METHOD DEVELOPMENT, VALIDATION OF STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHOD AND FORCE DEGRADATION STUDY OF PYRIMIDINE DERIVATIVE

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Abstract:- An isocratic reversed phase stability-indicating high-performance liquid chromatographic (HPLC) assay method was developed and validated for quantitative determination of pyrimidine derivative and the simultaneous stability indicating study was carried out by using gradient pump system with the mobile phase Methanol/ Water (55/45) was selected to achieve maximum detection. SHIMADZU-HPLC, phenomenex C_{18}, 250×4.6mm, 5µ column was used for the detection having flow rate 1ml/min, at 225 nm. The proposed method was found to be rapid, accurate and robust.

Keywords: HPLC method development, Stability indicating method, Pyrimidine Derivative.

Introduction

High performance liquid chromatography is an integral analytical tool in assessing product stability. HPLC method able to separate, detect and quantify the various products that can form on storage or manufacturing and also detect the impurity that may be introduce during synthesis. The experimental protocol for performing stability indicating method will depends on chemistry of each compound. HPLC is distinguished from ordinary liquid chromatography because the pressure of HPLC is relatively high, while ordinary liquid chromatography typically relies on the force of gravity to provide pressure. Due to the higher pressure separation conditions of HPLC, HPLC columns have relatively small internal diameter (e.g.4.6mm), are short (e.g.25mm), and packed more densely with smaller particles, which helps achieve finer separations of a sample mixture than ordinary liquid chromatography can. This gives HPLC superior resolving power when separating mixtures, which is why it is a popular chromatographic technique.

Ashihara *et.al*[1] reported that purine and pyrimidine nucleotides, extracted from cultured plant cells with 6% perchloric acid, were separated directly with HPLC using anion-exchange Shimpack WAX-1 column. More than fifteen nucleoside mono-, di-, and triphosphates and nucleotide sugars were clearly separated and quantified without any interference from plant phenolic compounds. Hartmann *et.al*[2] used reversed-phase HPLC electrospray ionization tandem mass spectrometry to investigate 24 metabolites of purine and pyrimidine metabolism in urine samples from healthy persons and from patients with confirmed diagnoses of inherited metabolic disorders.

Markelj *et.al*[3] reported that nine purine and pyrimidine bases were separated and determined simultaneously using reversed phase high performance liquid chromatography (RP-HPLC) in some food samples and biological fluids. Lenthe *et.al*[4] used urine or urine-soaked filter-paper strips as samples and measured thymine, uracil, and their degradation products dihydrothymine, dihydrouracil, N-carbamyl β-aminoisobutyric acid, and N-carbamyl β-alanine. Reversed-phase HPLC was combined with electrospray ionization tandem mass spectrometry, and detection was performed by multiple-reaction monitoring. Stable-isotope-labeled reference compounds were used as internal standards. All pyrimidine degradation products could be measured in one analytical run of 15 min.

Furthermore, Kuilenburg *et.al*[5] developed a fast and sensitive method, using HPLC-tandem mass spectrometry, allowing the detection of all pyrimidine de novo metabolites within a single analytical run of 14 min, using urine or urine soaked filter paper strips. Fan *et.al*[6] reported a method based on optimum acid hydrolysis followed by high performance liquid chromatography (HPLC) with diode array detection was developed for quantitative determination of bio-available nucleosides, present as purine and pyrimidine bases including adenine, cytosine, guanine, hypoxanthine, thymine and uracil, in natural and cultured *Cordyceps*. The chromatographic behavior of some purines and pyrimidines on a monolithic Chromolith Performance Si column under normal-phase high-performance liquid chromatography mode has been studied by Kazoka[7].

Tidke and Solanki[8] reported identification and quantification of benzoic acid from local market beverages of Amravati (M.S.) which was investigated at HPLC Shimadzu LCAT system. The HPLC analysis was performed by isocratic elution with mobile phase acetonitrile and ammonium acetate buffer 4.2 PH in 40:60 respectively. UV detection was carried out at 228nm by using phenomenex C18 column (250 × 4.6 mm, 5µ) at ambient temperature and flow rate of 1ml/min by external standard methods. Precision, Accuracy, Robustness, Stability, Sensitivity LOD and LOQ were established. The obtained results showed that the benzoic acid concentration varied between the different kinds of beverage samples with level lower than the maximum values established by national and international legislation.

Kamkhede and Solanki[9] reported the development and validation of thiazole derivatives under various stress conditions, as acid/base hydrolysis, oxidative stress degradations is carried out in this process. The simultaneous forced degradation study of thiazole derivatives, using gradient pump system the mobile phase water: methanol 30:70 was selected to achieve maximum detection, sensitivity at ambient temperature using phenomax c-18 column (250 mm × 4.5 mm, 5µl) flow rate 1.0 ml/min, at 238 nm. The proposed method was found to be rapid, accurate and consistent.

An isocratic reversed phase stability-indicating high-performance liquid chromatographic (HPLC) assay method was developed and validated Ughade and Solanki[10] for quantitative determination of Semicarbazone Derivative and the simultaneous stability indicating study was carried out by using gradient pump system the mobile phase methanol : water 70:30v/v was selected to achieve maximum detection, sensitivity at ambient temperature using Lichrospher C18 column (250 mm × 4.6 mm,5µl) flow rate 1.0 ml/min, at 285 nm. The proposed method was found to be rapid, accurate and consistent.

Method development, validation of stability indicating liquid chromatographic method and force degradation study of imidazole derivative of high pharmaceutical value was reported by Tidke and Solanki[11]. An isocratic reversed phase stability-indicating high-performance liquid chromatographic (HPLC) assay method was developed and validated for quantitative determination of Imidazole Derivative and the simultaneous stability indicating study was carried out by using gradient pump system with the mobile phase Acetonitrile/ Sodium dihydrogen orthophosphate buffer P^H-3 (55/45) was selected to achieve maximum detection. SHIMADZU-HPLC, phenomenex C_{18}, 250×4.6mm, 5µ column was used for the detection having flow rate 1.2 ml/min, at 225 nm. The proposed method was found to be rapid, accurate and robust.

