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DETERMINATION OF INTRINSIC STABILITY AND DEVELOPMENT OF VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR ANAGLIPTIN

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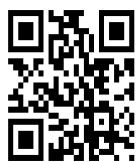

ABSTRACT

Pharmaceutical drugs are potential molecules with specific biological activity. However, long term use of these chemical molecules can affect the human physiological system because of their increased levels in the human body. Therefore, identification and structure elucidation of impurities or degradation products should be taken into consideration in order to assure drug safety. The present research work represents development and validation of simple, precise and rapid RP-HPLC method for the estimation of Anagliptin from its laboratory mixture. The chromatographic separation was achieved on Shimadzu HPLC series 1100 using ShodexC-18(250×4.6mm, 5μm) as stationary phase and Methanol and Acetonitrile (70:30 v/v) as mobile phase at flow rate of 0.8ml/min. The 246nm considered as detection wavelength for Anagliptin and was used throughout the experimentation. The developed method was found linear in the concentration range of 5-25μg/ml. The stress degradation study was performed by exposing the drug at various stress conditions. The proposed method was found to be superior with respect to resolution of drug from its degradation products under applied stress conditions. The developed RP-HPLC method was validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity and robustness.

INTRODUCTION

Chemically Anagliptin (ANG) is N-[2-({2-[(2S)-2-cyanopyrrolidin-1-yl]-2-oxoethyl} amino)-2-methylpropyl]-2-methylpyrazolo [1,5-a] pyrimidine-6-carboxamide (figure 1) used for the treatment of type 2 diabetes mellitus. ANG belongs to dipeptidyl peptidase 4 inhibitors or gliptins. The molecular formula of ANG is $C_{19}H_{25}N_7O_2$ and its molecular weight is 383.45g/mol.Stabilityof a pharmaceutical preparation can be defined as the capability of particular formulation in microbiological, therapeutic and toxicological specifications throughout its shelf life. Drug stability refers to the extent to which a drug Substance or product retains, the same properties within specified limit and characteristics that it possessed at the time of its manufacturing.[1]Various analytical methods are used to establish the identity, purity, physical characteristics of the drug and also the potency of the drugs that we use. Chemical properties of the analyte or drug and its concentration, sample matrix, the speed and cost of the analysis, type of measurements i.e., quantitative, qualitative or phase analysis are the parameters on which the analytical methodology are based.[2]The impurities formed under stress degradation studies or under storage conditions are the source responsible for toxicity or side effects. Thus, the structural

characterizations, activity and toxicity evaluation of drug products may give strong support to reduce the side effects and it also prevents recall the formulation or dosage form from the market.Several instrumental methods such as separation techniques, spectrometric techniques, electro-analytical techniques, thermo-analytical techniques, and modern hyphenated techniques was previously reported in pharmaceutical analysis.[3-4]Now a day the importance of method development by analytical methods using HPLC and impurity profiling has been increased significantly.[5-8] Literature survey revealed that very few methods of analysis reported for the estimation of selected drugs in single and also in combined dosage form by using UV spectrophotometer and HPLC. [9-12] To the best of our knowledge, a stability indicating method for ANG individually has not been located and its degradation products have not been identified by RP-HPLC. Our objective was to develop a stability indicating method under theconditions prescribed by ICH guidelines, to separate said drug from its degradation products and estimate drug in presence of impurities which has been generated under stress conditions by RP-HPLC and further validate the developed method.

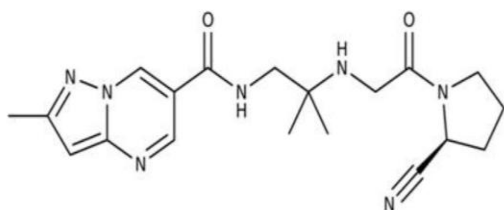

Figure 1: Structure of Anagliptin

MATERIAL AND METHOD

Chemicals and reagents: The drug Anagliptin was procured from INTAS Pharmaceutical (Ahmedabad, India). All solvents were used of HPLC and GR grade such as methanol, acetonitrile, ortho phosphoric acid, triethylamine, sodium hydroxide, hydrogen peroxide, microcrystalline cellulose, magnesium stearate, talc etc.

