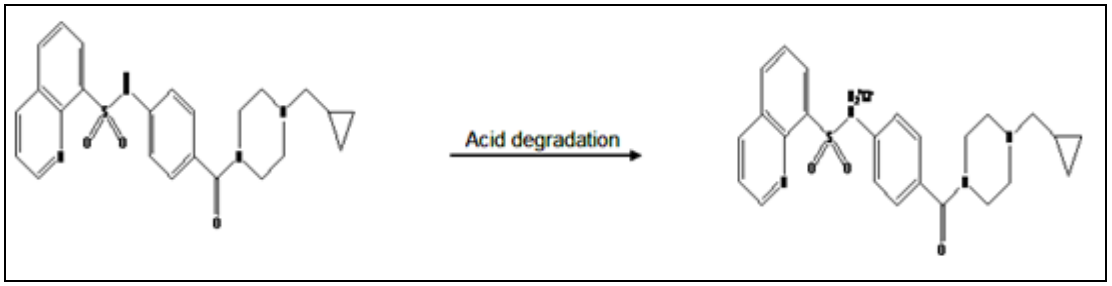

FIG. 21: REACTION OF MITAPIVAT WITH HCL

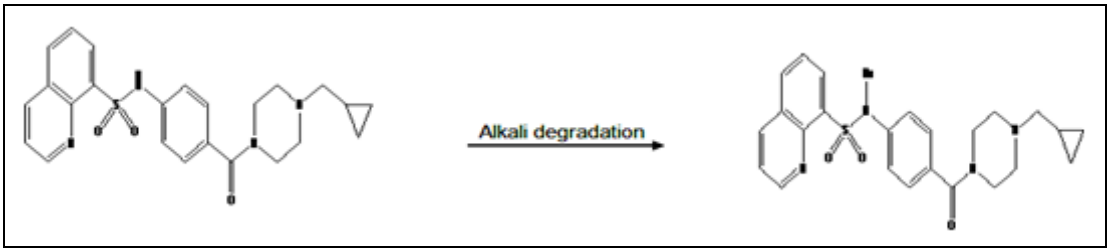

FIG. 22: REACTION OF MITAPIVAT WITH NAOH

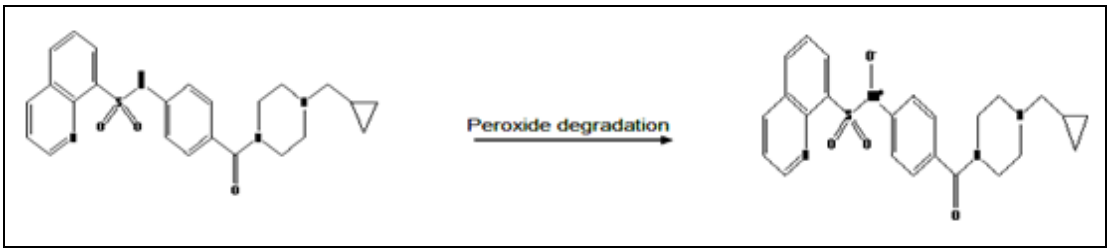

FIG. 23: REACTION OF MITAPIVAT WITH H2O2

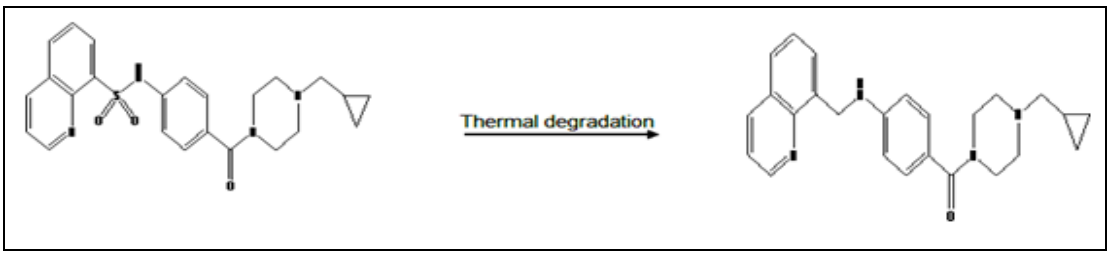

FIG. 24: REACTION OF MITAPIVAT ON HEATING

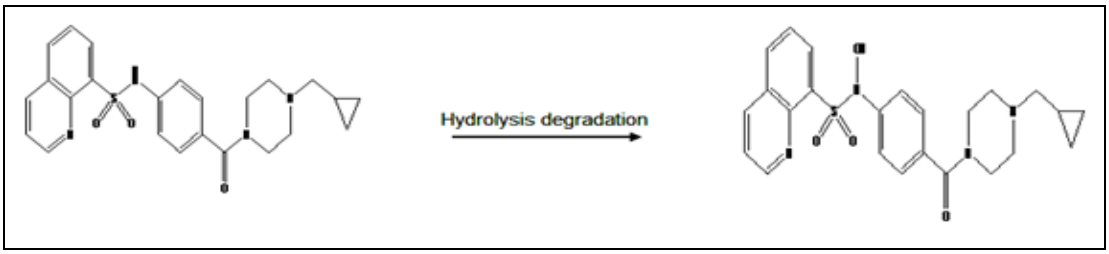

FIG. 25: REACTION OF MITAPIVAT WITH WATER

CONCLUSION: The current study found that the stability indicating assay method combining RP-HPLC and FD Characterization with LC-MS was simple, reliable and non-interfering with degradation products or placebo. Thus, this can be applied to regular Mitapivat analysis.

Further investigation of degradation products was done by using *in-silico* techniques. All the degradation products obtained from the degradation of Mitapivat were screened for toxicity and ADME properties using Swiss ADME, pkCSM web server, protox 2. The SWISS ADME web server and pkCSM web server were used for the prediction of pharmacokinetic properties and toxicity of the drug and its degradation products. The intestinal absorption of the drug was 94.859% where as the degradation products DP-1, DP-3, DP-5 shows drastic decrease in the absorption. Only DP-4 shows the property of BBB permeation. Along with the drug, Mitapivat DP-2, DP-3, DP-4, DP-5 are CYP 3A4 inhibitors while DP-1 does not show CYP 3A4 inhibition. The predicted LD-50 of DP-4 is very much less than the drug and the degradation products which shows that the margin of safety of DP-4 is very less. The drug and theDP-1 doesn't show any toxicity while DP-2, DP-3, DP-4, DP-5 shows immune toxicity. The volume of distribution is more for DP-4 while DP-2 has low volume of distribution.

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