Material and method

Chromatographic conditions

In this study high performance liquid chromatography system with LC solution data handling system (SHIMADZU-LCAT) was used for analysis. The system was controlled and data was recorded with spinchrome (RF software). The assays were performed on LC system consisting of SHIMADZU-LC 20 AT pump and SHIMADZU-UV detector. The injection volume was 20µl and it is injected in rhenodyne injector system. The detector was set at 225nm and peak area was integrated automatically by computer using spinchrome CRF software. Detection was carried out by using phenomenex C_{18} column (250mm × 4.6mm, 5µ (micron)) at ambient temperature all the calculation consisting quantitative analysis ware perform with external standardization by the measurement of peak area. The mobile phase was filtered through 0.45µ membrane and solution through 0.25µ, filtered and degassed. The injection volume was 20µl and analysis was performed at ambient temperature.

Preparation of stock solution

The standard solution of concentration 1mg/ml as of 7-amino-4-(2,4-dichlorophenyl)-4,4a,6,7-tetrahydrothieno[3,2-d] pyrimidin-2(3H)-one prepared by using mobile phase methanol / water (55/45). The stock solution was stored at ambient temperature, protected from air and sunlight from the standard stock solution working standard solutions was prepared using mobile phase to get 0.005-0.030µg/ml. The solution were stored at ambient temperature and protected from direct sunlight.

Optimum chromatographic conditions

Table -1

Parameter	Optimum condition
Chromatographic column	SHIMADZU-HPLC, phenomenex C_{18}, 250×4.6mm, 5µ
Mobile phase	Methanol/ Water (55/45)
Flow rate	1 ml/min
Detection	225nm
Injection volume	20 µl
Temperature	Ambient
Retention time pyrimidine	8.083

Standard chromatogram

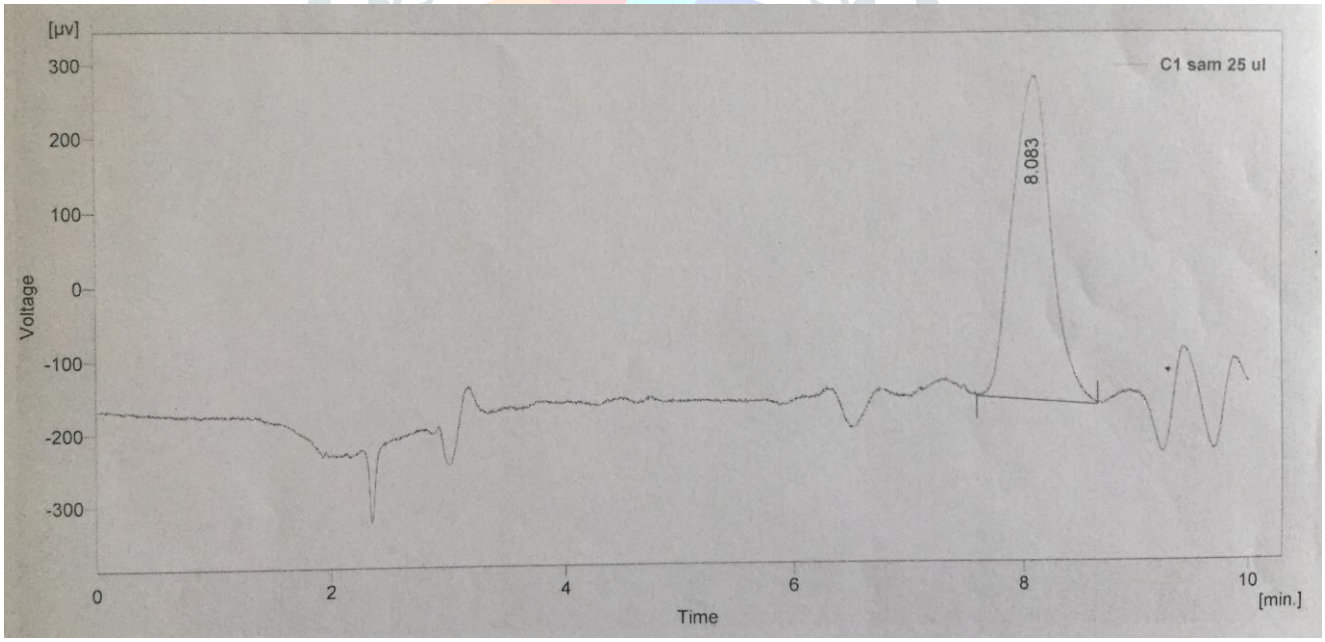
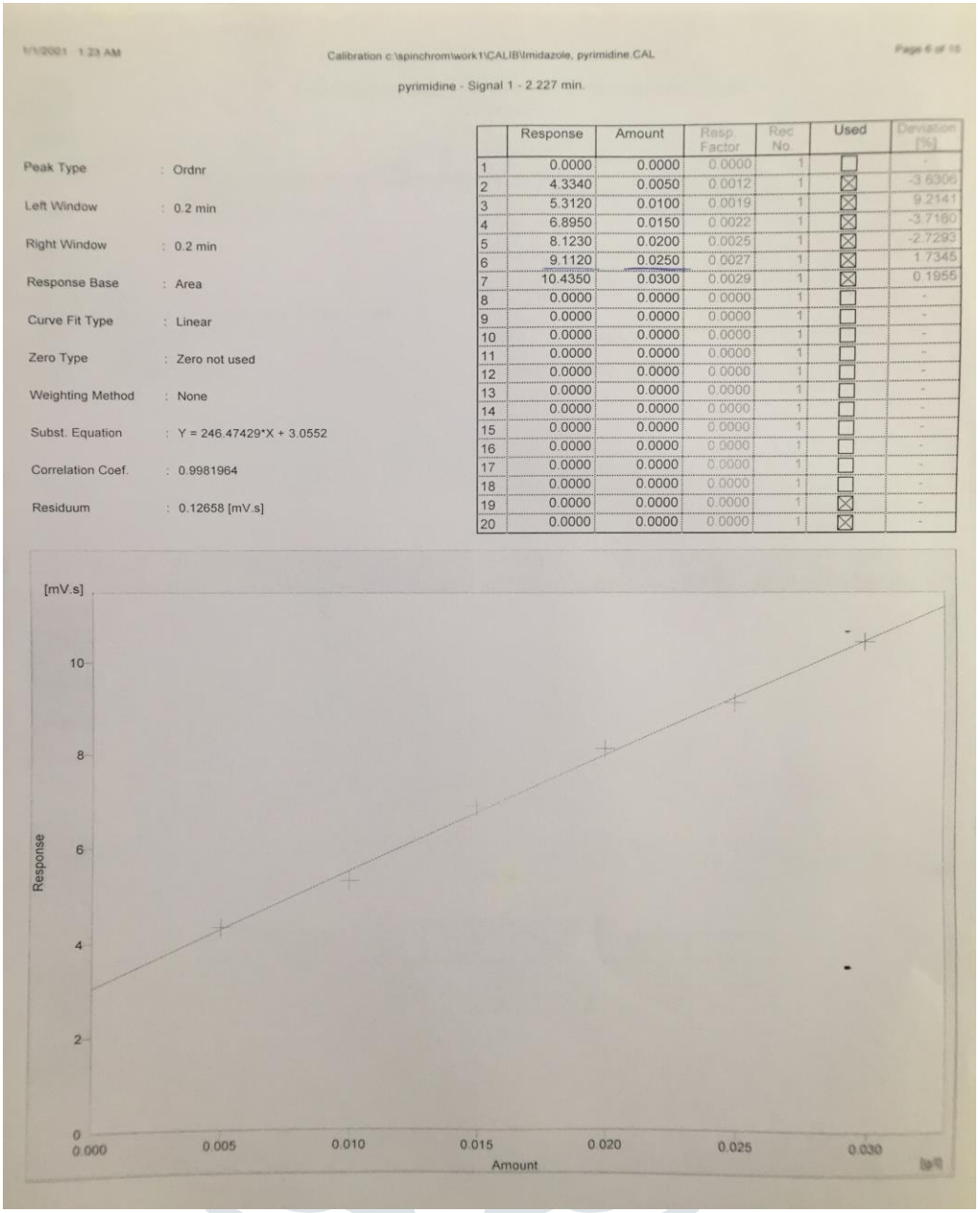


