5.1. SELECTION OF DRUG

The model drug selected for this study was Pomalidomide. It is a potential antineoplastic agent along with immunomodulatory action. It was approved by USFDA as orphan drug on 8th February 2013 for multiple myeloma for patients who fail to respond to previous therapies. It was also approved by European medicines agency on 31st May 2013 as Committee for medicinal products for human use recommended its use for a rare and incurable cancer of bone marrow which is multiple myeloma. It is a thalidomide derivative. Thus, it also engraves the life threatening congenital and birth defects of thalidomide if given to pregnant women. For maintaining the risk-benefit balance, its administration is limited to men and for women, except those having child bearing potential. For estimation of Pomalidomide in bulk as well as dosage form, many analytical methods are reported in literature. Despite of the fact, no DOE based stability indicating HPLC method along with degradation kinetics study and total error approach has been reported till date as per our knowledge.

5.2 DRUG PROFILE

Chemical name: Pomalidomide [1]

IUPAC Name: 4-amino-2-(2, 6-dioxopiperidin-3-yl) isoindoline-1,3-dione

Molecular Formula: $C_{13}H_{11}N_3O_4$

Log P value: 0.2

pKa: pKa1 value of 1.56 (aromatic amine) and pKa2 value of 11.59 (secondary amine)

Molecular Weight: 273.25 g/mol

Chemical Structure:

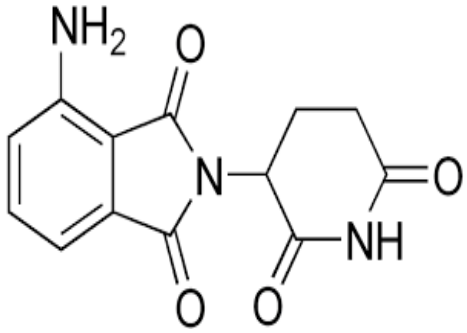


Figure 5.1: Structure of Pomalidomide

Appearance: Yellow fine crystalline solid powder

Melting point: 272-274°C

Solubility: Freely soluble in water, acetone, DMSO

Drug Category: Antineoplastic.

Mechanism of action: Pomalidomide appears to inhibit TNF-alpha production, enhance the activity of T cells and natural killer (NK) cells and enhance antibody-dependent cellular cytotoxicity (ADCC)

Uses: Immunomodulating, antiangiogenic and antineoplastic activities.

Marketed Formulation:
Pomalyst capsules containing 4 mg of Pomalidomide (Calgene corporatio)
Pomalid capsules containing 4 mg of Pomalidomide (Natco Pharma)
Pomalex capsules containing 4 mg of Pomalidomide (Sun pharma)

5.3 LITERATURE REVIEW

Various methods have been reported in literature such as RP-HPLC method for estimation of Pomalidomide in capsule and tablet dosage form [4, 6]. Bioanalytical HPLC method with Fluorescence detection as well as UPLC/MS/MS method for quantitation of Pomalidomide in human plasma was also found in the literature [5, 7]. Also, a LC-MS method for identification and characterization of Pomalidomide and its related substances was reported [8]. Also, recently a stability indicating HPLC method have been reported for estimation of POM, but it is not able to elute any degradation products formed by application of stress degradation conditions. Also, the reported method is not LCMS compatible and no DOE approach is used in it for development of a robust analytical method. [13] Therefore, instead of sufficient literature available for the selected drug, no stability indicating analytical method using DOE and total error approach is available for POM along with degradation kinetics study at various stressor conditions best to our knowledge.

5.4 SECTION –A

5.4.1 Experimental

Development and validation of HPLC method for Pomalidomide and application of total error approach for checking uncertainty of data distribution

5.4.1.1 Chemicals and materials

Pomalidomide was kindly supplied as a gift sample by Calida Pharmaceuticals, Ahemedabad, Gujarat. HPLC grade acetonitrile and methanol were procured from Fischer Scientific Pvt Ltd. (India). Formic acid was purchased from Merck (India). Double distilled water was prepared at laboratory premises. Unless otherwise specified, all solutions were filtered through a 0.2 µm Nylon 6, 6 membrane filter, Ultipor® N66® from Pall Life Sciences, USA; prior to use.

5.4.1.2 Equipments and analysis conditions

Equipments used were an ultrasonic bath (Analab Scientific Instruments Pvt Ltd, Vadodara), precision analytical balance (A X 120, by Shimadzu Corporation analytical and measuring Instruments division, Kyoto, Japan), pH Meter (Labindia Instruments Pvt Ltd, Navi Mumbai. UV Spectroscopy analysis for deriving the suitable wavelengths for estimation of drugs were identified by scanning over the range of 200–400 nm with a Shimadzu UV-1700 double beam spectrophotometer (Shimadzu, Japan).
Chromatographic analysis was carried out on a Waters, Ahmedabad (from Waters Acquity Corporation, Milford, MA, USA) and consisting of following components a gradient pump, PDA detector, a manual injection facility with 20 µl fixed loop, low pressure gradient flow control valve , column oven, solvent delivery module.

5.4.1.3 HPLC method and sample preparation

10mg of POM was weighed accurately and transferred into a 10 ml volumetric flask containing DDW. DDW was added up to the mark to produce a stock solution containing 1000 µg/ml of POM. From stock solution 100 µg/ml of working standard solution was prepared by 10 times dilution. Then appropriate aliquots of POM working standard solutions were taken in 6 different volumetric flasks each and diluted up to the mark with mobile phase to obtain final concentrations of 10-60 µg/ml. The chromatographic analysis was performed using Empower 3 software on a Hypersil BDS C_{18} column (250×4.6 mm, 5 µm particle size) keeping flow rate for mobile phase 1 ml/min and ratio of mobile phase to be 60:40 (0.2%Formic acid: ACN).

5.4.1.4 Analysis of Formulation

The developed HPLC method was applied for analysis of synthetic mixture prepared at laboratory premises using excipients and procedure as reported in literature. [4] An accurately weighed portion of the powder equivalent to 4 mg of POM was transferred to volumetric flask. DDW was added and sonicated for 10 min. The solution was filtered through Whatman filter paper (No. 42) into 10 ml volumetric flask and then diluted up to water to get stock sample solution. Appropriate dilution was carried out and final sample of 40 µg/ml was prepared for performing the assay of method. Six replicate samples were used for analysis.

5.4.1. 5 Method validation [9]

The method was validated using International Conference on Harmonization (ICH), Q2 (R1) guideline (ICH, 2005) for linearity, range, precision, limit of detection, limit of quantification and robustness.

5.4.1.6 Application of Total error approach

Quality of pharmaceutical product is based on how accurate the analytical method is utilized for its estimation; therefore Validation of developed analytical method has

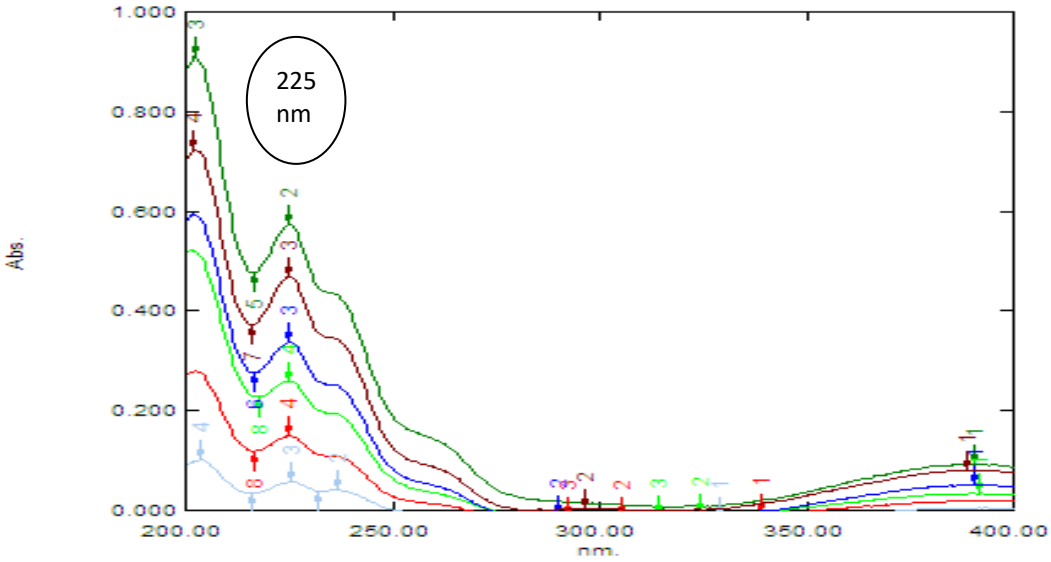
gained abundant importance for checking the reliability of product. Since publication of ISO 17025 [11] which is the main standard used by testing and calibration laboratories, uncertainty in results associated with pharmaceutical products has become utmost important. Accuracy of the method was confirmed by study of uncertainty profile using total error approach taking Beta value as 66.7% at 90% confidence interval. % Bias was calculated in the recovery study and based on that the upper and lower limit of tolerance was calculated. Uncertainty in results was checked within paradigm of 1% tolerance for beta expectance tolerance limit.

5.4.2 Results and discussion

5.4.2.1 Determination of suitable wavelength

The UV spectrum of POM is presented in figure 5.2. The spectrum indicates that λmax of POM is 225 nm. The UV spectra of POM and its impurities were extracted in PDA detector from 200-400 nm but sufficient absorption of drug as well as DP's was observed 225 nm itself.

Figure 5.2UV spectrum of Pomalidomide



5.4.2.2 Method optimization and development

For this study, one factor at a time approach was utilized. Various combinations of organic solvents and buffers were tried. Also the ratios of mobile phases were varied in numerous ways. The changes and flow rate and organic modifiers were also tested as shown in Table 5.1. Finally an optimized mobile phase ratio along with other parameters was selected as described in Table 5.2. Figure 5.3 represents the optimized overlay chromatogram for Pomalidomide. Table 5.3 represents the system suitability parameters for the developed chromatographic method.

Table 5.1: HPLC method development and optimization

Mobile phase	**Ratio (%v/v/v)**	**Column**	**Flow rate (ml/min)**	**Pomalidomide**	
				Rt (min)	Peak shape
methanol: water	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	4.1	Broad peak
methanol: water	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	4.4	Broad peak
methanol: water	40:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	4.0	Broad peak
ACN: water	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.5	Broad peak
ACN: water	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.8	Broad peak
ACN: water	40:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.1	Broad peak
0.2% formic acid: ACN(pH=3)	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.6	Symmetric peak
0.2% formic acid:ACN (pH=3)	40:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.5	Symmetric peak
0.2% formic acid:ACN (pH=3)	**60:40**	**BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)**	**1.0**	**3.8**	**Sharp and Symmetric peak**

Table 5.2: Optimized method parameters for developed HPLC method

Method parameter	**Optimized value**
Column	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)
Mobile phase	0.2% Formic acid:ACN(pH=3), 60:40
Retention time	3.80± 0.04 min
Detection wavelength	225 nm
Flow rate	1 ml/min
Temperature	Ambient

Figure 5.3 Overlay chromatogram for Pomalidomide by optimized method

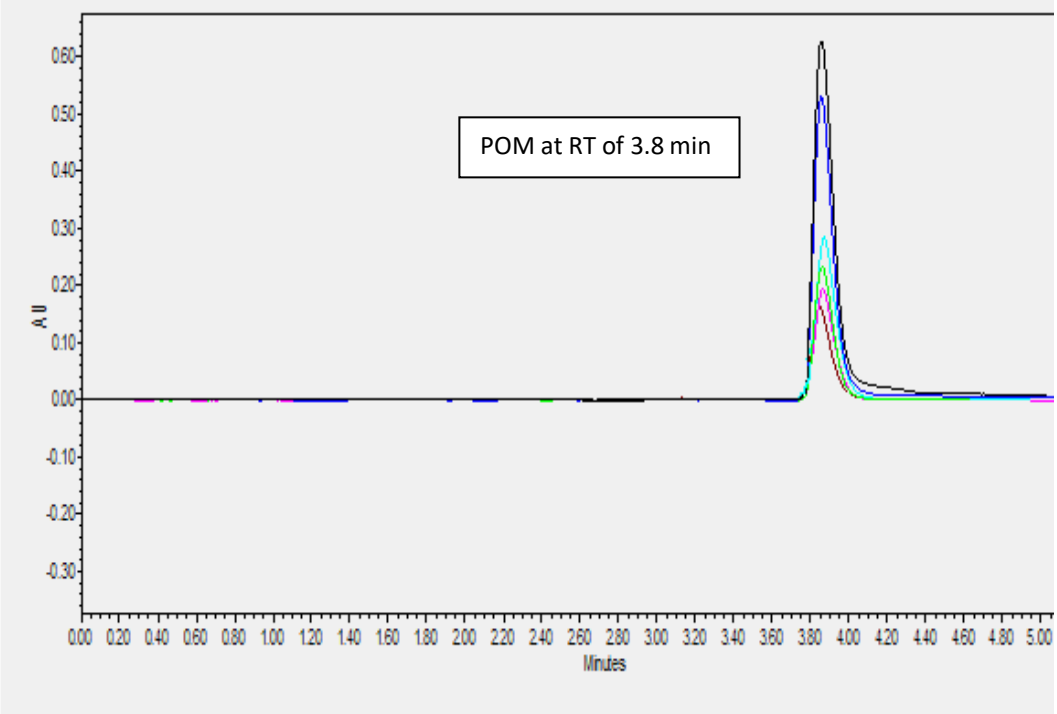


Table 5.3: Summary of system suitability parameters for Pomalidomide

Parameter	Data obtained* Pomalidomide
Retention time (min) ± SD	3.80 ± 0.04
Theoretical plate ± SD	4407± 365.39
Tailing factor ± SD	1.38 ± 0.09

5.4.2.3 Method validation using ICH Q2 (R1) guideline

The validation of developed HPLC-PDA method was done as per ICH Q2 (R1) guidelines. The method showed linearity in range of 10 - 60 µg/ml with correlation coefficient of 0.9991.
Figure 5.4 represents the calibration curve for Pomalidomide.
Intraday and interday precision studies were undertaken, low %RSD value signifies method to be precise. Accuracy studies were done using total error approach.
For LOD and LOQ, calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated.
Robustness was performed by making small deliberate changes in method like change in analytical wavelength, flow rate and change in % of organic phase by 2 unit plus and minus in selected variables of the method. No significant change in system suitability parameters of method was observed signifying method to be robust. Refer Table 5.4 shows the results of robustness studies.
Ruggedness was checked by change in operator in which also no significant change was observed in system suitability parameters of method signifying method to be rugged.
The summary of validation parameters is represented in Table 5.5.

