Research Article

A NOVEL STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF CLOPIDOGREL IN BULK AND ITS DOSAGE FORMS

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ABSTRACT
A Simple, rapid and sensitive stability indicating Reverse Phase High Performance Liquid Chromatographic method was developed for the estimation of Clopidogrel (CLG) in presence of its oxidative, acid, alkali and thermal degradation and neutrality of drug. The method was validated as per International Conference on Harmonization (ICH) guidelines. The mobile phase used in this study is a mixture of Acetonitrile: OPA - Ortho phosphoric acid buffer (50:50v/v). Stationary phase was Discovery AltimaC_{18} (150mm x 4.6 mm, 5µm) reverse phase column at 30°C ambient temperature. The analysis was performed with run time of 10.0 minutes at a flow rate of 1.00ml/min. The CLG was monitored at 240nm with UV detection and CLG was eluted at 2.7min. The method was linear (0.9998) at concentration ranging from 25to150µg/ml, precise (intra-day relative standard deviation (RSD) and inter-day RSD values < 1.0%), accurate (mean recovery = 99.41%), specific and robust. Detection and quantification limits were 18.7µg/ml and 112.5µg/ml, estimated from linearity by regression analysis. The results showed that the proposed method was suitable for rapid determination of clopidogrel in bulk dosage forms.

Keywords: Clopidorgel, RP-HPLC, Forced degradation, Validation, Dosage forms.

INTRODUCTION

Clopidogrel, chemically (+)-(s)-α-(2-chlorophenyl)-6, 7-dihydrothieno [3, 2-c] pyridine-5(4H)-acetic acid methyl ester sulphate is a potent oral antiplatelet agent often used in the treatment of coronary artery disease, peripheral vascular disease and cerebrovascular disease. It is white to off-white crystalline solid, freely soluble in water and methanol. It is also acts as an antihypertensive agent. The mechanism of action of Clopidorgel is irreversible blockade of the adenosine diphosphate (ADP) receptor P2Y12 and is important in platelet aggregation, the cross-linking of platelets by fibrin. The blockade of this receptor inhibits platelet aggregation by blocking activation of the glycoprotein IIb/IIIa pathway (1-5).The main objective of this study is to develop a new, simple and rapid analytical method by stability indicating RP-HPLC method to quantify CLG in bulk and its dosage forms. Validation study is carried out as per ICH guidelines (6). Analytical methods are essential to characterize drug substances and drug products composition during all stages of pharmaceutical development. For routine analytical purpose it is always necessary to establish methods capable of analyzing huge number of samples in a short time period with high accuracy and precision.

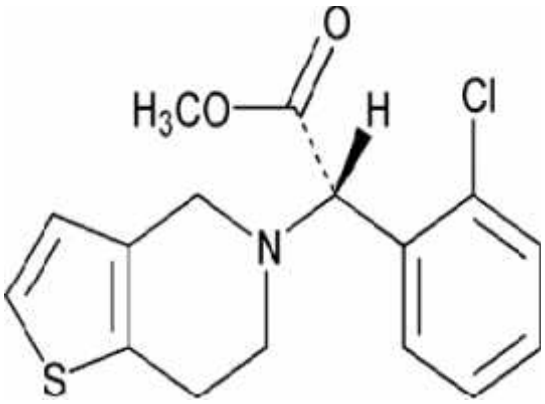

Fig: 1. Structure of Clopidorgel bisulphate

Very few analytical methods are reported for the quantification of Clopidorgel bisulphate in plasma by liquid chromatography, fluorescence detection, and UV detection. In the present study we propose a simple, sensitive and reproducible RP-HPLC method for the determination of Clopidorgel bisulphate. Comprehensive literature survey reveals the estimation of Clopidorgel bisulfate in pharmaceutical formulations by various chemometric; HPLC [7], HPTLC (8-11), and an LC-ESI-MS-MS (11) method were developed. The proposed method was validated with respect to selectivity, linearity, precision, and accuracy, limit of quantitation (LOQ) and limit of detection (LOD) according to ICH requirements (6) to show it could be used for determination of CLG in pharmaceutical formulations.