Fig-1

Calibration

Standard solutions of pyrimidine derivative were prepared in mobile phase i.e Methanol/ Water (55/45) having different concentration of 0.005-0.030µg/ml. Triplicate 20µl injection were made for each standard solution to see the reproducibility of detection response at each concentration level. The peak area of each solution was plotted against concentration to obtain the calibration graph. The five concentrations of compounds were subjected to regression analysis to calculate the calibration equation and correlation coefficients.



	Response	Amount	Resp. Factor	Rec No.	Used	Deviation [%]
1	0.0000	0.0000	0.0000	1	☐	-
2	4.3340	0.0050	0.0012	1	☒	-3.6306
3	5.3120	0.0100	0.0019	1	☒	9.2141
4	6.8950	0.0150	0.0022	1	☒	-3.7160
5	8.1230	0.0200	0.0025	1	☒	-2.7293
6	9.1120	0.0250	0.0027	1	☒	1.7345
7	10.4350	0.0300	0.0029	1	☒	0.1955
8	0.0000	0.0000	0.0000	1	☐	-
9	0.0000	0.0000	0.0000	1	☐	-
10	0.0000	0.0000	0.0000	1	☐	-
11	0.0000	0.0000	0.0000	1	☐	-
12	0.0000	0.0000	0.0000	1	☐	-
13	0.0000	0.0000	0.0000	1	☐	-
14	0.0000	0.0000	0.0000	1	☐	-
15	0.0000	0.0000	0.0000	1	☐	-
16	0.0000	0.0000	0.0000	1	☐	-
17	0.0000	0.0000	0.0000	1	☐	-
18	0.0000	0.0000	0.0000	1	☐	-
19	0.0000	0.0000	0.0000	1	☒	-
20	0.0000	0.0000	0.0000	1	☒	-

Fig -2

Result and discussion

Method validation

HPLC analysis was performed by isocratic elution with a series of mobile phase containing Methanol/ Water (55/45). The flow rate was determined by testing the effects of flow rate on a pick area and resolution i.e 0.9ml/min, 1ml/min, 1.1ml/min. 1ml/min was found to be optimum. All experiment carried out at ambient temperature. To determine appropriate wavelength for determination of pyrimidine in mobile phase several trails were carried out. The suitable wavelength selected for monitoring the pyrimidine derivative was 225nm. All the solutions were filtered, degassed.

It was observed that there was no interference from the mobile phase or baseline disturbance and all the analyte were observed at 225nm. The chromatographic run time was 10 min and column void volume 8.083 min. Through the study the stability of chromatograph i.e. system was monitored by calculating the resolution. Table no-1. Fig - 1

Validation of optimized HPLC method for pyrimidine derivative was carried out with respect to following parameters given in table format.