Figure 5.4: Calibration curve for Pomalidomide by optimized method

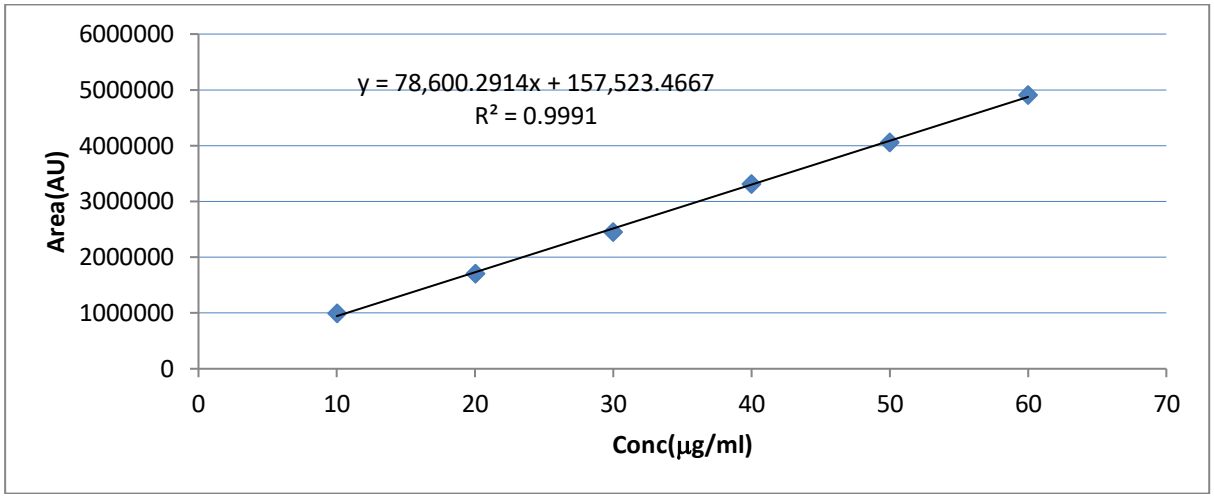


Table 5.4: Robustness study for the developed HPLC method

Factor	Retention time (min) POM	Peak area (AU)
A. Flow rate (ml/min)		
0.9	3.84	998085
1	3.80	998082
1.1	3.78	998089
Mean ±SD	3.81±0.03	998085±845
B. Ratio of ACN:		
38 %	3.81	998088
40%	3.80	998100
42%	3.81	998081
Mean ±SD	3.80±0.10	998089±708
C. Wavelength		
223 nm	3.82	998087
225 nm	3.80	998083
227nm	3.82	998080
Mean ±SD	3.82±0.09	998083±999

Table 5.5 Summary of validation parameters

Parameter	Pomalidomide
Analytical wavelength(nm)	225
Retention time (min)	3.8±0.04*
Linearity range (µg/ml)	10-60
Regression equation	Y = 78,600.2914x + 157,523.4667
Correlation coefficient	0.9991
Intraday precision (%RSD)	0.13%
Inter day precision (%RSD)	0.38%
LOD (µg/ml)	0.47

LOQ (µg/ml)	1.40
Assay %± SD	100.43±0.39*
Robustness	Robust
Ruggedness	Rugged

*Data obtained after 6 replicates

5.4.2.4 Total error approach

The model drug selected for our study was Pomalidomide which much more potent than thalidomide. The effective dose of Pomalidomide capsules available in market ranges from 1 mg, 2 mg to 4 mg. Thus it is of utmost importance that it remains within the therapeutic window and below the MTC (minimum toxic concentration in the body) otherwise side effects like tumour lysis syndrome (rapid destruction of cancer cells) may prevail which may be fatal for the health of patients. Therefore, accuracy checking in results for Pomalidomide formulations becomes very essential. Hence, total error approach was also applied for the results of accuracy in validation of assay method. The results of accuracy for the validation of the assay method of Pomalidomide before and after introduction of correction factor are represented in Table 5.6. Uncertainty profile of Pomalidomide where Beta is taken equal to 66.7% with a confidence level of 90% is shown in Figure 5.5. The figure interprets that the uncertainty contour falls within 1% (viz., % Recovery lies within range 99-101%) of acceptance criteria for accuracy of the developed HPLC method. The results of measurements uncertainty, expanded uncertainty, upper and lower uncertainty limits, upper and lower limits of tolerance for calculation of % uncertainty are represented in Table 5.8. The intermediate computations are represented in Table 5.7.

Table 5.6 Results of accuracy for the validation of the assay method of Pomalidomide before and after introduction of correction factor

Conc. (µg/ml)	**Cal Conc. (µg/ml)**	**% Recovery**	**% Bias**	**LT**	**UT**	**CF**	**% Recovery**	**% Bias**	**LT**	**UT**
10	10.09	101.51	1.51	-0.08	3.1	0.99	100.49	0.49	-1.1	2.08
20	20.054	100.45	0.45	-5.1	6.06		99.44	-0.56	-6.1	5.05
30	29.9	99.46	-0.54	-3.4	2.3		98.46	-1.54	-4.4	1.32
40	40.16	100.68	0.68	-2.01	3.37		99.67	0.33	-2.3	3.02
50	49.8	99.36	-0.64	-4.8	6.1		98.36	-1.64	-7.1	3.8
60	60.39	101.11	1.11	-2.6	4.9		100.09	0.09	-3.7	3.88

LT- lower tolerance, UT-upper tolerance, CF-correction factor

Table 5.7 Results of computation of some parameters necessary to estimate the two sided (0.667, 0.9) tolerance intervals

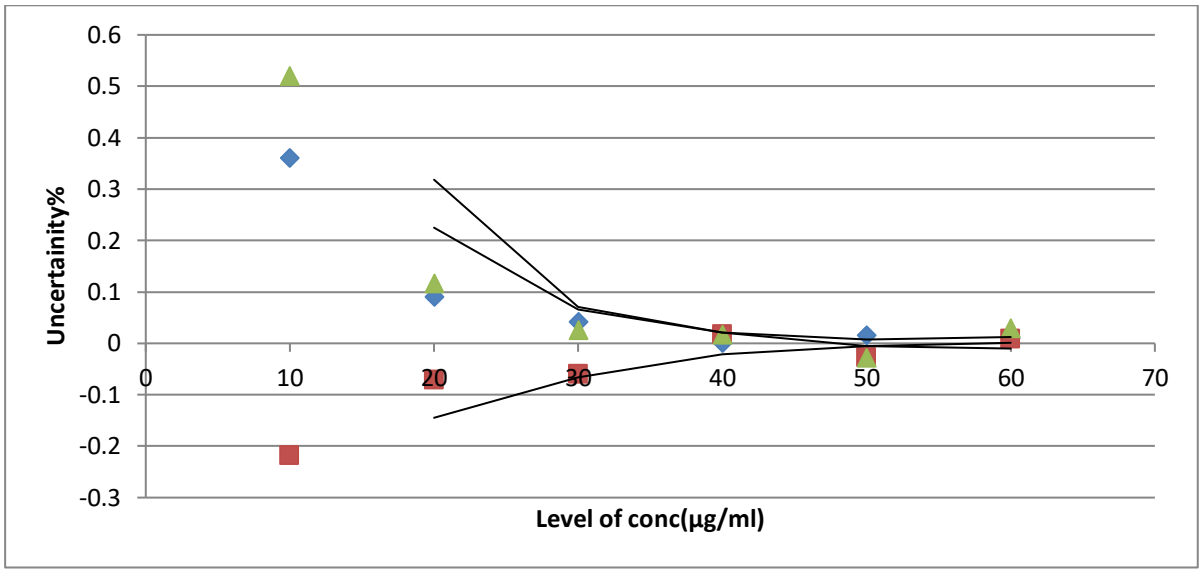
Fcritical value	Fn value	Accuracy LT (µg/ml)	Accuracy UT (µg/ml)	Accuracy LT %	Accuracy UT %
5.14	2.41	9.76	10.42	97.60	104.10
	2.56	19.72	20.38	98.60	101.90
	2.32	29.57	30.23	98.50	100.76
	3.01	39.83	39.83	99.57	99.57
	2.51	49.47	50.13	98.94	100.26
	2.69	60.06	60.72	100.1	101.20

Table 5.8 Calculation results of the measurements uncertainty, expanded uncertainty, upper and lower uncertainty limits and upper and lower limits of tolerance

Conc (µg / ml)	df (v)	U-L	Uncertainty	Expanded uncertainty	Upper Uncertainty limit (µg/ml)	Lower Uncertainty limit(µg/ml)	LT%	UT%
10	11.36	6.5	1.846590909	0.36	13.78318182	6.396818182	-0.21831818	0.520318182
20	6.696	3.3	0.9375	0.09	21.929	18.179	-0.07125	0.11625
30	6.145	2.26	0.642045455	0.042	31.18409091	28.61590909	-0.06080303	0.02480303
40	7.474	0	0	0	40.16	40.16	0.017	0.017
50	6.521	1.32	0.375	0.015	50.55	49.05	-0.0278	-0.0278
60	6.849	1.1	0.3125	0.0104	61.015	59.765	0.008	0.028916667

LT- lower tolerance, UT-upper tolerance, df (v)- degree of freedom, (U-L)-upper beta content tolerance interval-lower beta content tolerance interval

Figure 5.5 Uncertainty profile for method validation for the determination of Pomalidomide, Beta is taken equal to 66.7% with a confidence level of 90%



5.4.2.5 Applicability of developed method for analysis of formulation

Assay was performed on the laboratory mixture in 6 replicates which gave 100.43 %±0.387 signifying method can be extended on the marketed formulation. The procedure utilized for analysis of formulation was as mentioned in section 5.4.1.6. Assay results for the study of formulation are presented in Table 5.9.

Table 5.9: Assay results for Pomalidomide by developed HPLC method

Actual conc. (mg in 10 ml)	Amount of POM found (mg in 10 ml)	%Label claim	Standard Deviation	%RSD
4	4.08	102.20%	0.39	0.96 %
4	4.01	100.27%		
4	4.03	100.72%		
4	3.99	99.75%		
4	3.98	99.57%		
4	4.00	100.02%		

5.5 SECTION-B

5.5.1 Experimental

Development of stability indicating HPLC method for Pomalidomide and application to degradation kinetics study

5.5.1.1 Chemicals and materials

Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were procured from SD Fine chem. Ltd., Mumbai, India. Hydrogen peroxide (H_2O_2), Manganese oxide (MnO_2) was procured from Fischer Ltd., India. All other materials were as mentioned in section 5.4.1.1.

5.5.1.2 Equipments and analysis conditions

Photolytic degradation study was carried out in a photo-stability chamber (Thermo lab Scientific Equipments Pvt. Ltd, Vadodara) equipped with a light bank consisting of four UV (OSRAM L73) and fluorescent (OSRAM L20) lamps, that complied with specifications prescribed in the ICH guideline Q1B. The system is capable of controlling specific temperature and humidity ($\pm 2^0$C and ± 5% RH). Precision water/oil baths equipped with temperature controller were used for degradation studies for acidic, basic, neutral hydrolysis and oxidative degradation conditions. All other equipments were same as described under section 5.4.1.2.

5.5.1.3 Preparation of standard drug sample

10mg of POM was weighed accurately and transferred into a 100 ml volumetric flask containing DDW. DDW was added up to the mark to produce a stock solution containing 100 µg/ml of POM.

5.5.1.4 Preparation of Degradation Products (DP's)

Stress degradation studies were carried out as per ICH guidelines. A preliminary degradation study was performed to gather some basic information about the stability of the API and to determine number of DP's formed under different stress conditions. Four samples were generated for every stress condition for API

i) Blank solution stored at normal conditions
ii) Blank solution subjected to stress condition in the same manner as the drug
iii) Zero time sample containing the API which was stored at normal conditions and
iv) Drug solution subjected to stress treatment.

Then in the final study, POM was stressed to maximum condition where 05-80% decrease in peak area of API occurred.
Degradation samples generated for each stress condition were mixed to optimize and develop stability indicating assay method. The same stress degradation study was performed on formulation of POM to determine formation of any DP's due to drug-excipient interaction.

The specific protocols for stress conditions applied were as follows:

Hydrolytic degradation studies were performed in acidic, basic and neutral conditions by heating or refluxing API in HCl, NaOH and H_2O.

For acid hydrolytic degradation, about 0.1 g of POM was dissolved with 100 ml of water (1000 µg/ml), 1 ml of this stock solution was taken in a round bottom flask (RBF) and 4.5 ml of 0.003M HCl was added to it. The flask was kept at 30°C for 15 min. After completion of stressor interval, 4.5 ml of 0.003M NaOH was added to make up volume up to 10 ml to produce 100 µg/ml of sample. This was done to neutralize the sample.
For base hydrolytic degradation, about 0.1 g of POM was dissolved with 100 ml of water (1000 µg/ml), 1 ml of this stock solution was taken in a round bottom flask (RBF) and 4.5 ml of 0.001M NaOH was added to it. The flask was kept at 30°C for 10 min. After completion of stressor interval, 4.5 ml of 0.003M HCl was added to make up volume up to 10 ml to produce 100 µg/ml of sample. As mentioned above it was done to neutralize the sample.

For neutral hydrolysis, about 0.1 g of POM was dissolved with 100 ml of water (1000 µg/ml); 1 ml of this stock solution was taken in a round bottom flask (RBF) and 9 ml of DDW solution added to it. The flask was kept at 30°C for 15 min.

For oxidative degradation, about 0.1 g of POM was dissolved with 100 ml of water (1000 µg/ml); 1 ml of this stock solution was taken in a round bottom flask (RBF) and 9 ml of 6% H_2O_2 solution added to it. The flask was kept at 30°C for 30 min.

Thermal degradation was carried out on solid POM 100 mg spreaded on glass petridish as a thin layer about 1mm thickness in high precision oven at 60°C for up to 15 days.

For photolytic degradation also the solid drug was (100 mg) spreaded as a thin layer about 1 mm thickness in a petridish, were exposed to the UV light in the photo stability chamber. The samples were placed for 07 days during which total exposure given was UV light of about 144 Wh/m^2 and 5382 Lux.

Each degraded drug solution was diluted up to 10 times using mobile phase as a diluent. The solid drug samples exposed to thermal and photolytic conditions were rendered into solutions (1 mg/ml) in a DDW before final dilution by mobile phase for chromatographic analysis.

5.5.1.5 Method optimization and development utilizing Design of experiments

The required ATP (Analytical target profile) for the developed method required symmetric peaks and good resolution between the peaks of drug substance and its degradation products. For achieving the ATP, preliminary trials were taken on isocratic mode of HPLC, but we were not able to achieve the ATP thus further trials were taken using gradient mode of HPLC. Based on initial trials of drug in presence on its degradation products, the resolution between peak of drug substance and DP2

and asymmetry of DP2 was not as required as per ATP, thus to fulfill the criteria of ATP and for obtaining a robust analytical method, the experimental design was implemented for optimization of method using Design expert 7.0 software. The factors for the experimental design were decided as per the gradient trials taken for achievement of the ATP. Preliminary experiments were performed by using Central composite design (CCD) of Response surface methodology (RSM) to identify the critical factors and to set their levels (maximum and minimum) for the experimental design as represented in Table 5.11.

5.5.1.6 Preparation of Degradation kinetics study samples

Degradation reactions are affected by conditions such as solvent, concentration of reactants, time of exposure, temperature, pH of the medium, radiation energy and presence of catalysts. Thus degradation kinetics studies are essential for determining its order of reaction. Degradation kinetics of POM was done by gradually increasing the stress conditions such as concentration of reactants, time of exposure and temperature as stressor conditions.

For acid induced degradation the sample was exposed to stressor at 3 levels (viz., 0.001, 0.003 and 0.005 N HCl) at 3 temperature conditions (viz., 30, 50 and 70 °C) and at 5 time points (viz., 15, 30, 60, 120 and 180 mins).

For base induced degradation the sample was exposed to stressor at 3 levels (viz., 0.001, 0.003 and 0.005 N NaOH) at 3 temperature conditions (viz., 30, 50 and 70 °C) and at 5 time points (viz., 10, 20, 30, 40 and 50 mins).

For neutral hydrolysis the sample was exposed to stressor DDW at 3 temperature conditions (viz., 30, 50 and 70 °C) and at 5 time points (viz., 15, 30, 60, 120 and 180 mins).

For oxidation induced degradation the sample was exposed to stressor at 3 levels (viz., 6%, 15% and 30% H_20_2) at 3 temperature conditions (viz., 30, 50 and 70 °C) and at 5 time points (viz., 30, 60, 90, 120 and 150 mins).