Instruments: UV-visible spectrophotometer, Shimadzu: High performance liquid chromatography, pH-meter, sonicator, filter, weighing balance, photo stability chamber, oven, etc.

Preparation of standard solutions of Anagliptin: Standard stock solution (A1) prepared by accurately weighed about 10.0mg ANG was transferred in a 10.0ml volumetric flask, dissolved in sufficient quantity and volume was made up to the mark with mobile phase. (Conc. 1000µg/ml). Working stock solution (A2) was prepared by 1.0ml of stock solution (A1) was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase (conc. 100µg/ml) and then working standard solution (A3) was prepared by appropriate diluted working stock solution (A2) with mobile phase to get the final concentration of 10µg/ml.

Selection of wavelength: The working standard solution of ANG (10µg/ml) was scanned in the range of 400-200nm in 1.0cm cell against solvent blank (methanol) and the spectra was recorded as shown in Figure 2.From the recorded UV spectra, ANG shows maximum absorbance at 246nm.

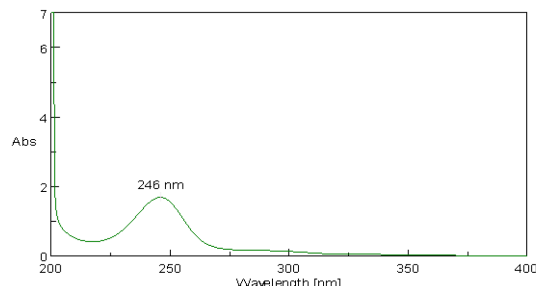

Figure 2: Spectrum of Anagliptin in methanol

Preliminary optimization of mobile phase and chromatographic conditions: In order to achieve the optimized chromatographic conditions, one or two parameters was modified at each trial and chromatograms were recorded with all specified chromatographic conditions. The standard solutions were prepared in different solvents and various trials was taken for the selection of mobile phase.

Study of system suitability parameters: After equilibration of column with mobile phase, five replicate injections of 20µl solution was injected through the manual injection and the chromatogram was recorded and the peak area was measured.

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Forced degradation study: In stress testing a drug substance and laboratory mixture exposed to an environment, vigorous than the normal sometimes called as accelerated stability conditions like high temperature, high humidity over the period of time, hydrolysis of drug with base or acids, and photo stability. This study was carried out via; Alkaline hydrolysis, Acidic hydrolysis, Oxidative study, Neutral hydrolysis, Thermal studies and Photochemical studies under UV light.

Acid Hydrolysis Studies:

An accurately weighed quantity about 10.0mg of Standard and sample equivalent to 10.0mg of ANG was transferred into two different 10.0ml volumetric flasks. To each flask 3.0ml of reagent (0.1N HCl) was added and kept at room temperature for a period of 4hrs. The stressed solution was withdrawn after 4 hrs and neutralisation was carried out. The content of each flask was sonicated for 15 min, volume was made up to the mark with mobile phase and filtered (1000µg/ml). A 1.0ml portion of the filtrate was further diluted to 10.0ml with mobile phase. Further 1.0ml of solution was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase (10µg/ml). A 20µl volume of each final diluted stressed solution was injected separately and chromatograms were recorded.

Alkali Hydrolysis Studies:

An accurately weighed quantity about 10.0mg of Standard and sample equivalent to 10.0mg of ANG was transferred to two different 10.0ml volumetric flasks. To each flask 3.0ml of reagent (0.1N NaOH) was added and kept at room temperature for 3 hrs. The stressed solution was withdrawn after 3 hrs and neutralisation was carried out. The content of each flask was sonicated for 15 min, volume was made up to the mark with mobile phase and filtered (1000µg/ml). A 1.0ml portion of the filtrate was further diluted to 10.0ml with mobile phase. Further 1.0ml of solution was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase (10µg/ml). A 20µl volume of each final diluted stressed solution was injected separately and chromatograms were recorded.

Neutral Hydrolysis Studies

An accurately weighed quantity about 10.0mg of Standard and sample equivalent to 10.0mg of Anagliptin was transferred to two different 10.0ml volumetric flasks. To each flask 3.0ml of distilled water was added and kept at room temperature for 3hrs. The stressed solutions were withdrawn after 3 hrs. The content of each flask was sonicated for 15 min, volume was made up to the mark with mobile phase and filtered (1000µg/ml). A 1.0ml portion of the filtrate was further diluted to 10.0ml with mobile phase. Further 1.0ml of solution was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase (10µg/ml). A 20µl volume of each final diluted stressed solution was injected separately and chromatograms was recorded.