Precision

It can be defined as "response to the repeated injection of the same standard solution under normal operating conditions". The % RSD value for this method should be ≤ 1.0%. The precision studies were performing by the analysis of five different concentrations i.e 0.005-0.030µg/ml for pyrimidine derivative six times on same day. The method passed the test for repeatability as determined by %RSD of the peak of six replicate injections at test concentration. The results are shown in following table - 2

Table – 2

Compound name	λmax	Peak area	% RSD
of 7-amino-4-(2,4-dichlorophenyl)-4,4a,6,7-tetrahydrothieno[3,2-d] pyrimidin-2(3H)-one	225	9.112	0.18

Accuracy

Accuracy was determined by the comparison of spike concentration of pyrimidine derivative with the measured concentration. A standard working solution containing pyrimidine derivative yielding final concentration of 0.005, 0.010, 0.015, 0.020, 0.025 and 0.030µg/ml respectively were prepared. The prepared standards were injected six times as a test sample. The result for accuracy is given in the table bellow, reviewed that the method was accurate. Table - 3

Table – 3

Compound name	Spik concentration	Measured concentration	%RSD	% Daviation
of 7-amino-4-(2,4-dichlorophenyl)-4,4a,6,7-tetrahydrothieno[3,2-d] pyrimidin-2(3H)-one	0.025µl	0.024 µl	0.024	4

LOD and LOQ

The standard stock solution was further diluted to get pyrimidine derivative concentration in the range 0.005-0.030µg/ml. Linearity of the method was studied by injecting five concentration of the pyrimidine derivative prepared in the mobile phase in triplicate in to LC system, keeping the injection volume constant. The peak areas were plotted against corresponding concentration to obtain calibration graph. Table presents the equation of the regression line, correlation coefficient (r^2). Relative standard deviation (RSD) values of the slope and intercept for pyrimidine derivative. Excellent linearity was obtained for compounds between the peak area and concentration of 0.005-0.030µg/ml with r^2=0.9981964. Fig - 2

Dilution of pyrimidine derivative was made from standard stock solution. The samples of pyrimidine derivative were injected in LC system LOD and LOQ were experimentally verified by six injections of at the LOD and LOQ concentration. By using standard deviation of response and slope the LOD was calculated to be 0.066mg/ml and LOQ was calculated 0.202 mg/ml for pyrimidine derivative respectively. Table – 4

Table – 4

Compound name	λmax	Equation	R^2	LOQ mg/l	LOD mg/l
of 7-amino-4-(2,4-dichlorophenyl)-4,4a,6,7-tetrahydrothieno[3,2-d] pyrimidin-2(3H)-one	225	Y=246.47429 X + 3.0552	0.9981964	0.202	0.066

Ruggedness

The method ruggedness is defined as the reproducibility of results when then the method is performed under different condition such as different operator in same laboratories, different equipment in same laboratory, different source of reagent and solution, different source of column, etc.

The ruggedness of HPLC method was evaluated by carrying out analysis using standard working solution the same chromatographic system and same column for different days the prepared solution was injected six times as test sample. Small difference in area and good consistency in retention time were obtained. The comparable detector responses obtain on different day indicated that the method is capable of producing result with high precision of different days. Table - 5

Table – 5

Date	7 Nov	8 Nov	9 Nov
Name	Pyrimidine	Pyrimidine	Pyrimidine
Area	9.112	9.090	9.224
SD	0.034	0.040	0.043
%RSD	0.142	0.175	0.184

Robustness

To evaluate the robustness of HPLC method, few parameters were change. The parameter includes variation of flow rate of mobile phase and solvent from different lots were taken. Robustness of the method was carried out in triplicate only one parameter was change in experiment time. The differentiation of 0.025µg/ml pyrimidine derivative under various conditions was performed. Each mean value was compared with mean value obtained by optimum condition. The statically comparison was done with the test and no difference was found between result. Therefore the result shows that method is robust to the small change in experimental condition. Table - 6

Table – 6

Flow rate	1.1 ml/min	1 ml/min	0.9 ml/min
Mean	8.012	8.083	8.097
SD	0.043	0.036	0.038
%RSD	0.212	0.173	0.185

Force Degradation Study

Forced degradation studies show the chemical behaviour of the molecule which in turn helps in the development of formulation and package. A forced degradation study is an essential step in the design of a regulatory compliant stability program for both drug substances and products, and formalized as a regulatory requirement in ICH Guideline Q1A in 1993. Forced degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions. It is required to demonstrate specificity of stability indicating methods and also provides an insight into degradation pathways and degradation products of the drug substance and helps in elucidation of the structure of the degradation products. Thus, this review discusses the current trends in performance of forced degradation studies by providing a strategy for conducting studies on degradation mechanisms

Alkaline hydrolysis

At height temperature

1 mg/ml of pyrimidine derivative solution was prepared in 10 ml volumetric flask. In a round bottom flask 1 mg/ml of pyrimidine derivative solution was reflux with 1 ml of 0.1 M NaOH at 80^0 C for 30 minutes. After cooling

the reaction mixture was neutralised, diluted, filtered and sonicated. 20 µl of this reaction mixture was injected to HPLC system in triplicate to observe degradation at high temperature.

At room temperature

Equal volume of reactant and 0.1 M NaOH solution was treated in volumetric flask and kept 24 hrs at room temperature. After 24 hrs reaction mixture was neutralized, diluted, filtered and sonicated. 20 µl of this reaction mixture was injected to HPLC system to analyze the degradation of product. The study was carried out in triplicate.