For photolytic degradation study, the sample was exposed to the stressor condition as described above in the photo stability chamber for exposure up to 4 time points (viz., 1, 3, 5, 7 days).

For thermal dry heat degradation study, the sample was exposed to the stressor condition as described above in high precision oven for exposure up to 6 time points (viz., 1, 3, 5, 7, 10, 15 days).

The results would provide information on half life & shelf life of drug and its formulation respectively.

5.5.2 RESULTS AND DISCUSSION

5.5.2.1 Stability studies

Drug stability is of utmost importance in pharmaceutical drug development and thus studies related to stability, shelf life monitoring and degradation kinetics play a significant role during the process. Under different stress conditions, various degradation products are formed which needs to be analysed by the developed chromatographic analytical method. Adequate resolution and symmetric peaks of drug along with the peaks of degradation product formed in stressor conditions is quite arduous task. Method development utilizing Design of experiments acts as a good solution for this task which provides ample design space for the study. Extrapolating the stability studies to degradation kinetics helps in elucidation of order of reaction with half life and shelf life parameters which are essential for understanding mechanism of the drug decomposition. The results of stability studies are utilized for taking very important decisions during product lifecycle, reliability of the results obtained is of utmost importance and thus possible uncertainty contour is determined using total error approach.

5.5.2.2 Stress degradation studies

The present investigation was aimed to develop a stability indicating analytical method for the analysis of POM in presence of its degradation products.
Stability protocols were initiated by applying stressor conditions as prescribed by ICH Q1 (R2) guidelines.
%Degradation was calculated using formula as below:

$$\%\text{Degradation} = \frac{\text{Initial area of untreated stock solution} - \text{reduced area of treated stock solution}}{\text{Actual area of untreated stock solution}} \times 100$$

For acid hydrolysis study, HCl with stressor concentration of 0.1 N was applied initially. Similarly for alkali hydrolysis study, NaOH with stressor concentration of 0.1 N was used initially. Acidic and basic solutions were neutralized prior to be injected into the system. For oxidation 3% H_2O_2 was used initially. Time interval for all the stressor conditions initially was 15 min.

In acidic as well as alkali hydrolysis, 100% degradation of drug was found whereas no degradation of drug occurred in oxidative stress condition. Thereby, it was decided to reduce the level of stress conditions for acid and alkali hydrolysis conditions and enlarge the stressor conditions for oxidative condition. Further for acid and base stress condition keeping similar stressor and time interval, concentration of stressor was reduced to 0.01 N but still 100% degradation of drug was observed and thus further the stressor concentration was reduced to 0.001 N for 15 min which gave only 3.27% drug degradation in acidic condition and 1.55% drug degradation in basic condition. DP1 at retention time of 4.8 min was obtained on applying stressor HCl at concentration of 0.003N for 15 min. (Figure 5.7 a) DP2 at retention time of 3.4 min

was obtained on applying stressor NaOH at concentration of 0.001N for 10 min. (Figure 5.7 b) But for reasonable chromatographic assay [12] time interval was increased up to 180 min which gave 12.16% drug degradation in acidic condition and 15.75% drug degradation in basic condition.

For oxidative stress condition, stressor concentration was increased to 6% H_2O_2 with 15 min interval at 30°C which gave only 0.77% drug degradation. On increasing time interval up to 150 min, %drug degradation of 9.59 was achieved. Significant % drug degradation was now achieved but no DP was seen as it was getting merged with H_2O_2 peak at RT of 2 min. To resolve the problem, 6% MnO_2 was added to the sample. Now DP3 was visible at RT of 2.3 min on applying stressor H_2O_2 at concentration of 6% for 30min and adding 6% MnO_2 at the end of 30 min. (Figure 5.7 c)

In neutral hydrolysis of drug solution at 30°C for 15 min, only 0.63% degradation was observed and also no degradation product was observed. (Figure 5.7 d)
In dry heat degradation of drug powder at condition of 60°C in dark gave only 3.11% degradation on day 1 whereas 16.52% degradation on day 15. (Figure 5.7 f) In photolytic stress condition, drug is comparatively very stable which under standard photolytic conditions gave only 2.57% degradation even after 7 days of exposure. (Figure 5.7 e)

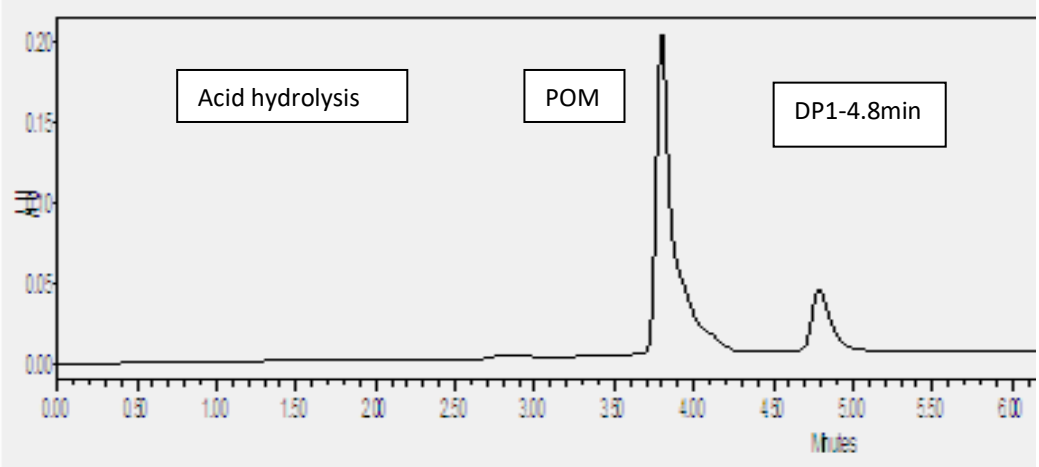
Comprehensive from the results, drug shows enormous change in stability with slight change in stress conditions, thus acid, base, neutral hydrolysis and oxidation study stability protocols were further undertaken at elevated temperature.

In thermal degradation condition and photolytic degradation conditions, drug degradation was observed as discussed above but did not any show degradation products.

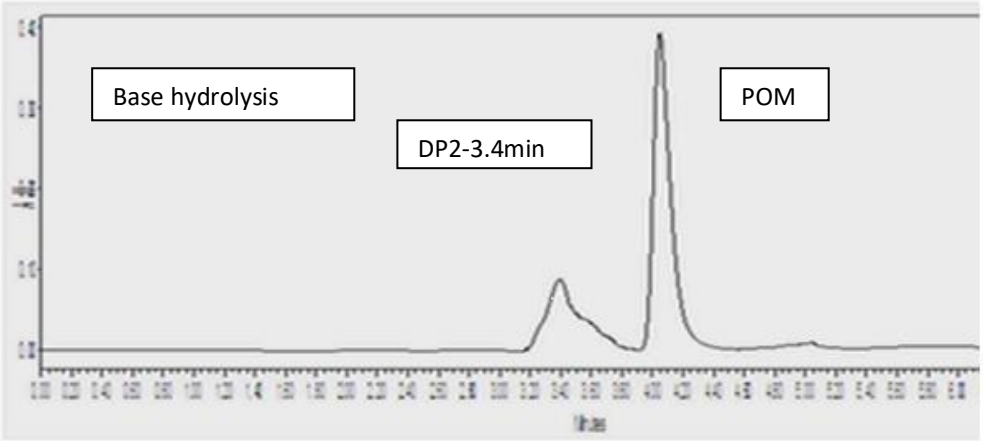
Each degraded drug solution was diluted up to 10 times with mobile phase rendered as diluents. The acid and alkali hydrolysed solutions were neutralized before dilution. The solid drug samples exposed to thermal and photolytic conditions were rendered into solutions (1 mg/ml) in a DDW before final dilution by mobile phase for chromatographic analysis.

Figure 5.7 HPLC chromatogram of POM with stressor conditions of acid, base and oxidation degradation.

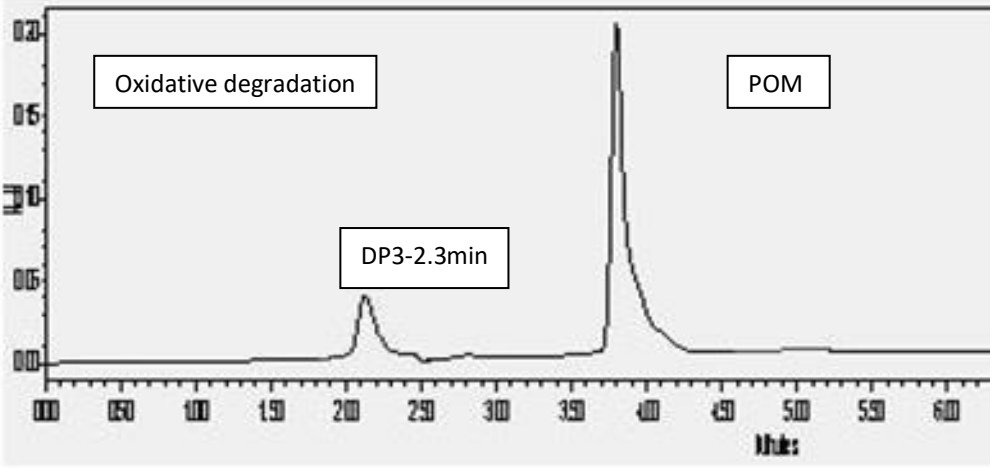
a) POM and DP1 by acid hydrolysis for 15 min with stressor condition of 0.003 M HCl at room temperature



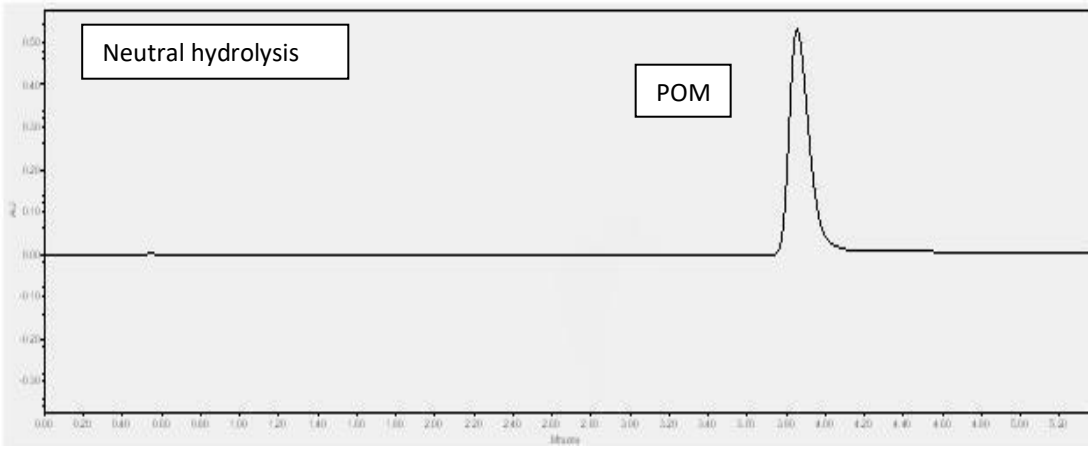
b) POM and DP2 by base hydrolysis for 10 min with stressor condition of 0.001 M NaOH at room temperature



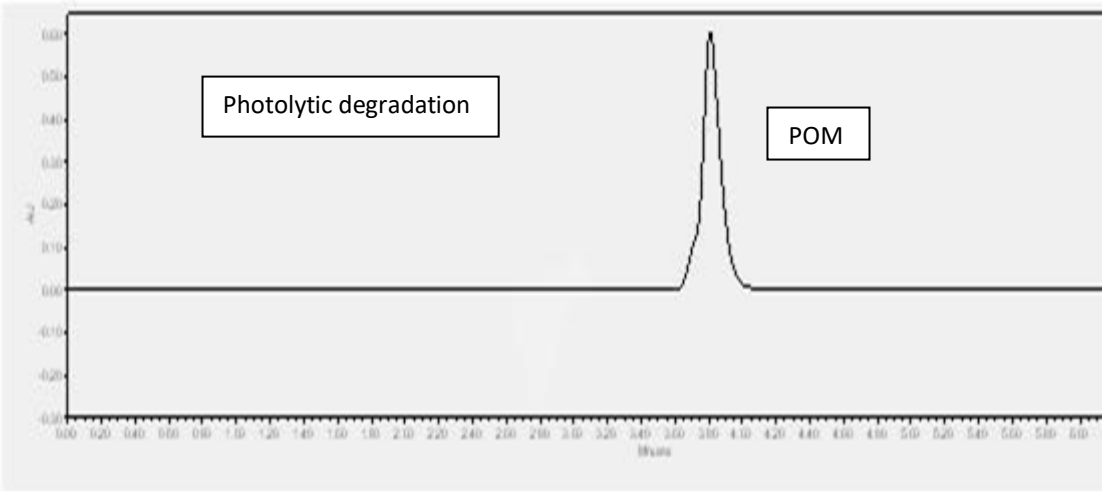
c) POM and DP3 by oxidation for 30 min with stressor condition of 6% H_2O_2 at room temperature



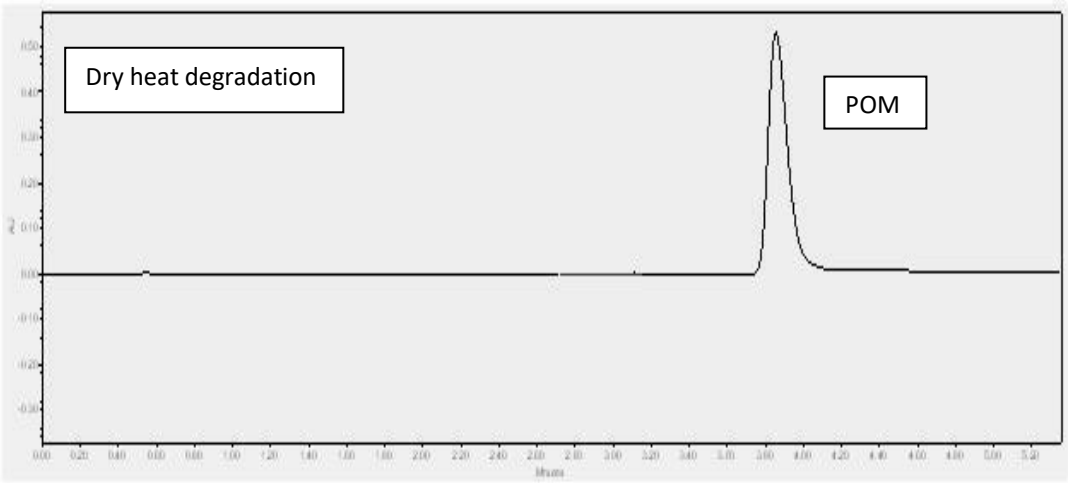
d) POM by neutral hydrolysis for 15 min with stressor condition in DDW at room temperature



e) POM by photolysis stressor condition on exposure to the UV light in the photo stability chamber for 7 days



f) POM by dry heat degradation in high precision oven with stressor condition at 60°C for up to 15 days



5.5.2.3 HPLC method optimization using Design of experiments

HPLC-PDA detection method by application of Design of experiments approach utilizing Central composite design of Response surface methodology was used as a tool for obtaining a robust analytical method. The sample utilized for development of optimized analytical method contained mixture from all degradation stress conditions. As previously discussed in section 5.5.1.5, gradient mode of HPLC was needed for achievement of ATP. The CCD design matrix was generated using three factors at five levels resulting in a total of 20 analytical runs as it considers –α, -1, 0, +1, + α levels. (Table 5.11). Quadratic model and polynomial model were suggested on evaluation of design. Linear model was suggested on analysis of response resolution as per lack of fit tests, whereas quadratic model was suggested on analysis of response asymmetry as per lack of fit tests. By assessing these results, it was possible to elaborate mathematical models that attempted to describe the relationship between the independent variables studied and the responses of interest. The ANOVA table and its statistics for responses such as resolution as well as asymmetry are represented in Table 5.12 and 5.13 respectively.