Materials And Methods

Instrumentation

Quantitative HPLC was performed on a high performance liquid chromatography equipped with Waters HPLC 2965 System with Auto Injector 2996 photodiode array detector (PDA) Detector and 2695pump was used. The output of signal was monitored and integrated using EMPOWER PRO software.

Reagents and chemicals

HPLC grade acetonitrile and water as well as ortho phosphoric acid buffer, A.R. grade were purchased from Fisher scientific, Mumbai, India. All other chemicals used were of HPLC grade.

Chromatographic conditions

The mobile phase used in this study is a mixture of Acetonitrile and Ortho phosphoric acid buffer (50:50v/v). Stationary phase was Altima C18 column (150mm x 4.6 mm, 5μm) as a stationary phase and dimensions at ambient temperature 30°C. The contents of the mobile phase were filtered before use through a 0.22μ membrane filter. The mobile phase was pumped from the solvent reservoirs to the column at a flow rate of 1.0ml/min for 10.0min. The elution was monitored at 240 nm using PDA detector. The retention time of the drug was found to be 2.7min.

Preparation of buffer

0.1%OPA Buffer: 1ml of Perchloric acid was diluted to 1000ml with HPLC grade water.

Preparation of mobile phase

Mobile phase was prepared by mixing 0.1%OPA and Acetonitrile HPLC Grade in the ratio of 50:50%v/v. The prepared mobile phase was sonicated for 15min. and filtered through 0.22µm membrane filter to remove the impurities which may interfere with final chromatogram.

Preparation of Standard stock solutions

Accurately weighed 18.75mg of Clopidogrel transferred 25ml and volumetric flasks, 3/4 th of diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labeled as Standard stock solution (750μg/ml of Clopidogrel).

Preparation of Standard working solutions (100% solution)

1ml of Clopidogrel from each stock solution was pipette out and taken into a 10ml volumetric flask and made up with diluent. (75μg/ml of Clopidogrel).

Preparation of Sample stock solutions

5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100 ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (750 μg/ml of Clopidogrel).

Preparation of Sample working solutions (100% solution)

1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (75μg/ml of Clopidogrel)

Method validation

System suitability parameters

The system suitability parameters are determined by preparing standard solution of Clopidogrel and the solutions were injected six times and the parameters like retention time, peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

Specificity (system suitability)

Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Linearity

To demonstrate the linearity of assay method, inject 6 standard solutions with concentrations of about 18.75 ppm to 112.5ppm of Clopidogrel. Plot a graph to concentration versus peak area. Slope obtained was 22553 Y-Intercept was 2641and Correlation Co-efficient was found to be 0.999 and Linearity plot was shown.

Accuracy

Three Concentrations of 50%, 100%, 150% are Injected in a triplicate manner and %Recovery was calculated as 99.41. And chromatograms were shown in fig 6.11-6.13.

Precision

Precision is the degree of repeatability of an analytical method under normal operation conditions.

Repeatability

Six working sample solutions of 75ppm are injected and the % Amount found was calculated and %RSD was found to be 0.7.

Intermediate precision

Five working sample solutions of 75ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be 0.5 and chromatogram was shown in Figure 6.3.

LOD and LOQ

Limit of detection is the lowest concentration in a sample that can be detected, but not indispensably quantified under the verbally expressed experimental conditions. The circumscription of quantitation is the lowest concentration of analyte in a sample that can be resolute with acceptable precision and precision. Limit of detection and circumscribe of quantitation were calculated utilizing following formula LOD = 3.3 σ/S and LOQ = 10 σ/S, where, σ is the standard deviation of replication and S is the slope of the calibration curve. The LOD and LOQ values are 0.318μg/ml& 0.964μg/ml.

Robustness

Small Deliberate change in the method is made like Flow minus, flow plus, Mobile phase minus, Mobile phase plus, Temperature minus, Temperature Plus. %RSD of the above conditions is calculated.

Forced degradation tests

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation.The specificity of the method can be demonstrated through forced degradation studies conducted on the sample using oxidation, acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions main peak of the drug was studied for peak purity, that indicating the method effectively separated the degradation products from the pure active ingredient.