Table – 7

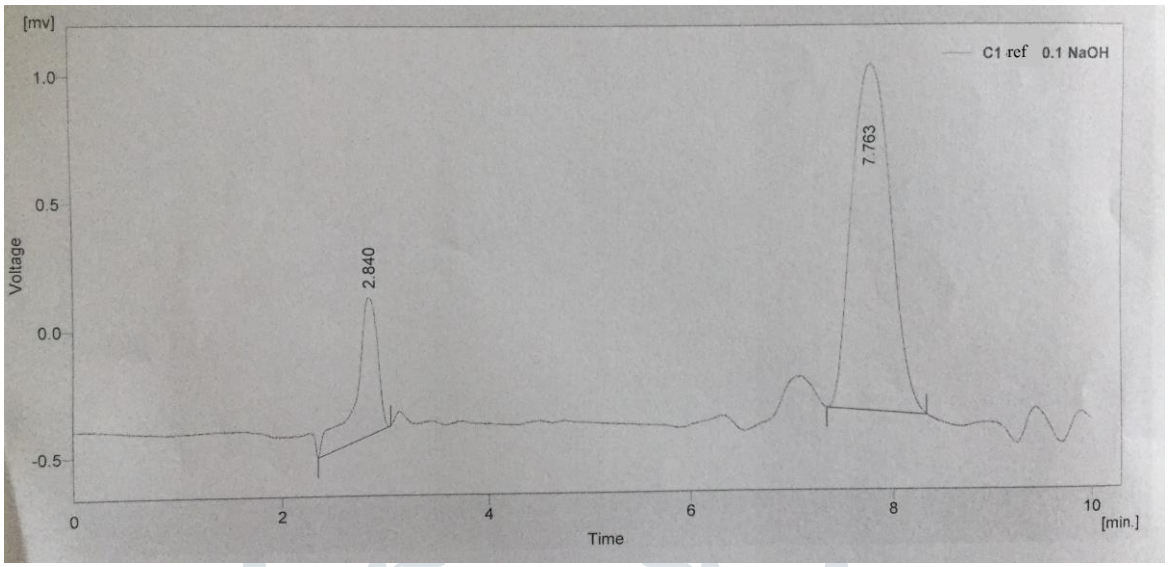


Fig - 3

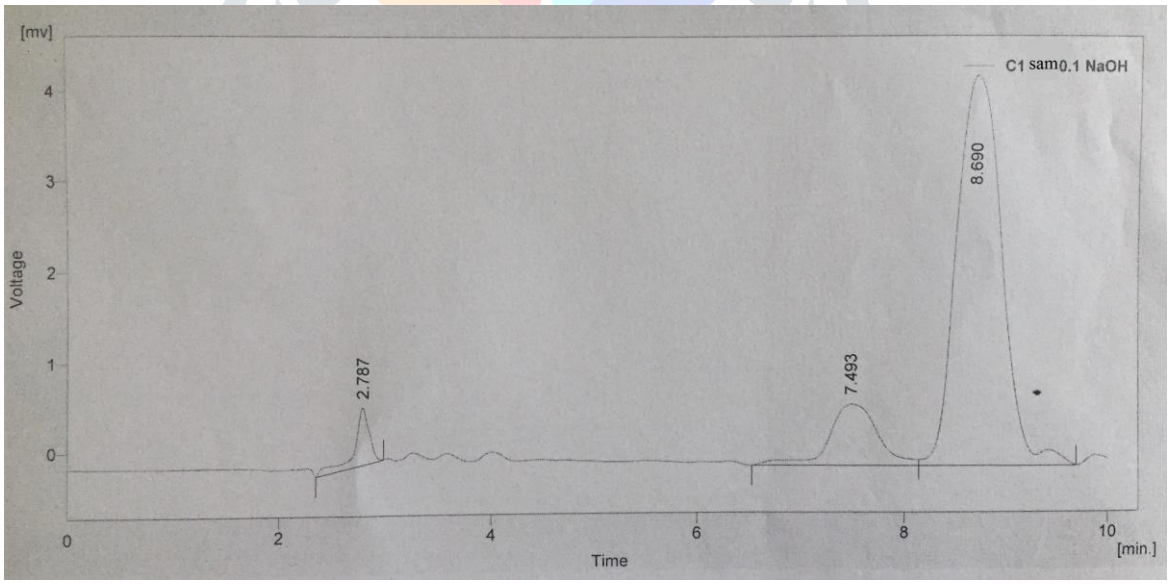


Fig - 4

Table - 7

Sr. No	Reaction condition	Retention time	λmax	Peak Area	Concentration	Degradation %	Recovery %
1	T.P 80^{0}C Base	7.767	225	36.980	0.137	85.31	14.67
2	R.T 24 hr Base	8.690	225	133.589	0.52	46.95	53.05

Acid Hydrolysis

At height temperature

1 mg/ml of reactant solution was reflux with 1 ml of 0.1 M HCl at 80^0 C for 30 minutes. After cooling the reaction mixture, was processed as above. 20 µl of this reaction mixture was injected to HPLC system in triplicate to observe degradation at high temperature.

At room temperature

Equal volume of drug and 0.1 M HCl solution was kept in volumetric flask at room temperature. After 24 hrs reaction mixture was processed as above and 20 µl of this reaction mixture was injected to HPLC system to analyze the degradation of product. The study was carried out in triplicate. Table - 8