5.5.2.3.1 Equations from ANOVA Analysis

When there are three or more means being compared, statistical significance can be ascertained by conducting one statistical test: ANOVA.
Final ANOVA Equations in Terms of Coded Factors:
Resolution=3.39+1.04*A-0.057*B+0.039*C
Asymmetry=1.16-0.019*A+0.081*B+4.054*C, where
A= % organic phase- A (acetonitrile) at hold time in the analytical method (ml)
B= Hold time in the gradient elution method (min)
C= pH of aqueous solvent in the mobile phase (0.2% formic acid- B)

The quadratic polynomials for each response variable along with their ANOVA generated statistical parameters are tabulated in Table 5.14. The ANOVA table for response resolution states the model F-value to be 62.45 which implies the model to be significant and shows only 0.01% chance of noise. Also lack of fit value of 46.29 implies it to be significant and stands only 0.03% chance of noise. The predicted R^2 is also in reasonable agreement with the Adjusted R^2. The ANOVA table for response asymmetry states the model F-value to be 5.07 which implies the model to be significant and shows only 0.91% chance of noise. Also lack of fit value of 76.73 implies it to be significant and stands only 0.01% chance of noise. The value of Adequate precision 27.99 for response resolution and Adequate precision 7.048 for response asymmetry much greater than 4 which is minimum desirable states that the model can be used to navigate the design space. The pertubation plots as well as the 2D contour plot for response resolution are represented in Figure 5.8 and Figure 5.9 respectively. The pertubation plots of residuals as well as the 2D contour plot for response asymmetry are represented in Figure 5.10 and Figure 5.11 respectively.

5.5.2.3.2 Contour plots for different responses

A contour plot is a graphical representation of the relationships among three numeric variables in 2-dimensions. These are geometric illustration of responses obtained by plotting one independent variable vs. another while holding the magnitude of response level and other variables constant. (Figure 5.8 and 5.10)

5.5.2.3.3 Perturbation plots

The perturbation plots helps to compare the effects of all the factors at a particular point in the design space. The response is plotted by changing only one factor over its range while holding all the other factors constant. (Figure 5.9 and 5.11)

The models generated for both response variables were found to be highly significant statistically ($p < .0001$). Both showed coefficient of determination values (R^2) above 0.90 for these models and the ratio between the mean square of regression and the residual mean square (MSR/RMS) greater than F which demonstrates significance of the regressions indicating excellent fit of the generated polynomials to the response data ($p < .0001$ in all the cases). All the models were found to have insignificant values of ''lack of fit, '' ($p > .005$) ratifying that the proposed model was appropriate. Close proximity in the magnitude of adjusted (Adj) and predicted (Pred) R^2 to the actual model R^2 also confirm excellent fit to the data (Table 5.14).

5.5.2.3.4 Optimization Criteria

For every response, a desired target or range must be specified along with their degree of importance. Based on our input required goals for the responses, software generates various optimized solutions. After analysis, numerical as well graphical optimization was done which gave 8 solutions.

5.5.2.3.5 Point Verification and Working Point selection

For optimization as per ATP, 8 solutions were obtained. Out of the 8 optimized solutions generated by the software, 4 solutions were selected for checkpoint analysis i.e. to verify whether the predicted and experimental results are closely correlated. All these 4 predictions are tested by experimental trials and the responses observed must lie within 95% confidence interval of their predicted values. One of these solutions was also selected as the final optimized working point for the proposed method as the working point. On implementation of solution, 3D overlay plot (Figure 5.12) showing predicted desirability to be 1 in derringer desirability plot (Figure 5.13) optimum design space was obtained for development of chromatographic method. In the design space, yellow region shows the workable region for getting robust analytical results whereas gray region indicates the nonworkable region for optimum robust results. Table 5.14b shows the point verification and working point selection for the proposed working point. The optimized chromatographic conditions used were A=acetonitrile-0.2% and B=Formic acid (pH- 2.84) for mobile phase by establishing a gradient elution technique. Gradient set for mobile phase composition was (time/%B): 0.01/55, 2.6/62, 4/ 70, 5/60, 10/55, detection wavelength used was 225nm. The mobile phase flow rate and typical pressure of the system were maintained at 1.00 ml/min and 2000

psi respectively. The analysis was performed at ambient temperature with injection volume of 20 µl. The mobile phase was filtered through 0.2 µm disposable filters from Ultipore®, PALL life sciences (40 mm) and degassed by ultrasonic vibrations prior to use. The optimized chromatogram is presented in Figure 5.14.

Table 5.11 Variables and their levels for Central composite design

Factors	Coded values	Actual values
% organic phase- A (acetonitrile) at hold time in the analytical method (ml)	-α	23
	-1	30
	0	40
	+1	50
	+ α	56
Hold time in the gradient elution method (min)	-α	2.6
	-1	2.8
	0	3
	+1	3.2
	+ α	3.4
pH of aqueous solvent in the mobile phase (0.2% formic acid- B)	-α	2.1
	-1	2.5
	0	3
	+1	3.5
	+ α	3.8
Responses		Constraints
R1-Resolution between peak of Drug and DP2		2≤R1≥5
R2- Asymmetry of DP2		0.7≤R2≥1.8

Figure 5.8 Contour plots for response resolution

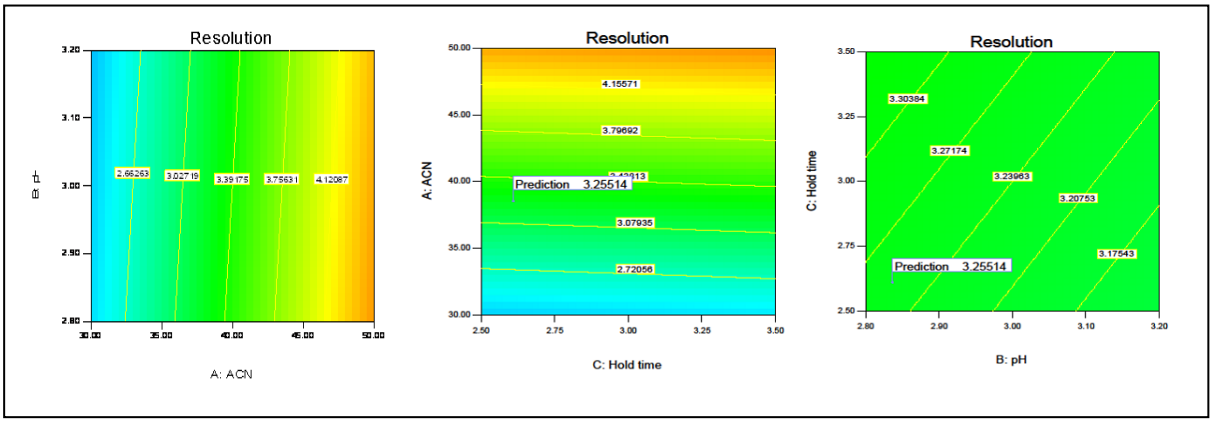


Figure 5.9 Perturbation plot for response resolution

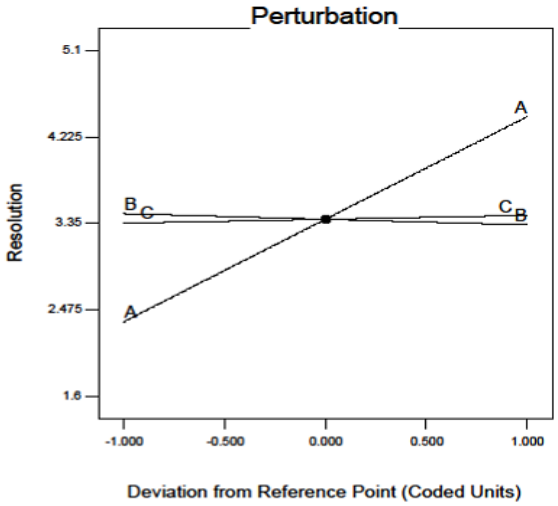


Figure 5.10 Contour plots for response asymmetry

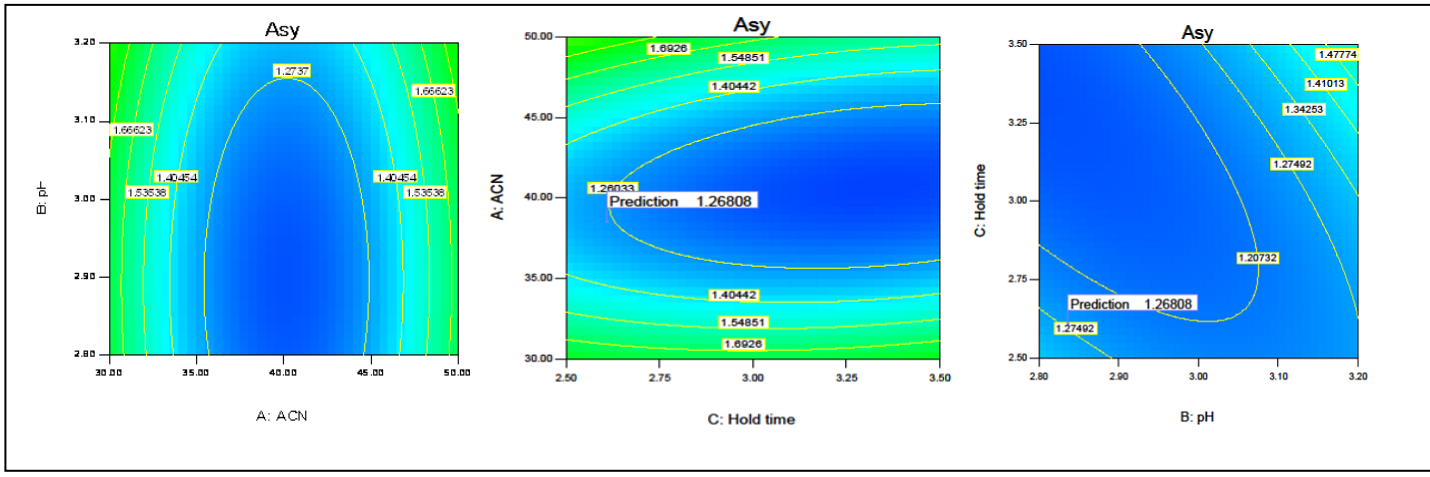


Figure 5.11 Perturbation plot for response asymmetry

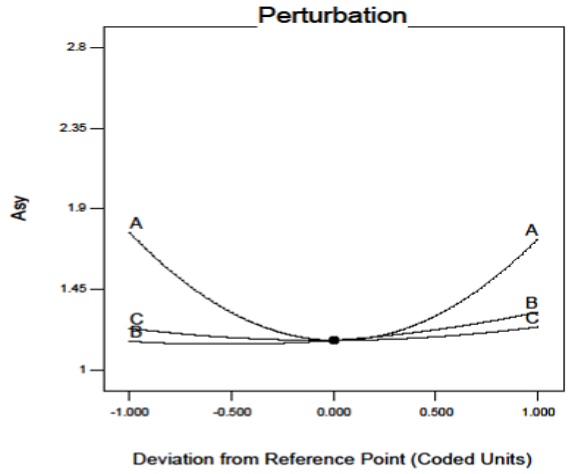


Table 5.12: ANOVA table for Response - Resolution, Model - Linear

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	14.85177148	3	4.950590494	62.45208476	< 0.0001	significant
A-ACN	14.60062104	1	14.60062104	184.1879719	< 0.0001	
B-pH	0.043835749	1	0.043835749	0.552991386	0.4679	
C-Hold time	0.026480734	1	0.026480734	0.334056524	0.5713	
Residual	1.268323519	16	0.07927022			
Lack of Fit	1.255990186	11	0.114180926	46.28956458	0.0003	significant
Pure Error	0.012333333	5	0.002466667			
Cor Total	16.120095	19				

Table 5.13: ANOVA table for Response - Asymmetry, Model – Quadratic

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	5.225737094	9	0.580637455	5.070370226	0.0091	significant
A-ACN	0.004728647	1	0.004728647	0.041292536	0.8430	
B-pH	0.086792351	1	0.086792351	0.757907278	0.4044	
C-Hold time	0.000241295	1	0.000241295	0.002107087	0.9643	
AB	4.16517E-05	1	4.16517E-05	0.00036372	0.9852	
AC	0.091746603	1	0.091746603	0.80116989	0.3918	
BC	0.126480475	1	0.126480475	1.104480647	0.3180	
A^2	4.964730643	1	4.964730643	43.35411403	< 0.0001	
B^2	0.083494527	1	0.083494527	0.729109289	0.4132	
C^2	0.09324776	1	0.09324776	0.81427869	0.3881	

	9		9	9		
Residual	1.145157906	10	0.114515791			
Lack of Fit	1.130424572	5	0.226084914	76.72564971	0.0001	significant
Pure Error	0.014733333	5	0.002946667			
Cor Total	6.370895	19				

Table 5.14a ANOVA statistics after analysis by central composite design

Coefficient code	**Resolution**	**Asymmetry**
Std. Dev.	0.281549676	0.338401818
Mean	3.3895	1.6645
C.V. %	8.306525322	20.33053879
PRESS	2.297322847	8.947061742
R-Squared	0.921320345	0.820251644
Adj R-Squared	0.906567909	0.658478123
Pred R-Squared	0.857487016	0.404364966
Adeq Precision	27.69850117	7.047796432

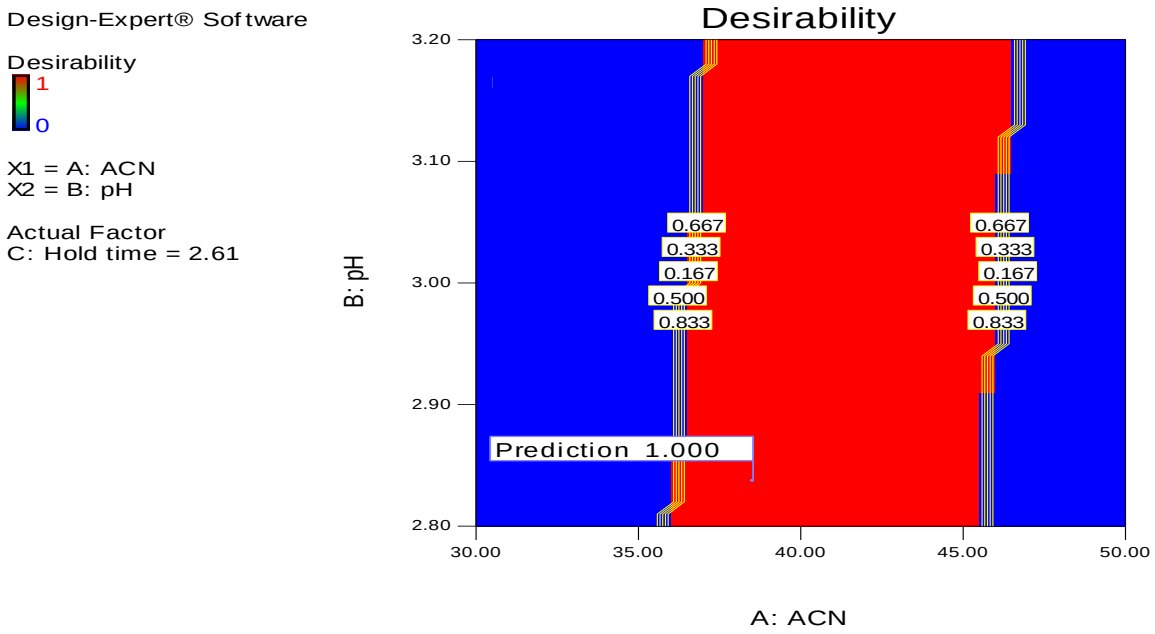
C.V. %-Coefficient of variation, PRESS-predicted residual error sum of squares, R-Squared- correlation coefficient