Thermal Degradation: An accurately weighed quantity about 10.0mg of Standard and sample equivalent to 10.0mg of ANG was spread on petri dish separately and exposed to dry heat in an oven at 80 □C for 4 hrs. The stressed solution was withdrawn after 4 hrs. The content of each flask was sonicated for 15 min, volume was made up to the mark with mobile phase and filtered (1000µg/ml). A 1.0ml portion of the filtrate was further diluted to 10.0ml with mobile phase. Further 1.0ml of solution was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase (10µg/ml). A 20µl volume of each final diluted stressed solution was injected separately and chromatograms were recorded.

Photochemical Degradation:

An accurately weighed quantity about 10.0mg of Standard and sample equivalent to 10.0mg of Anagliptin was transferred to two different 10.0ml volumetric flasks. To each flask 3.0ml of stock solution was added and kept in UV Chamber for 48 hrs. The stressed solutions were withdrawn after 44 hrs. The content of each flask was sonicated for 15 min, volume was made up to the mark with mobile phase and filtered (1000µg/ml). A 1.0ml portion of the filtrate was further diluted to 10.0ml with mobile phase. Further 1.0ml of solution was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase (10µg/ml). A 20µl volume of each final diluted stressed solution was

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injected separately and chromatograms were recorded.

Application of the proposed method to laboratory mixture: An accurately weighed quantity of laboratory mixture equivalent to 10.0mg of ANG was transferred to 10.0ml of volumetric flask, sonicated for 15 min with sufficient quantity of diluent (mobile phase) and volume was made up to mark with diluent. The content of flask was filtered through 0.45µm filter paper. A 1.0ml portion of the filtered was further diluted to 10.0ml with diluent. Further 1.0ml of solution was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase. After equilibration of stationary phase, such five sample solutions were prepared from same working stock solution, injected separately and chromatogram was recorded.

Recovery Studies: It was carried out by standard addition method (SAM). An accurately weighed quantity of laboratory mixture equivalent to 10.0mg of ANG was transferred to 10.0ml volumetric flask and to it standard drug was added at three different levels and sonicated for 15 min, with sufficient quantity of diluent and volume was made up to the mark with mobile phase. The content was filtered through 0.45µ Whatman filter paper. A 1.0ml portion of the filtrate was further diluted to 10.0ml with diluents. Further 1.0ml of solution was transferred in 10.0ml volumetric flask and volume was made up to the mark with mobile phase. A 20µl volume of each final diluted solution was injected separately and chromatogram was recorded and amount contributed by formulation and % recovery was calculated.

VALIDATION OF METHOD

Validation of the proposed method was carried out as per ICH guideline.

Accuracy: Accuracy of the proposed method was established on the basis of recovery studies performed by using standard addition method.

Precision: Precision of any analytical method was expressed as SD and % RSD of series of measurements. Precision of estimation of Anagliptin by proposed method was ascertained by replicate analysis of homogeneous samples of laboratory mixture.

Linearity and Range: An accurately weighed laboratory mixture equivalent to 80, 90, 100, 110 and 120% of label claim was taken and dilutions were made as described under estimation of laboratory mixture. Then each solution was injected and chromatogram was recorded.

RUGGEDNESS

Different Analyst: The ruggedness of the proposed method has been verified by analysing the laboratory mixture used for precision method by two different analyst using same instrument. The ruggedness result was compared with method precision data.

Intraday and Interday Variation: After equilibration of stationary phase, sample solution was injected separately at 0 Hr, 3 Hr, 5 Hr and chromatograms was recorded. Similarly, the same solutions were injected on 1st, 2nd, 3rd day. The chromatograms so recorded and results was calculated. The content of ANG in each solution was calculated by comparing the peak area of sample.

Robustness: The robustness of the method was evaluated by injecting the sample at deliberately varied chromatographic conditions, composition of mobile phase, wavelength, pH and flow rate. The system suitability parameters were evaluated and amount of Anagliptin was calculated from sample solution in each varied condition.