Degradation procedure :oxidation

To 1 ml of stock solution of Clopidogrel 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 1c. For HPLC study, the resultant solution was diluted to obtain (75ppm) solution and 10 μl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies

To 1 ml of stock ssolution Clopidogrel 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 1c .The resultant solution was diluted to obtain (75ppm) solution and 10 μl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies

To 1 ml of stock solution Clopidogrel 1 ml of 2 N sodium hydroxide was added and refluxed for 30mins at 1c. The resultant solution was diluted to obtain (75ppm) solution and 10 μl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies

The standard drug solution was placed in oven at 105^0c for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to (75ppm) solution and10μl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability studies

The photochemical stability of the drug was also studied by exposing the (750ppm) solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain (75ppm) solutions and 10 μl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to (75ppm) solution and 10 μl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Result And Discussion

HPLC method development and optimization

The chromatographic method was optimized by changing various parameters, such as the mobile phase composition. Different mobile phases were tried, but satisfactory separation and good symmetrical peak were obtained with the mobile phases consisting of Acetonitrile and Ortho phosphoric acid buffer (50:50v/v). A typical chromatogram obtained by using the aforementioned mobile phase and 10μl of the injected assay preparation is illustrated in **Fig: 2.**

Method validation

The objective of method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines Q2A and Q2B (2009). Method validation characteristics were tested in accordance with ICH guidelines. Method specificity was verified by comparing the chromatograms of sample of pharmaceutical preparation, standard solution and blank. Method precision, recovery in the range of 50% to 150% of label claim of the drug using the blend, Linearity was tested in the range 25–150 μg/ml. Intra and inter-day instrumental system precision as well as repeatability and intermediate method precision were obtained using six replicates per day. Limits of detection [LOD] and limit of quantification [LOQ] were provided for CLG. Calculation was made by means of RSQ (Residual Square of regression).