Table 5.14b Point verification and working point selection

Response	**Prediction**	**Observed**	**95% CI-high**	**95% CI-low**	**SE Pred**
Resolution	3.255142	3.34	3.03481	3.475475	0.300121
Asymmetry	1.268079	1.19	0.90679	1.629369	0.375244

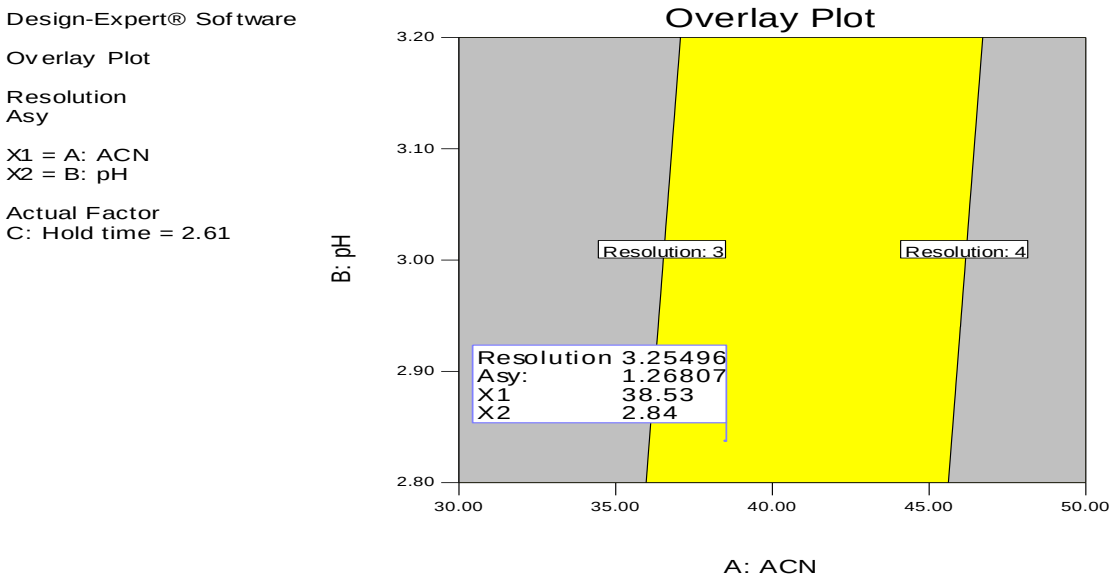
Confidence interval (95%) for the responses obtained from the selected working point

Figure 5.12 Desirability plot



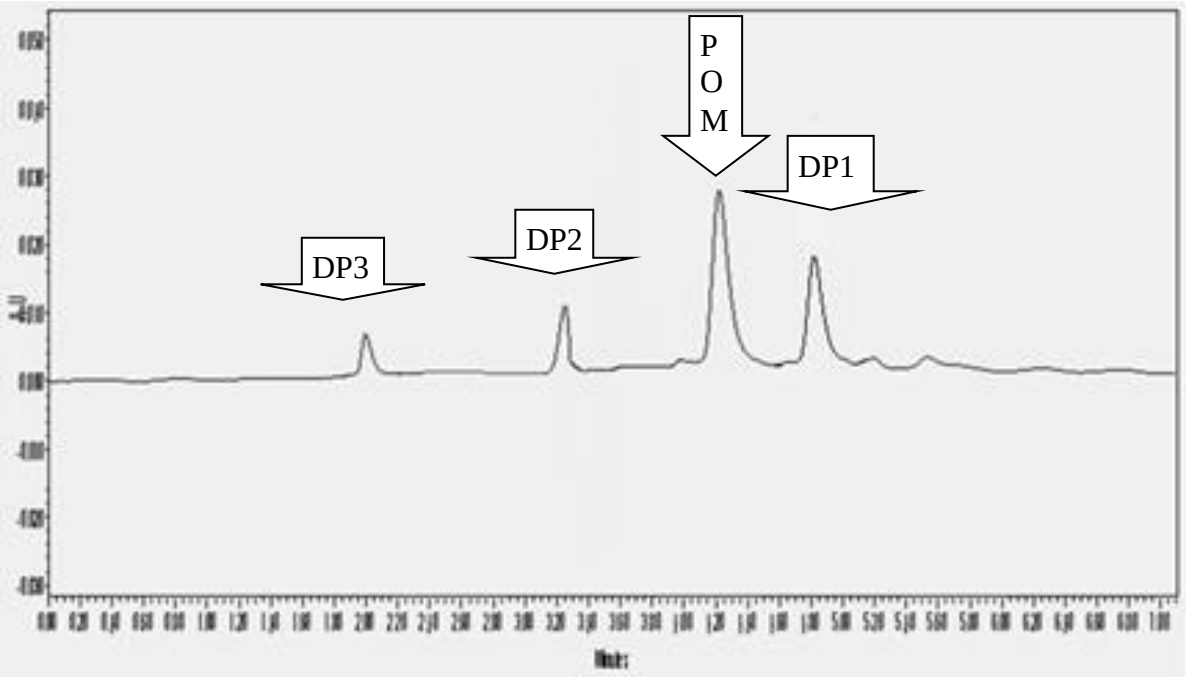
In Desirability plot (desirability increases from blue to red region; blue region indicates 0 and red region indicates 1 desirability)

Figure 5.13 3D Contour plot representing the design space



In overlay plot (yellow region is design space; gray region is undesirable region) for optimized chromatographic condition

Figure 5.14 Optimized chromatogram of POM along with DP1, DP2 and DP3 in acid, base and oxidative stress conditions



POM-Pomalidomide, DP1-Degradation product1, DP2-Degradation product2, DP3-Degradation product3

Table 5.14c Summary of validation and system suitability parameters for developed chromatographic method.

Parameter (Units)	**POM**	**DP1**	**DP2**	**DP3**
Linearity range (µg/ml)	10-60			
Correlation coefficient	0.9993±0.03			
Recovery (%)	99.27			
Precision (%RSD)				
Interday (n=3)	0.26			
Intraday (n=3)	0.45			
Robustness	Robust			
RT (min)	3.8±0.07	4.8±0.09	3.4 ±0.01	2.3 ±0.03
LOD (µg/ml)	0.48			
LOQ (µg/ml)	1.44			
Asymmetry	1.4	1.21	1.19	1.08

Theoretical plates	4029	3092	2450	4800
Resolution	3.34	3.12	6.39	-

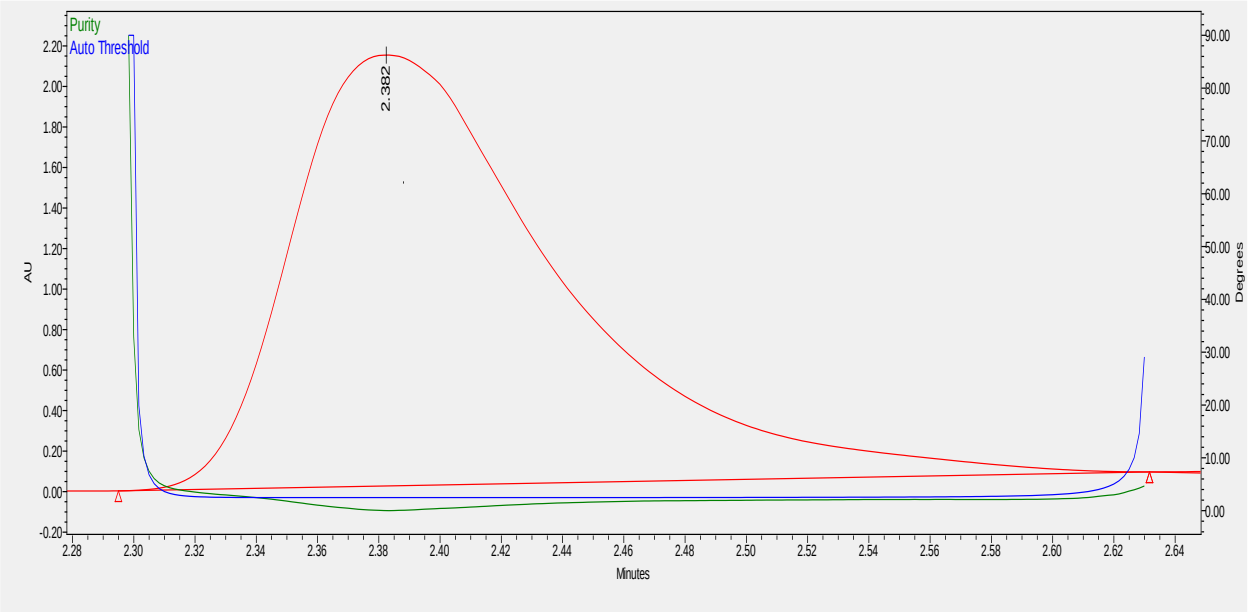
RSD-Relative standard deviation, SST- System suitability test parameters, Retention time-RT

5.5.2.4 Peak purity studies

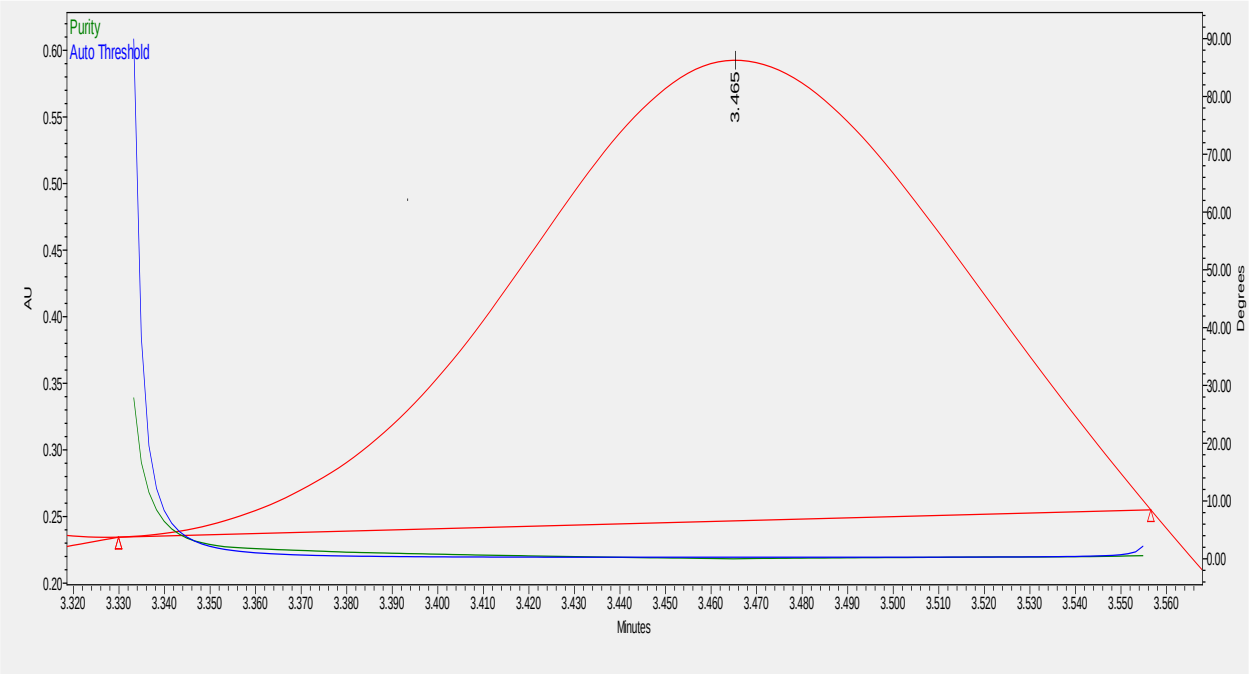
Peak purity studies were undertaken for confirming the presence of only one analyte at the retention time of peaks obtained by HPLC-PDA method. The extracted peak purity plots of standard drug along with the DP's are presented in Figure 5.15 and further the values of purity threshold higher than the values of purity angles for each peak signify the peaks to be pure. (Table 5.15)