RESULTS AND DISCUSSION

The chromatographic conditions were set as per the optimized parameters; mobile phase was allowed to equilibrate with stationary phase as indicated by steady baseline. A 20µl of solution was injected through manual injector and chromatogram was recorded. A mobile phase containing Methanol: Acetonitrile (70:30v/v) gave well-resolved peak and reasonable retention time as shown in Figure 3.

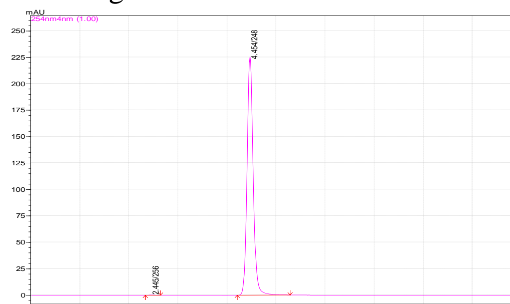

Figure 3: Chromatogram of standard ANG

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System suitability parameters

After equilibration of column with mobile phase, five replicate injections of 20µl solution were injected through the manual injection and the chromatograms were recorded and the peak area was measured. The recorded results of system suitability parameters are shown in Table 1.

Study of linearity: Aliquots of working stock solution were diluted in range of 0.5 - 2.5ml in 10.0ml volumetric flask with mobile phase and volume was made up to mark with diluent to obtained concentration ranging from 5-25µg/ml of Anagliptin. The mobile phase was allowed to equilibrate with stationary phase till steady baseline was obtained. Each of the final diluted solution was injected separately and recorded the chromatogram. The observations of area under curve are shown in Table 2.

Estimation of Anagliptin: The content of Anagliptin in each sample was calculated by comparing the peak area of sample with that of standard, the results obtained and statistical data are shown in Table 3.

Recovery study: This study was carried out by standard addition method. Amount contributed by formulation and % recovery was calculated using the formulae; the results are shown in Table 4.

VALIDATION OF METHOD: The assay test method is validated for Specificity, Linearity, Precision, Accuracy (Recovery), Stability of Analytical Solution and Robustness and was found to be meeting the predetermined acceptance criteria.

Precision and Accuracy: The developed method was found to be precise as the % RSD values were found to be less than 2%. Good recoveries (100.61%) of the drug were obtained at each added concentration, indicating that the method was accurate.

The linearity and range: The linearity and range study is indicative of accurate estimation of drug in over range of at least 80-120% of label claim as summarized in Table 5.

Ruggedness

Different Analyst: The ruggedness of the proposed method has been verified by analysing the laboratory mixture used for method precision by two different analysts using same instrument. The ruggedness results were compared with method precision data. Analyst to Analyst Variation obtained through different analyst study was found much acceptable are summarized in Table 6.

Intraday and Interday Variation: Intraday variation and the value of SD and % RSD are within the limits whereas interday values of SD and % RSD are within the limits are summarized in Table 7 and 8.

Robustness: The developed method was found to be robust when deliberate changes were carried out. The results are shown in Table 9.

Forced degradation study: In stress testing a drug substance and laboratory mixture exposed to an environment, vigorous than the normal sometimes called as accelerated stability conditions like high temperature, high humidity over the period of time, hydrolysis of drug with base or acids, and photo stability. Solution State Analysis of Stress degradation study, solution hydrolysis was performed in acidic, basic, neutral conditions at room temperature and the chromatograms of sample material are shown in figure 4, 5, and 6, respectively. Oxidative stress testing was performed using 3% H_2O_2 at room temperature, for 2 hrs and the chromatogram are shown in figure 7. The results of solution state analysis obtained are summarized in Table 10. The observations and results of solid state analysis are summarized in Table 11. The results obtained under thermal and photochemical study indicated the variable stability of laboratory mixture. In thermal study Standard and sample are more degraded than photochemical study.

CONCLUSION

The validated RP-HPLC method is sensitive, precise and robust. Moreover, the developed method was found to be more selective and rapid with respect to shorter run time. The stability study was performed by exposing the drug at various stress conditions. It shows degradation up to certain extend. The proposed method was found to be superior with respect to resolution of drug from its degradation products under applied stress conditions. Hence developed RP-HPLC method said to be stability indicating method and can be employed for the routine quality control analysis of Anagliptin in laboratory mixture.