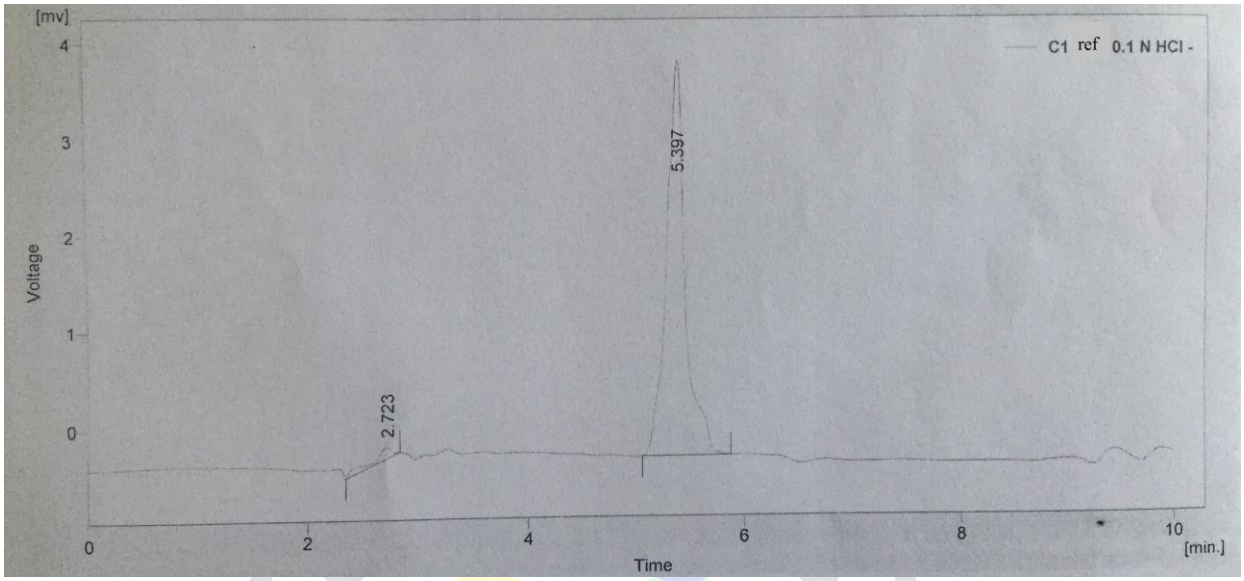


Fig - 5

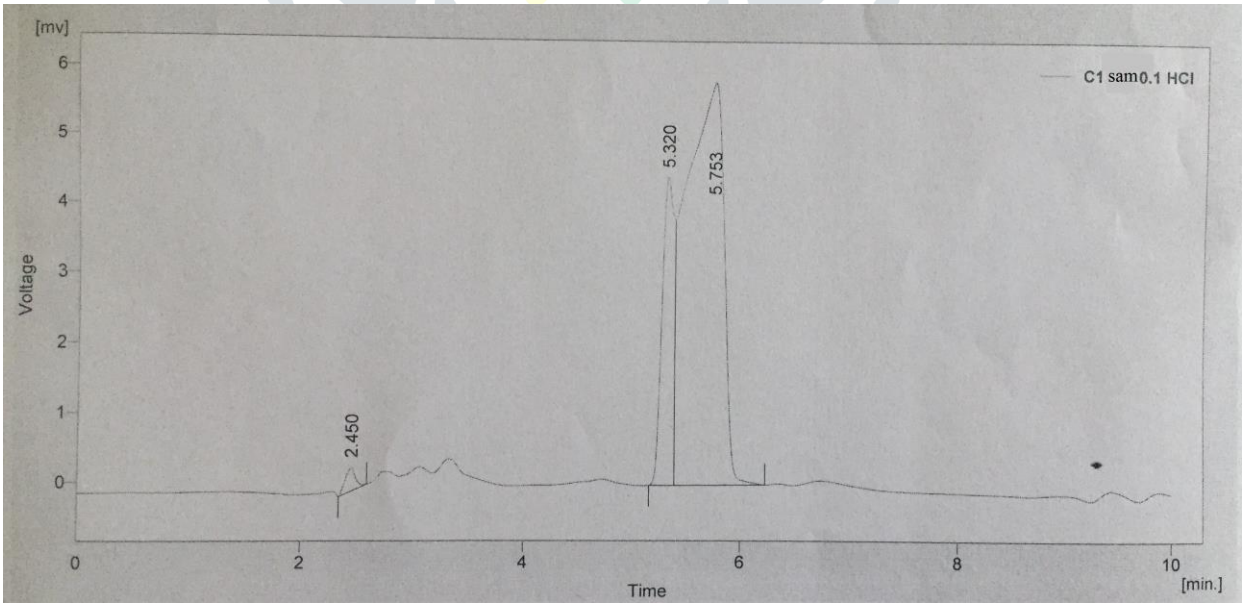


Fig - 6

Table – 8

Sr. No	Reaction condition	Retention time	λmax	Peak Area	Concentration	Degradation %	Recovery %
3	T.P 80^{0}C Acid	No peak found	225	0	0	100	0
4	R.T 24 hr Acid	No peak found	225	0	0	100	0

Oxidative Degradation 3% H_2O_2

At height temperature

1 mg/ml of pyrimidine derivative solution was reflux with 1 ml of 3% of H_2O_2 at 80^0 C for 30 minutes. After cooling the reaction mixture diluted with mobile phase filtered and sonicated. 20 µl of this reaction mixture was injected to HPLC system in triplicate to observe degradation at high temperature.

At room temperature

1ml of this solution was treated with 1 ml 3% of H_2O_2 solution in volumetric flask and kept 24 hrs at room temperature. After 24 hrs reaction mixture was processed as above.20 µl of this reaction mixture was injected to HPLC system to analyze the degradation of product. The study was carried out in triplicate. Table – 9