Figure 5.15 Peak purity studies

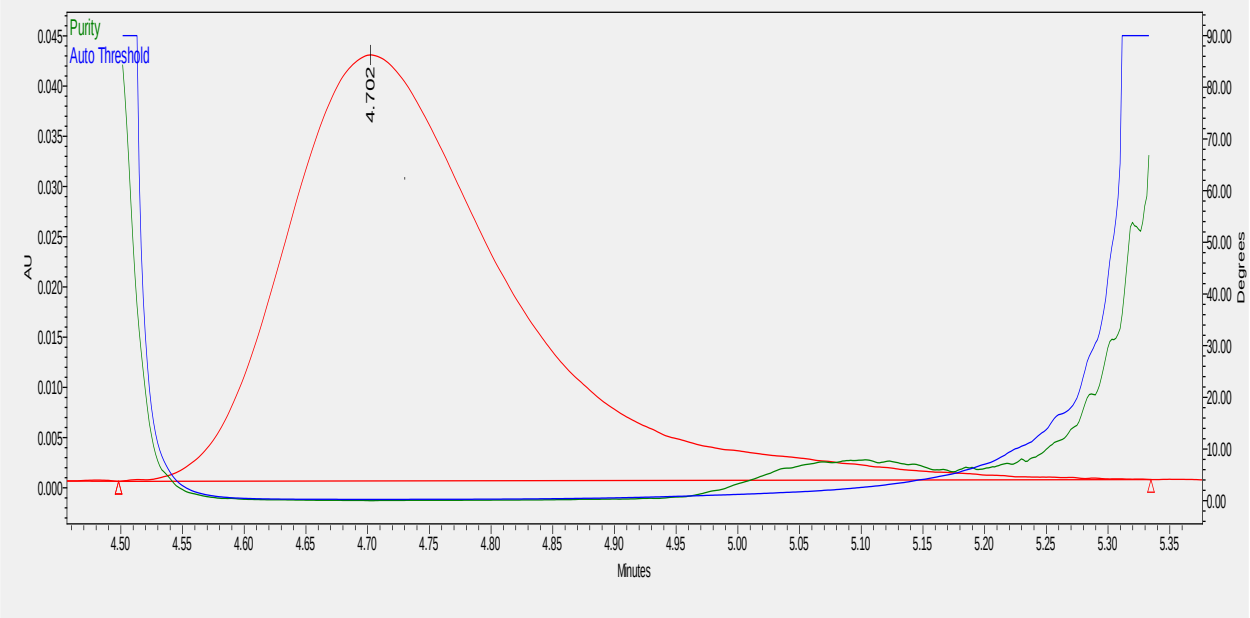
a) Peak purity plot of DP3



b) Peak purity plot of DP2



C) Peak purity plot of DP1



d) Peak purity plot of standard drug

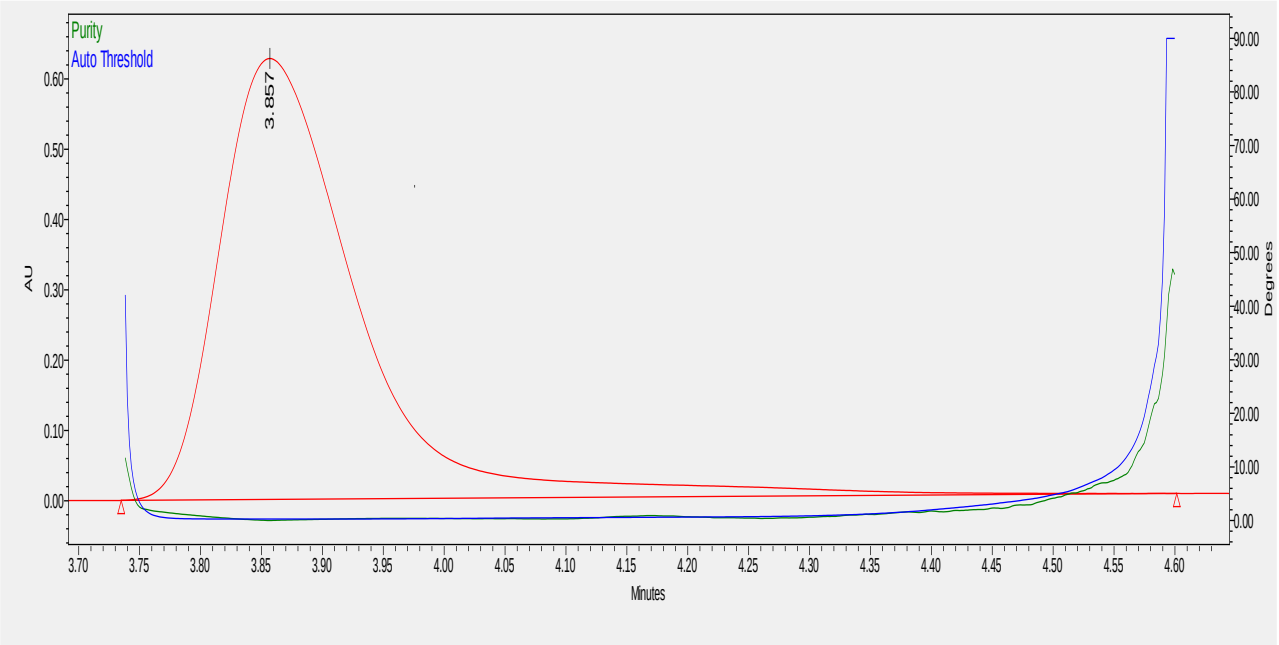


Table 5.15 Peak purity studies

Sr. No	Peak	Peak RT	Peak purity angle	Purity threshold
1	Standard drug	3.80	1.142	1.503
2	DP1	2.30	0.922	2.475
3	DP2	3.40	0.289	0.302
4	DP3	4.70	0.309	0.328

5.5.2.5 Degradation kinetics study

For acid, alkali and neutral hydrolysis, oxidative study, and photolysis as well as for dry heat degradation protocols were also extended for degradation kinetics study. It was done for determining of the order of reactions of the stability study and for acquiring the data such as half life and shelf life of the drug substance. Stressor conditions were applied as discussed in section 5.5.1.6.

At 30°C hydrolysis stress condition especially acid hydrolysis has a major impact on stability of the drug with a drug content of 20.12% drug remaining after 3 hours at 0.005N stressor condition whereas photolytic stress condition hardly has any impact on stability of drug with 97.43% drug remaining at standard stress condition.

Whereas on elevated temperature studies it was observed that alkali hydrolysis conditions have a massive influence on stability of drug giving only 3.63% drug remaining after 50 min on application of 0.005N NaOH stressor at 70°C.

Degradation kinetics plots for zero order, first and second order were plotted for inferring order of reaction in various stress conditions.

For zero order reaction, %degradation vs. time graphs were plotted, for first order reaction, log% degradation vs. time graphs were plotted and for second order reaction,

1/%degradation vs. time graphs were plotted. Figure 5.16 to Figure 5.27 depict the degradation kinetics plots. Based on higher values of correlation coefficient at various stress levels in kinetics plots it was inferred that acid hydrolysis, base hydrolysis and oxidation degradation conditions follows first order kinetics albeit dry heat degradation, photolytic degradation, neutral hydrolysis degradation conditions follows zero order kinetics. Also rate constants values were obtained from slope of the graphs. Rate constant values were utilized for calculation of half-life and shelf-life. To analyze the effect of temperature on the rates of chemical reactions Arrhenius plots were plotted. Logarithm of K values vs. 1/Temperature applied as stressor conditions consisted as X and Y axis in graphs of Arrhenius plots. From the slope of Arrhenius plots, values of activation energy for the chemical reaction were calculated. Refer Table 5.17 for degradation kinetics study parameters.

Finally after studying different parameters that affected the rate of the degradation it was concluded that the degradation rate is directly proportional to temperature, time interval and strength of stressor. (Figure 5.28 to Figure 5.31 for Arrhenius plots). Experiments were done in triplicate and average values were taken. Table 5.16 gives a brief summary of %degradation at different concentration, time interval and temperature of stressor conditions. Table 5.17 represents summary of degradation kinetics parameters obtained after applying stress degradation in acid, base, neutral, oxidation, photolytic and dry heat conditions.

Table 5.16 Summary of % degradation after different stressor conditions

a) In acid, base, neutral, oxidative condition at 30 °C at different time interval and for photolytic and thermal degradations at mentioned conditions

Time Pts (min)	% deg at x Conc. of HCl			Time Pts (min)	% deg at x Conc. of NaOH			Time Pts (min)	% deg at x % of H_2O_2			Time Pts (min)	% deg in neutral	Time Pts (Days)	% DR 5382 LUX and 144 UW/cm2	Time Pts (Days)	% deg 60 ºC at Dry heat condition in dark
	X=0.001 N	X=0.003	X=0.005		X=0.001 N	X=0.003 N	X=0.005		X=6%	X=15	X=30%					1	3.11

		N	N				N			%							
15	3.27	5.93	14.6	10	1.55	4.88	8.26	30	0.77	0.86	1.53	15	0.63			3	5.44
30	4.48	8.52	15.13	20	4.55	9.53	14.55	60	1.86	5.59	5.64	30	1.8	1	0.22	5	8.56
60	6.82	17.42	28.55	30	8.77	16.55	21.52	90	2.55	8.78	9.64	60	4.55	3	0.48	7	11.35
120	9.81	27.88	63.18	40	12.64	39.45	48.34	120	7.46	11.36	14.23	120	5.89	5	1.82	10	13.77
180	12.16	39.28	79.88	50	15.75	46.44	59.74	150	9.59	15.85	18.55	180	9.99	7	2.57	15	16.52

b) In acid, base, neutral, oxidative condition at 50 ℃ at different time interval

Time Pts (min)	% deg at x Conc. of HCl			Time Pts (min)	% deg at x Conc. of NaOH			Time Pts (min)	% deg at x % of H_2O_2			Time Pts (min)	% deg in neutral
	X=0.001N	X=0.003N	X=0.005N		X=0.001N	X=0.003N	X=0.005N		X=6%	X=15%	X=30%		
15	4.86	11.01	19.53	10	3.9	7.93	12.29	30	1.34	1.65	2.54	15	5.66
30	7.42	12.7	24.99	20	8.29	11.37	28.34	60	3.54	6.39	7.48	30	9.54
60	9.63	22.17	32.38	30	12.83	20.09	47.32	90	4.67	9.89	12.86	60	13.46
120	13.52	30.98	65.46	40	17.49	43.29	67.19	120	8.4	13.76	18.66	120	17.77
180	17.51	45.8	84.07	50	19.3	54.29	73.29	150	10.35	17.49	22.33	180	21.22

c) In acid, base, neutral, oxidative condition at 70 ℃ at different time interval

Time Pts (min)	% deg at x Conc. of HCl			Time Pts (min)	% deg at x Conc. of NaOH			Time Pts (min)	% deg at x % of H_20_2			Time Pts (min)	% deg in neutral
	X=0.001N	X=0.003N	X=0.005N		X=0.001N	X=0.003N	X=0.005N		X=6%	X=15%	X=30%		
15	12.17	22.43	25.64	10	10.29	12.46	21.12	30	2.89	3.34	4.54	15	12.63
30	16.67	25.46	39.25	20	16.23	21.36	35.37	60	5.37	7.43	9.02	30	16.71
60	20.11	26.69	53.11	30	35.32	40.22	69.32	90	6.38	11.11	14.22	60	24.62
120	27.33	35.88	67.11	40	45.3	56.38	88.37	120	10.1	16.55	23.12	120	30.54
180	31.1	54.11	89.44	50	58.29	70.32	96.37	150	13.37	19.29	26.51	180	36.14

Pts-points, RT-30°C, %deg-% degradation

Table 5.17 Summary of Degradation kinetics parameters obtained after applying stress degradation conditions (Retention time of DP obtained, rate constants, half-lives, shelf lives, correlation coefficient, order of reaction and activation energy)

Stressor conditions	**Conc. of stress**	**R^2 (temp in °C)**									**K (moles /ltr/min)**			**$t^{1/2}$ (min)**			**T^{90} (min)**			**Ea**
		Zero order			First order			Second order			30	50	70	30	50	70	30	50	70	**Kj /mol**
		30	50	70	30	50	70	30	50	70										
Acid (HCl) DP1 -4.8 min	0.001 N	0.9909	0.8895	0.9907	0.9980	0.9984	0.9959	0.8110	0.7669	0.7944	0.0053	0.0072	0.1110	3.991	2.991	1.990	0.779	0.558	0.338	-0.46
	0.003 N	0.9909	0.9449	0.9777	0.9889	0.9933	0.9941	0.8896	0.8554	0.754	0.2008	0.2002	0.1822	1.0	1.03	1.15	0.20	0.20	0.23	-0.32
	0.0005 N	0.9666	0.8833	0.9411	0.9779	0.9866	0.9669	0.7433	0.9000	0.857	0.4299	0.4007	0.3555	0.48	0.51	0.59	0.09	0.10	0.11	1.85
Base (NaOH) DP2 -3.4 min	0.001 N	0.9115	0.9112	0.9552	0.9996	0.9822	0.9981	0.8700	0.7119	0.778	0.3364	0.4000	1.2500	0.557	0.52	0.16	0.11	0.10	0.03	1.01
	0.003 N	0.9772	0.9665	0.9776	0.9932	0.9933	0.9990	0.8664	0.8667	0.941	1.1330	1.2466	1.5007	0.18	0.16	0.13	0.03	0.03	0.02	0.71
	0.0005 N	0.9777	0.9908	0.9188	0.9333	0.9978	0.9960	0.8336	0.7611	0.906	1.3667	1.6088	2.0335	0.15	0.13	0.10	0.03	0.02	0.02	2.84
Neutral		0.9633	0.9399	0.9422	0.7911	0.8222	0.8667	0.6699	0.7770	0.5122	0.0522	0.0088	0.1377	4.003	2.388	1.553	0.81	0.47	0.30	2.55
Oxidation (H_2O_2) DP3 -2.3 min	6 %	0.9400	0.9666	0.9772	0.9110	0.9775	0.9667	0.8711	0.7622	0.8288	0.0777	0.0076	0.0885	2.772	2.776	2.488	0.554	0.555	0.449	0.81
	15 %	0.8100	0.9355	0.8100	0.9889	0.9922	0.9977	0.7866	0.5887	0.6555	0.1199	0.1366	0.1330	1.776	1.554	1.611	0.335	0.330	0.332	0.29
	30 %	0.8	0.9	0.9	0.9	0.9	0.9	0.8	0.7	0.67	0.1	0.1	0.1	1.4	1.2	1.0	0.2	0.2	0.2	0.21

		97	04	57	99	95	82	32	13	4	42	69	93	74	8	941			
Photo	5382 LUX	0.958			0.946			0.880			0.189			3.66			0.55		-
Dry heat	60 ºC	0.953			0.836			0.669			0.975			0.21			0.04		-

K-rate constants, $t^{1/2}$-half-lives, t^{90}- shelf-life, Ea-activation energy R^2-correlation coefficient

Figure 5.16 Plot of first order kinetics of 0.001N HCl hydrolysis at 5 time points (viz., 15, 30, 60, 120, 180 min) at 30, 50, 70 °C

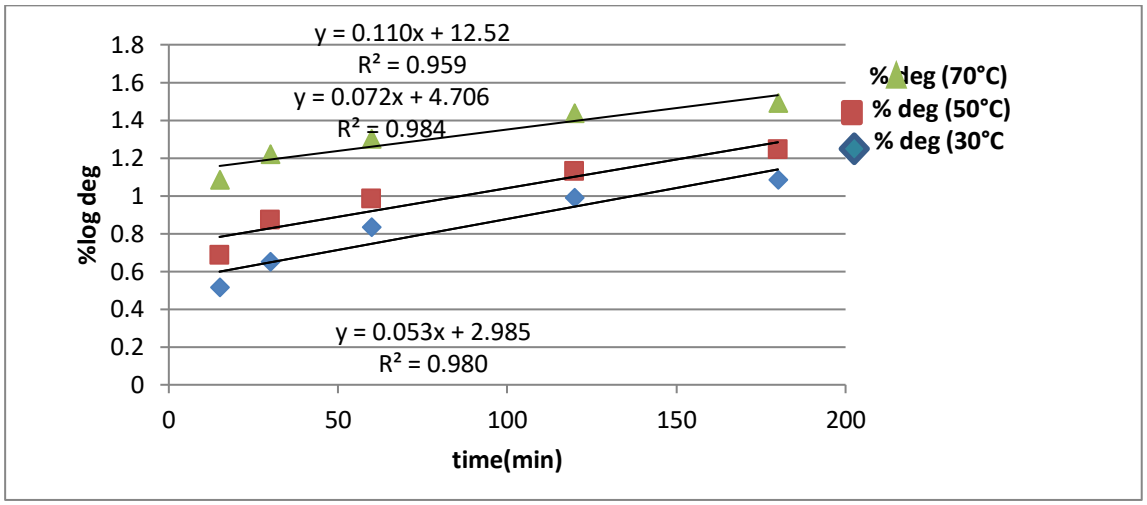


Figure 5.17 Plot of first order kinetics of 0.003N HCl hydrolysis at 5 time points (viz. 15, 30, 60, 120, 180 min) at 30, 50, 70 °C

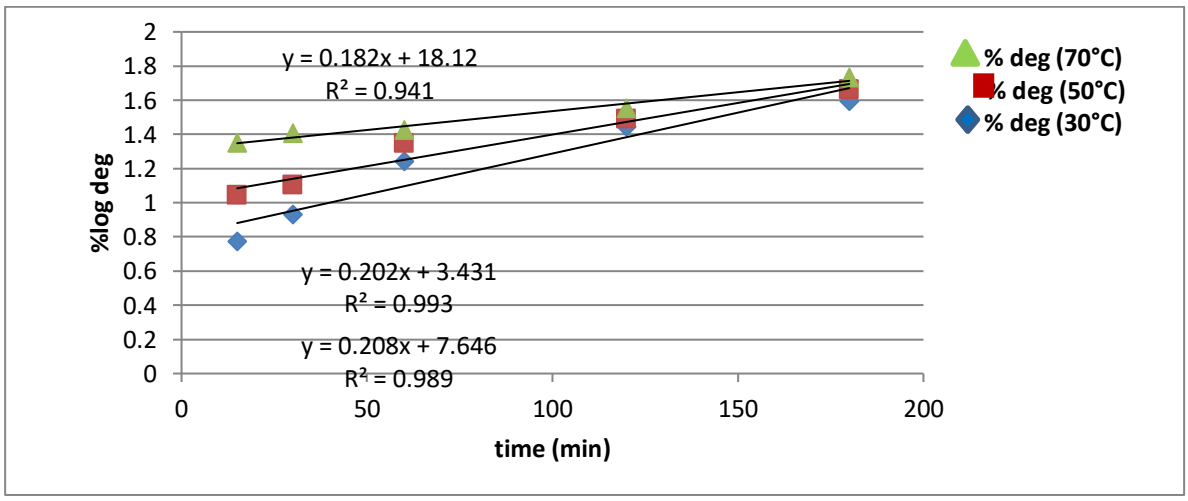


Figure 5.18 Plot of first order kinetics of 0.005N HCl hydrolysis at 5 time points (viz. 15, 30, 60, 120, 180 min) at 30, 50, 70 °C