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Table 1: Observations of system suitability parameters

Sr. No.	Wt. of Std. drug taken (mg)	AUC (mAU)
1.	10.06	1980398
2.		1927628
3.		1989550
4.		1992784
5.		1921027
	Mean	1962277
	%RSD	1.784
	Theoretical plate	24947.8
	Retention time	4.454
	Asymmetry	1.034

Table 2: Observations for linearity study

Sr. No.	Wt. of Std. ANG (mg)	Conc. (µg/ml)	AUC (mAU)
1.	10.14	5	1232541
2.		10	1996043
3.		15	2743205
4.		20	3567207
5.		25	4530214
Correlation Coefficient			0.9972

Table 3: Observations for estimation of anagliptin

Sr. No.	Wt. of Std. taken (mg)	AUC of Std. (mAU)	AUC of Sample (mAU)	Amt. of drug estimated (mg)	% Labelled claim
1.	10.04	1989263	2005123	10.12	99.79
2.			2006897	10.13	99.89
3.			2009125	10.14	99.99
4.			2023657	10.21	100.68
5.			2010977	10.15	100.09
				Mean	100.09
				±S.D.	0.349
				%RSD	0.348

Table 4: Observations of recovery study

Sr. No.	Wt. of sample (mg)	Amt. of std. drug added (mg)	AUC (mAU)		Amt. estimated (mg)	Amt. recovered (mg)	% Recovery*
			Std.	Sample			
1.	20.15	5.02	1988596	2998952	15.08	5.00	99.60
2.	20.30	10.08		4059786	20.41	10.26	101.78
3.	20.08	15.20		5033214	25.31	15.27	100.46
						Mean	100.61
						±SD	1.098
						%RSD	1.091

**Each value is mean of three observations*

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Table 5: Observations and results for linearity and range study

Sr. No.	Wt. of sample taken (mg)	% of level	AUC (mAU)
1.	16.10	80	1427817
2.	18.05	90	1684716
3.	20.40	100	1920880
4.	22.60	110	2110436
5.	23.85	120	2374457
Correlation Coefficient			0.9976

Table 6: Observations and results for different analyst study

Sr. No.	% Estimation of ANG	
	Analyst-I	Analyst-II
1.	100.95	101.08
2.	102.06	102.45
3.	101.91	101.45
Mean	101.64	101.66
±SD	0.6022	0.7087
%RSD	0.592	0.697

Table 7: Observations and results of intraday study

Sr. No.	Time (Hrs)	Wt. of sample taken(mg)	AUC (mAU)	% Labelled claim
1.	0th	19.78	1979496	101.00
2.	3rd		1969339	100.48
3.	5th		1988168	101.44
			Mean	100.97
			±SD	0.480
			%RSD	0.475

Table 8: Observations and results of interday study

Sr. no.	Deliberate condition	Wt. of sample taken (mg)	Retention time (min)	AUC (mAU)	Asymmetry	Theoretical plate
1.	Standard	20.15	4.481	2059341	1.258	27110.993
2.	Mobile Phase (Methanol: ACN) (75:25)		4.408	2063870	1.234	27125.448
3.	Mobile phase (Methanol: ACN) (65:35)		4.426	2047058	1.241	27106.599
4.	Wavelength (241nm)		4.494	2051639	1.233	26945.131
5.	Wavelength (251nm)		4.397	2036257	1.249	27654.835
8.	Flow rate (1.0ml/min)		4.301	2006854	1.255	27208.709
9.	Flow rate (0.6ml/min)		4.521	2092546	1.203	25977.709
		%RSD	1.67	1.28	1.502	1.88

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Table 9: Observations of robustness study

Sr no.	Day	Wt. of sample taken(mg)	AUC (mAU)	% Labelled claim
1.	1st	19.51	2074532	100.80
2.	2nd		2033966	101.17
3.	3rd		2022532	98.28
			Mean	100.08
			±SD	1.572
			%RSD	1.57