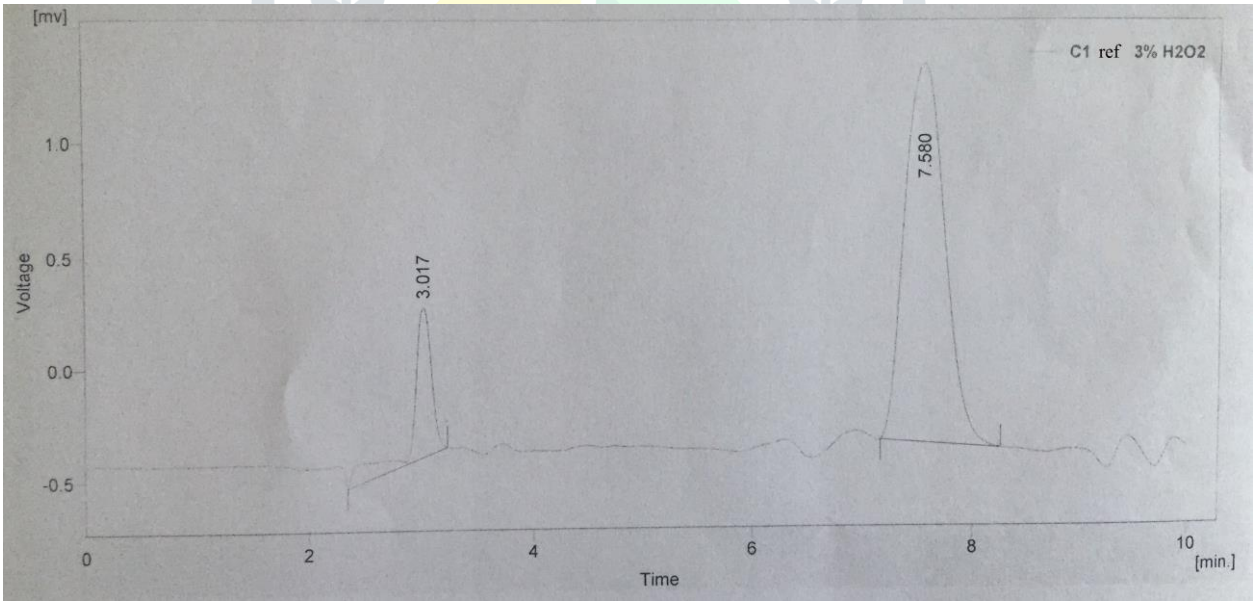


Fig - 7

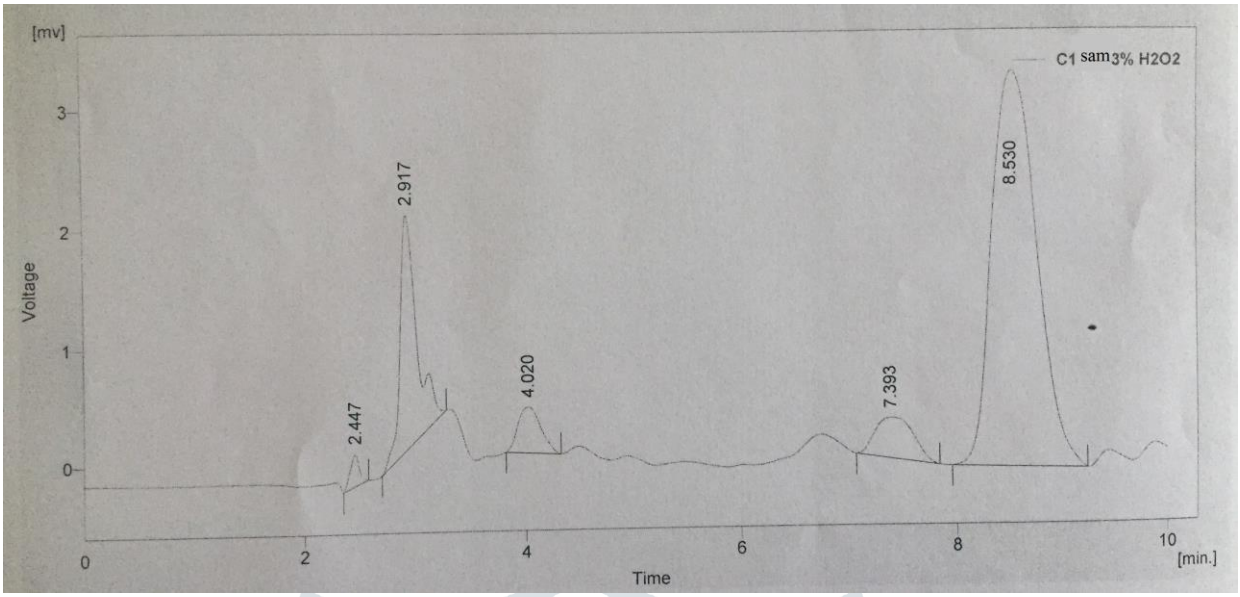


Fig - 8

Table – 9

Sr. No	Reaction condition	Retention time	λmax	Peak Area	Concentration	Degradation %	Recovery %
5	T.P 80^0C 3% H_2O_2	7.580	225	43.217	0.16	82.83	17.17
6	R.T 24 hr 3% H_2O_2	8.530	225	100.077	0.39	60.25	39.75

Oxidative Degradation 5% H_2O_2

At height temperature

1 mg/ml of pyrimidine derivative solution was reflux with 1 ml of 5% of H_2O_2 at 80^0 C for 30 minutes. After cooling the reaction mixture diluted with mobile phase, filtered and sonicated. 20 µl of this reaction mixture was injected to HPLC system in triplicate to observe degradation at high temperature.

At room temperature

Equal volume of reactant and 5% of H_2O_2 solution kept in volumetric flask at room temperature. After 24 hrs reaction mixture was diluted with appropriate mobile phase, filtered and sonicateed. 20µl of this reaction mixture was injected to HPLC system to analyze the degradation of product. The study was carried out in triplicate. Table - 10