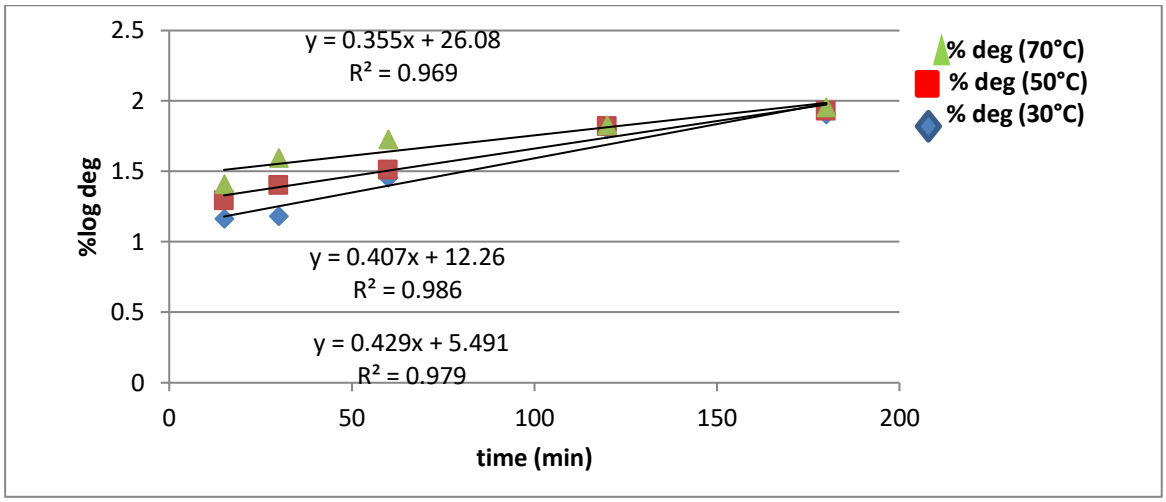


Figure 5.19 Plot of first order kinetics of 0.001N NaOH hydrolysis at 5 time points (viz., 10, 20, 30, 40, 50 min) at 30, 50, 70 °C

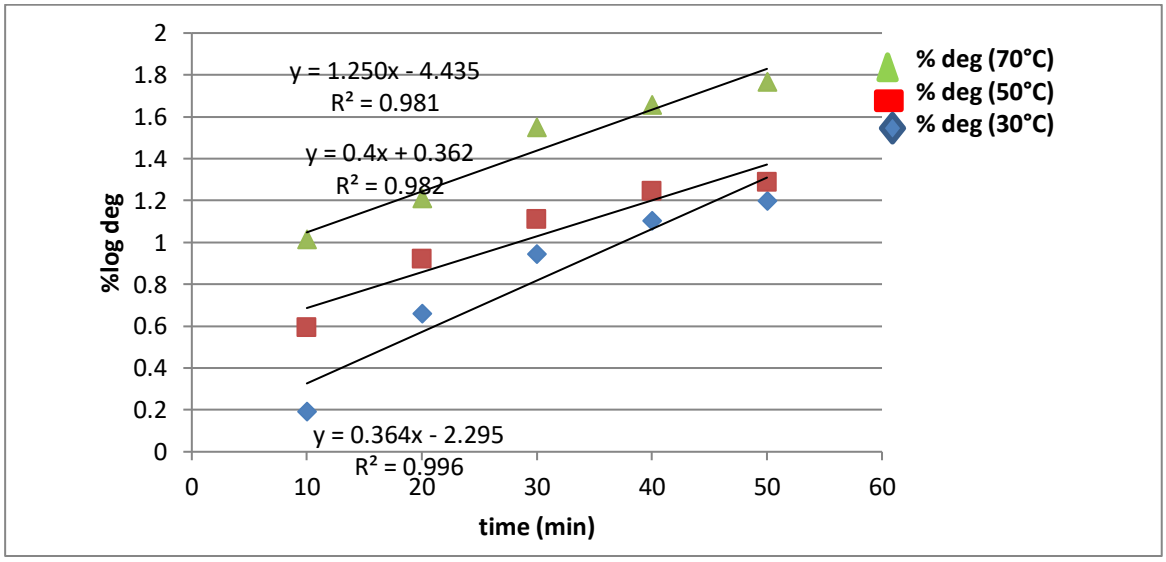


Figure 5.20 Plot of first order kinetics of 0.003N NaOH hydrolysis at 5 time points (viz., 10, 20, 30, 40, 50 min) at 30, 50, 70 °C

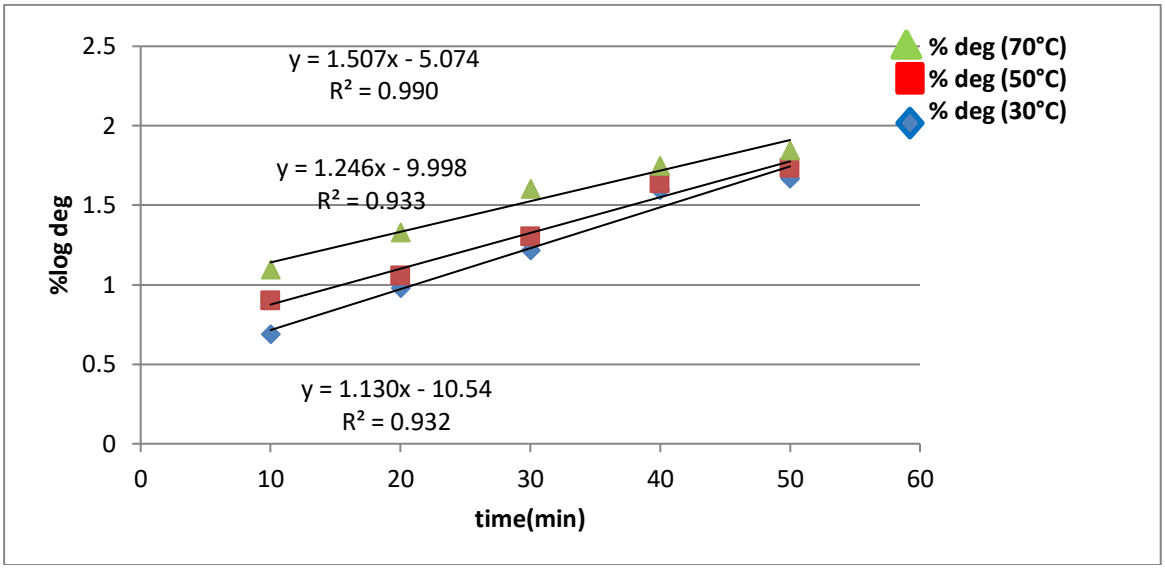


Figure 5.21 Plot of first order kinetics of 0.005N NaOH hydrolysis at 5 time points (viz., 10, 20, 30, 40, 50 min) at 30, 50, 70 °C

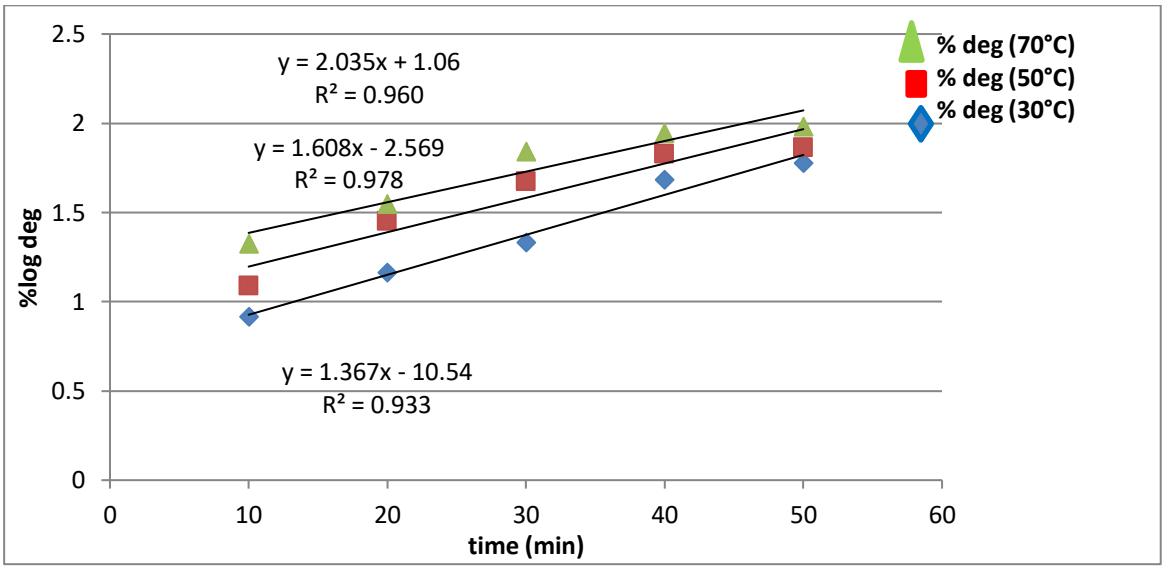


Figure 5.22 Plot of first order kinetics of 6% N H_2O_2 oxidation at 5 time points (viz., 30, 60, 90, 120, 150 min) at 30, 50, 70 °C

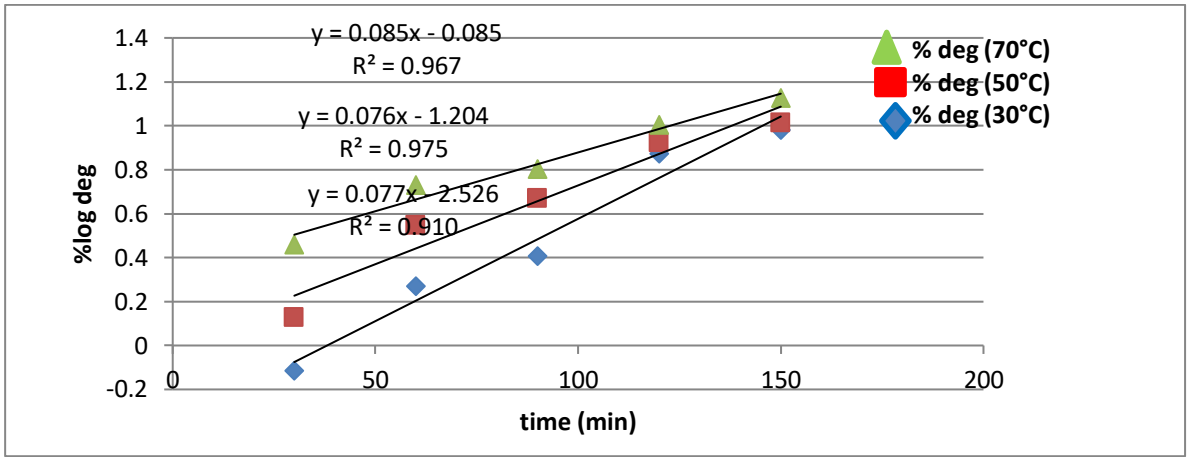


Figure 5.23 Plot of first order kinetics of 15% N H_2O_2 oxidation at 5 time points (viz., 30, 60, 90, 120, 150 min) at 30, 50, 70 °C

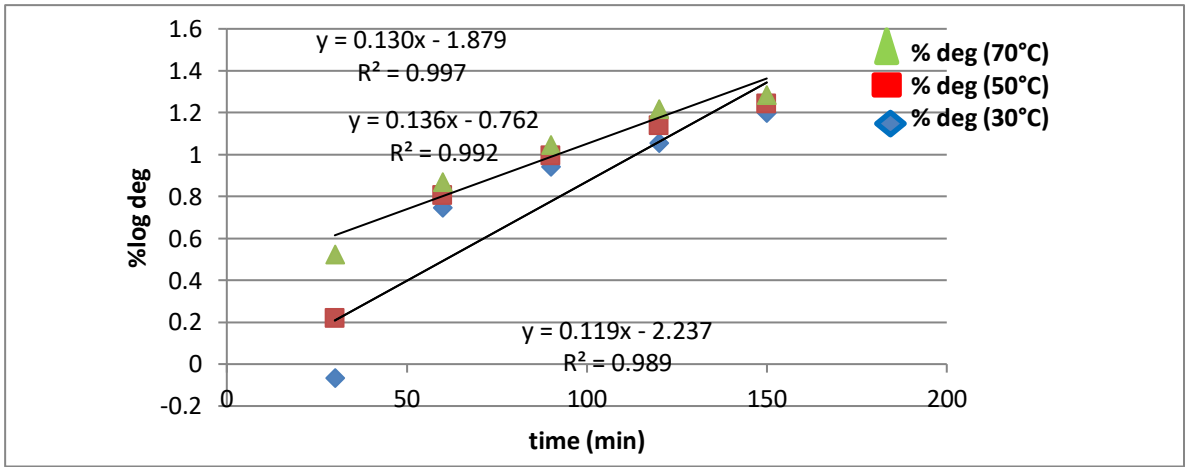


Figure 5.24 Plot of first order kinetics of 30% N H_2O_2 oxidation at 5 time points (viz., 30, 60, 90, 120, 150 min) at 30, 50, 70 °C

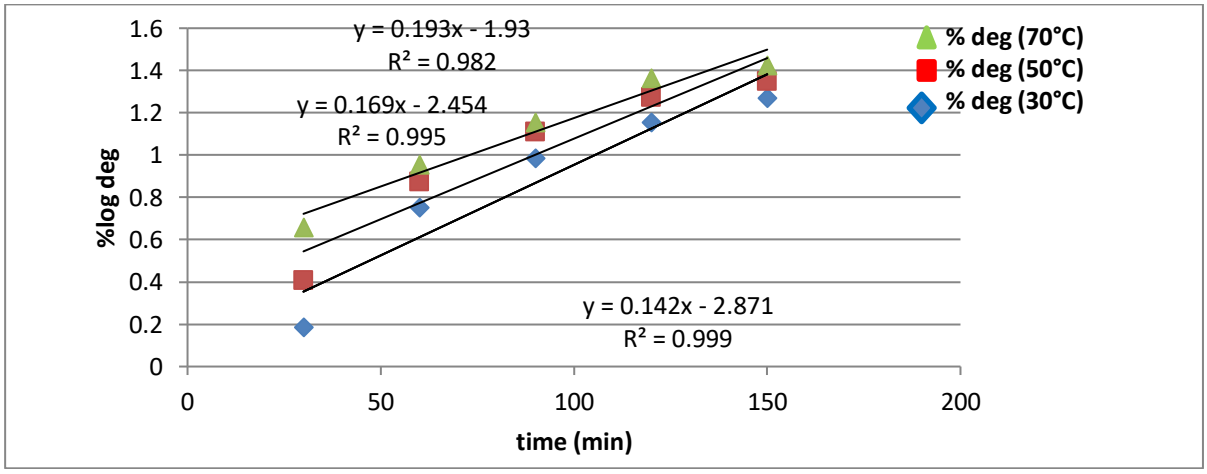


Figure 5.25 Plot of zero order kinetics for neutral hydrolysis at 5 time points (viz., 15, 30, 60, 120, 150 min) at 30, 50, 70 °C