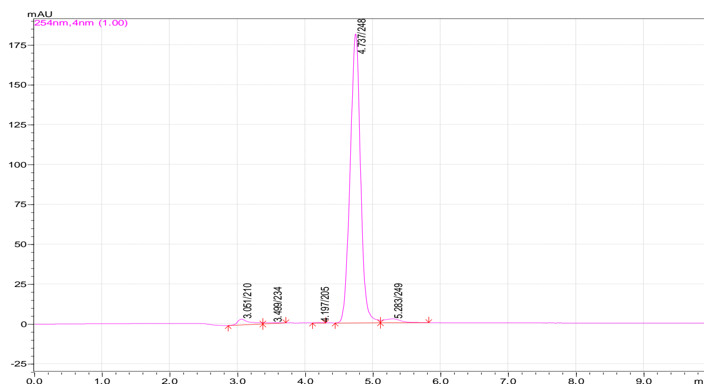

Figure 4: Chromatogram of sample in 0.1N HCl

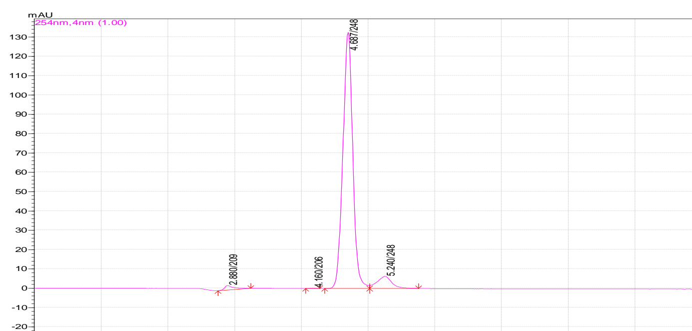

Figure 5: Chromatogram of sample in 0.1N NaOH

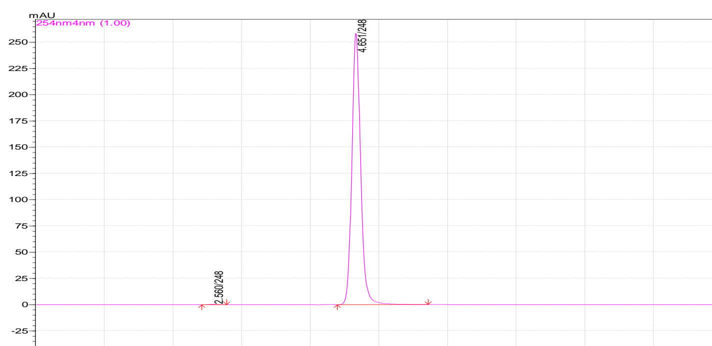

Figure 6: Chromatogram of sample in distill water

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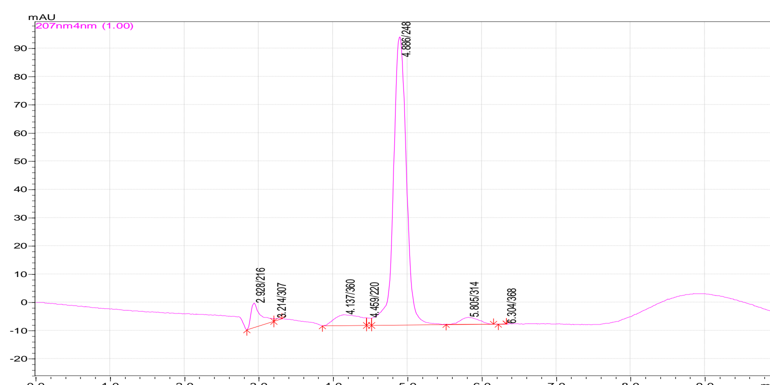

Figure 7: Chromatogram of sample in 3% H_2O_2

Table 10: Summary of the results of solution state analysis

Degradation medium	Conditions	% Un-degradation		% Degradation	
		Std.	Sample	Std.	Sample
Acid	0.1N HCl	85.74	86.23	14.26	13.77
Alkali	0.1N NaOH	70.38	69.58	29.62	30.42
Neutral	Distill water	98.89	98.39	1.11	1.61
Oxidation	3% H_2O_2	80.35	81.35	19.65	18.65

Table 11: Summary of the results of solid-state analysis

Conditions	% Un-degradation		% Degradation	
	Std.	Sample	Std.	Sample
Thermal (80°C)	70.15	69.35	29.85	30.65
Photochemical (254nm)	91.90	90.71	8.1	9.29

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