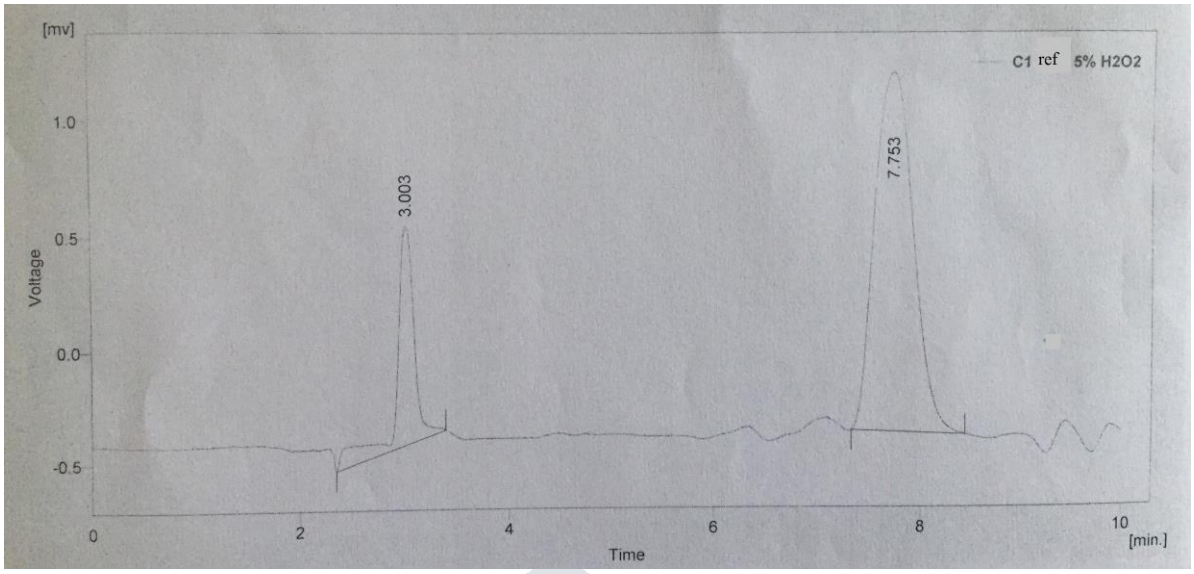


Fig - 9

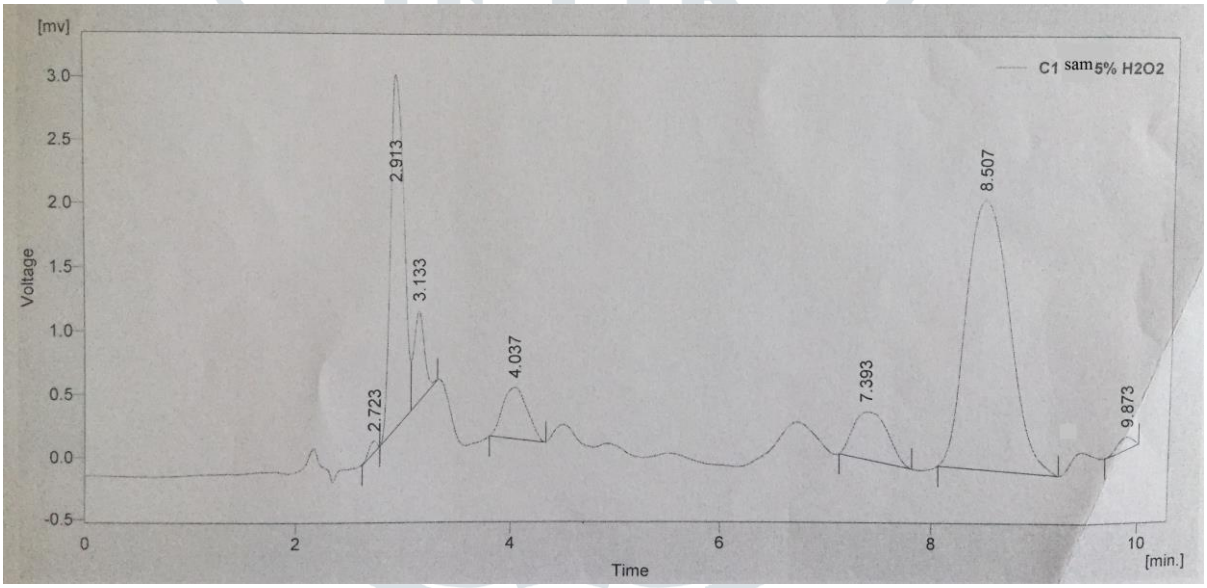


Fig - 10

Table – 10

Sr. No	Reaction condition	Retention time	λmax	Peak Area	Concentration	Degradation %	Recovery %
7	T.P 80^{0}C 5% H_2O_2	7.753	225	41.357	0.15	83.57	16.43
8	R.T 24 hr 5% H_2O_2	8.507	225	61.506	0.23	75.57	24.43

Conclusion

The method developed for pyrimidine derivative is having Chromatographic column SHIMADZU-HPLC, phenomenex C_{18}, 250×4.6mm, 5µ and Mobile phase Acetonitrile/ Sodium dihydrogen orthophosphate buffer P^H-3 (55/45) and methanol/water (55/45) on flow rate 1ml/min. The detection is carried out on wavelength 225nm. The

injection volume of sample is 20 µl. The readings were taken on ambient temperature. The retention time of pyrimidine derivative is 8.083.

The %RSD value for pyrimidine derivative is 0.18 shows that the method is precise. The method is accurate by comparing the spike concentration and measured concentration. The calibration plot of response v/s concentration shows a straight line showing five different concentrations. The correlation coefficient value for pyrimidine derivative is r^2=0.9981964 shows the method is linear.

The limit of quantification LOQ for pyrimidine derivative is 0.202mg/l. The limit of detection and for pyrimidine derivative is 0.066mg/l. Reading taken of different dates gives %RDS ≤ 1.0% shows that the method is rugged. The results of readings taken on different flow rate shows the method is robust.

RP-HPLC method was successfully developed for the determination of stability of pyrimidine derivative. The developed method is selective precise, accurate and linear. From all the results of method validation of pyrimidine derivative it is conform that the methods is standard and accurate method for the HPLC study and useful for the further study.

In force degradation study under various stress condition shown that the sample is get completely hydrolysed in acidic and alkaline condition at RT 24 hr, on reflux at 80^0C, on oxidising with 3% H_2O_2 and 5% H_2O_2as well. In acidic condition compound do not show any peak in standard range of retention time so, we can say that compound is 100 percent degraded with zero percent recovery. In other conditions also pyrimidine shows more percentage of degradation with less recovery.

Forced degradation data proved that the method is specific for analysis and free from interference of blank unknown degradation products. The result indicate the prevent stability of compound under various strains conditions. The method is suitable for the analysis forced degradation study of pyrimidine derivatives.

This study acquainted us with the degradation pathway of drug in extreme chemical and environmental conditions and chemical behaviour of the molecule which helps in the development of formulation and packaging of potent drug to researchers and may be beneficial to society.

Quantification of pyrimidine

Table – 11

Sr.No.	Reaction condition	% Degradation	% Recovery
1	Alkaline hydrolysis		
	T.P 80^0 C	85.31	14.69
	R.T 24 hr	46.95	53.05
2	Acid hydrolysis		
	T.P 80^0 C	100	0
	R.T 24 hr	100	0
3	Oxidative degradation 3% H_2O_2		
	T.P 80^0 C	82.83	17.17
	R.T 24 hr	60.25	39.75
4	Oxidative degradation 5% H_2O_2		
	T.P 80^0 C	83.57	16.43
	R.T 24 hr	75.57	24.43

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