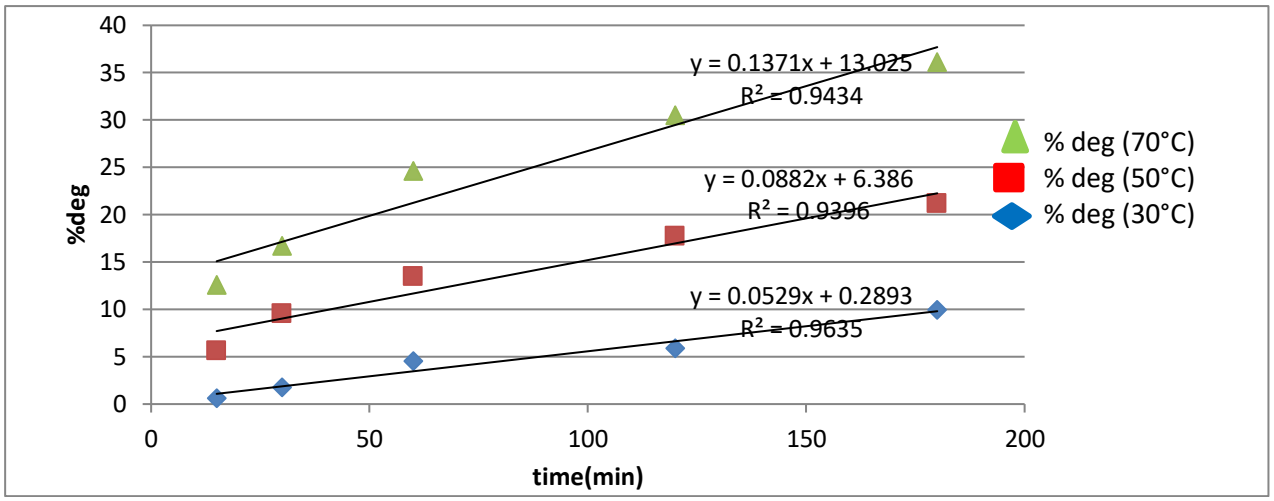


Figure 5.26 Plot of zero order kinetics for dry heat degradation at 60° C in dark for 1,3,5,7, 10, 15 days

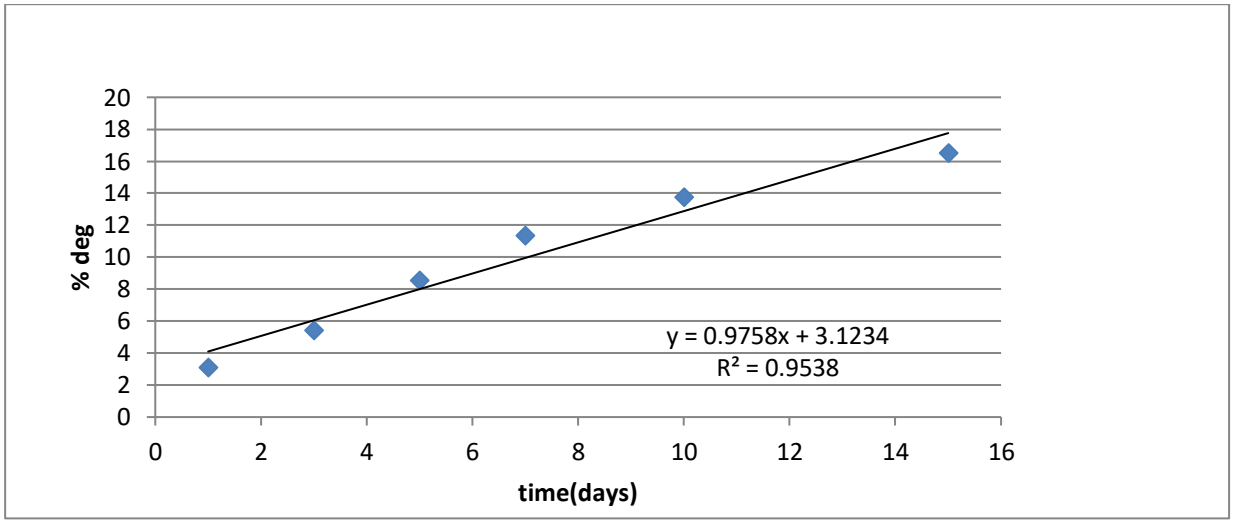


Figure 5.27 Plot of zero order kinetics for photolytic degradation at 5382 LUX and 144UW/cm2 for 1,3,5,7 days

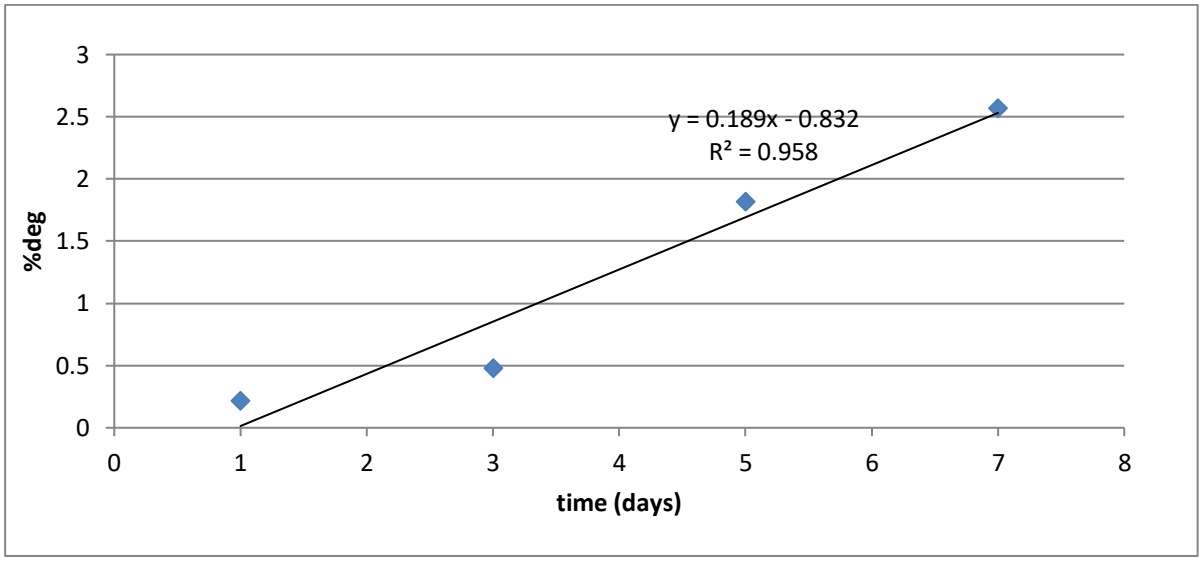


Figure 5.28 Arrhenius plot for acid degradation

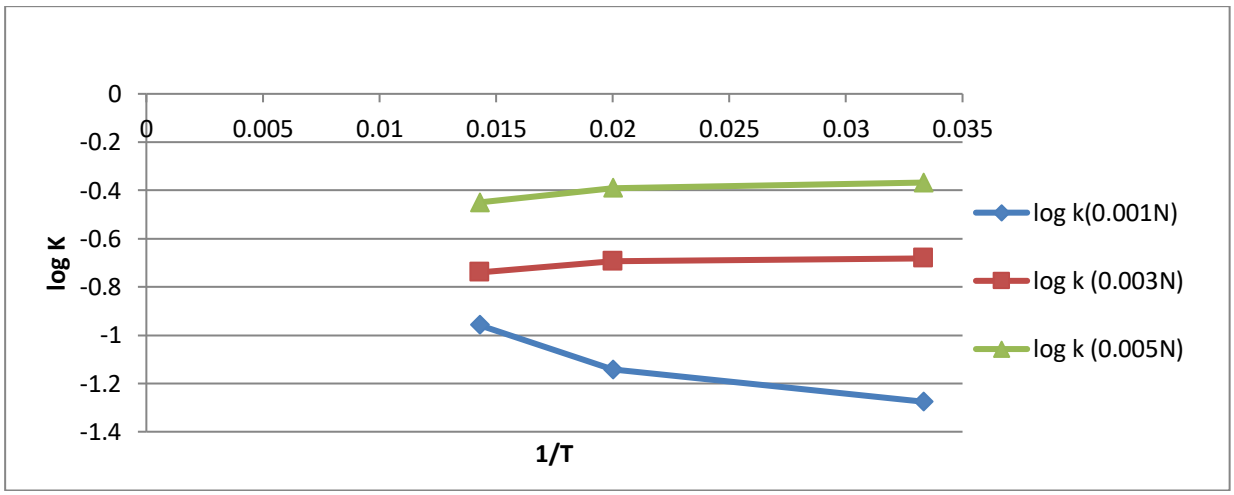


Figure 5.29 Arrhenius plot for base degradation

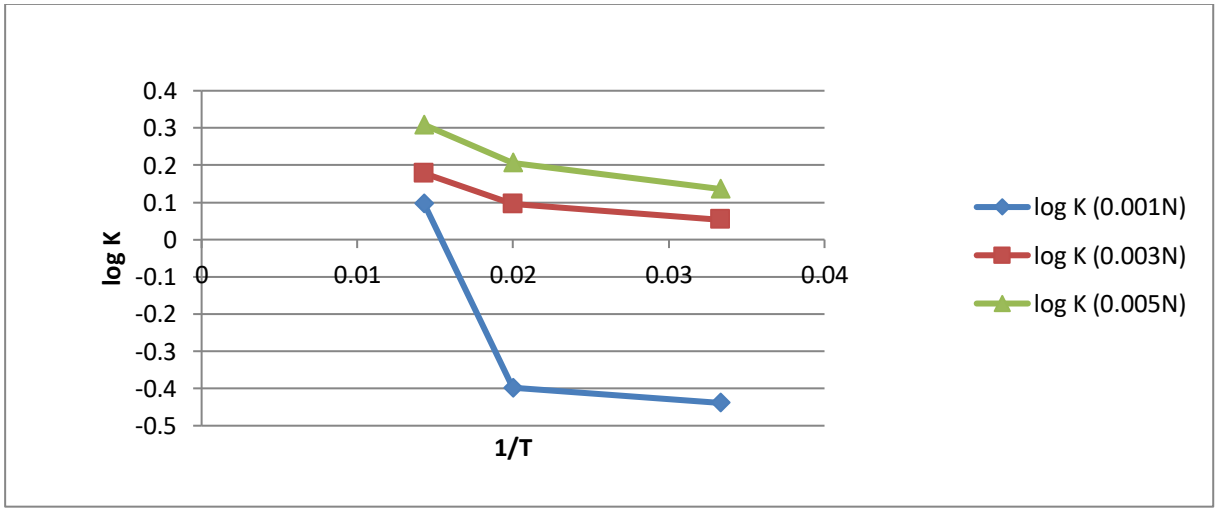


Figure 5.30 Arrhenius plot for oxidative degradation

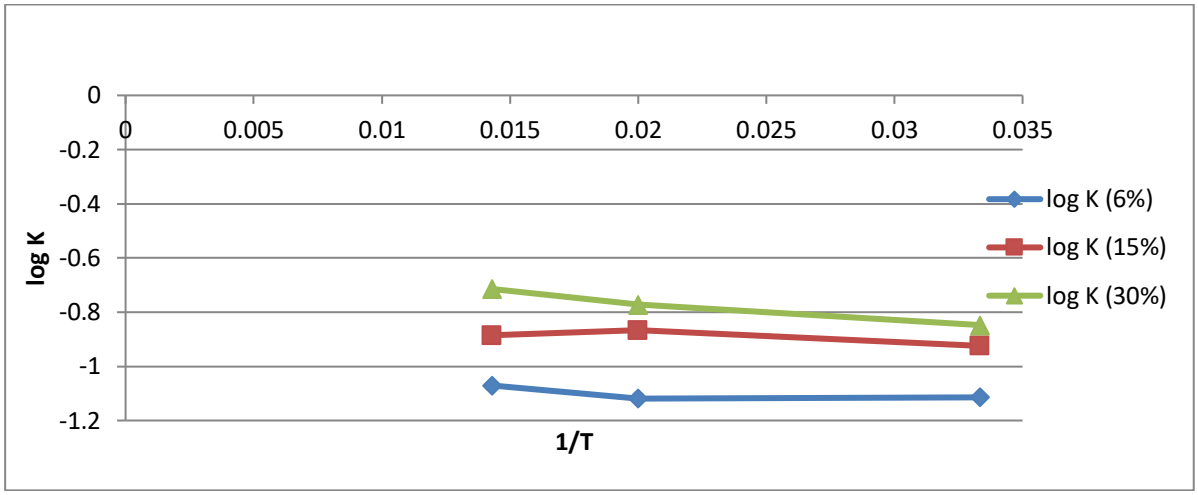
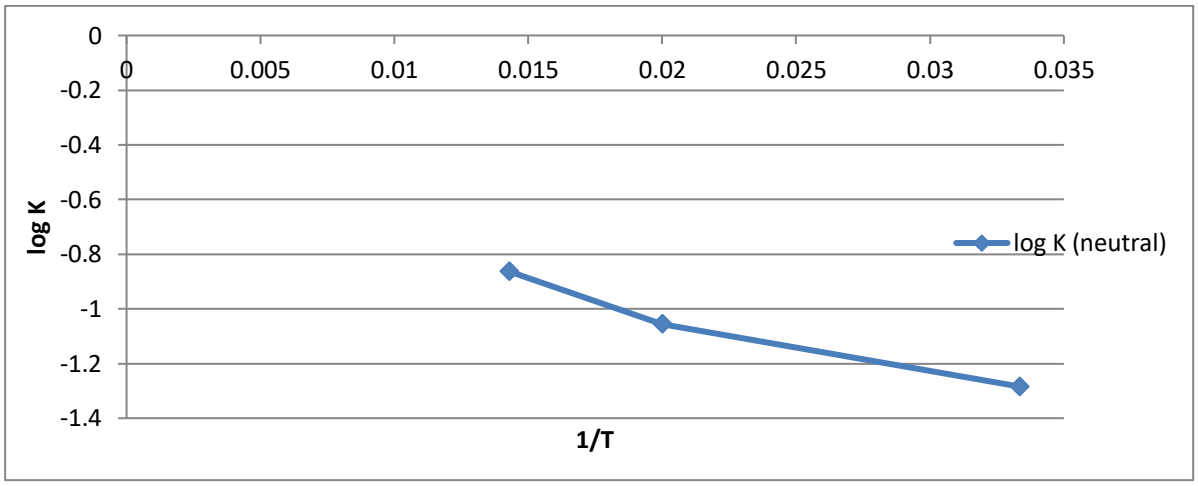


Figure 5.31 Arrhenius plot for neutral degradation



5.6 CONCLUSION

A simple, precise, and robust HPLC-PDA, DOE based gradient Stability indicating assay method have been developed and validated for the estimation of Pomalidomide in presence of its degradation products. Application of DOE helped in achievement of ATP and also provides with ample design space for future analytical conditions. The accuracy of the developed method was validated by total error approach taking uncertainty contour to be 1% and statistical analysis. Verification of accuracy results by total error approach strengthens the trust for inferring reliability of analytical method. Degraded matrix from acid, alkali, neutral hydrolysis, oxidation, photolysis and dry heat degradation showed degradation profile highlighting drug instability in acid and alkali hydrolytic conditions. Degradation kinetic studies gave the order of reaction which can be used for the prediction of degradation rate constants, half lives, shelf lives and activation energy of the chemical reaction. From our study we can suggest that caution should be taken to avoid exposure to acid and alkali hydrolytic stress conditions while formulating and analyzing Pomalidomide at laboratory

premises. The method is sensitive, accurate and fast which is applicable for the assay of POM in presence of its degradation products in bulk as well as dosage form.

5.7 REFERENCES

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