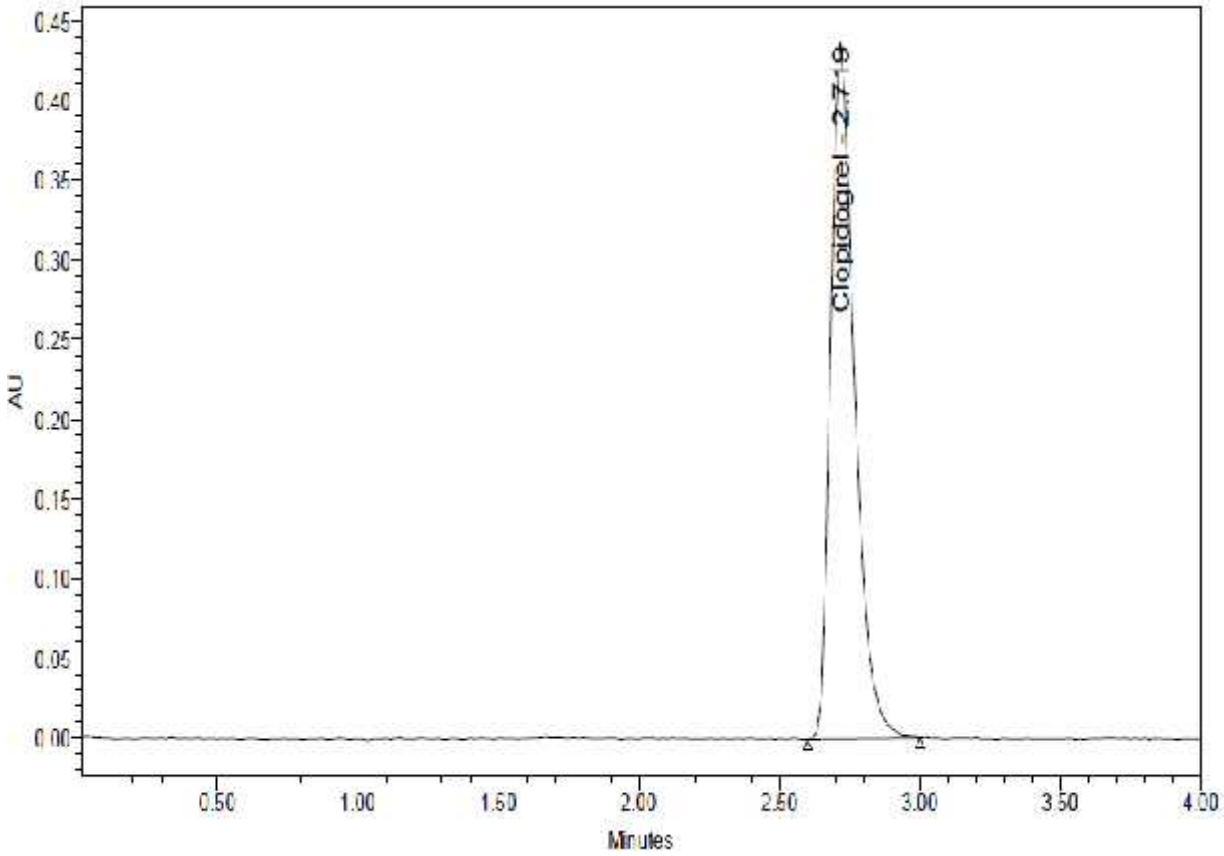

Fig : 2 A typical chromatogram showing the peak of Clopidogrel

Table no: 1 Optimized Chromatographic Conditions

Column	AltimaC18 150mm x 4.6 mm, 5μm.
Mobile phase	0.1% OPA: Acetonitrile (50:50)
Flow rate	1.0 ml/min
Detector	PDA 240nm
Temperature	30^{0}C
Injection Volume	10μL

Table no: 2 Table for system suitability

S.No	Peak name	Retention time	Area	%area	Usp plate count	Usp tailing
1	Clopidogrel	2.702	4876454	100.00	4848	1.38
2	Clopidogrel	2.709	4876281	100.00	4770	1.42
3	Clopidogrel	2.709	4816510	100.00	4993	1.42
4	Clopidogrel	2.713	4825890	100.00	4916	1.37
5	Clopidogrel	2.715	4812154	100.00	4934	1.37
6	Clopidogrel	2.719	4872032	100.00	4801	1.38
Mean			4846554			
Std deviation			31431.9			
%rsd			0.6			

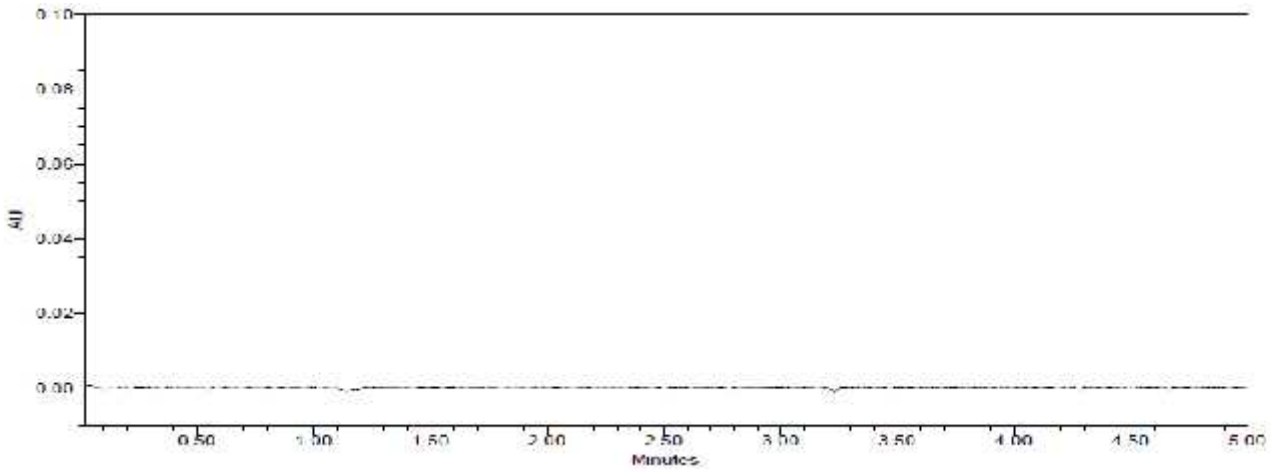

Fig no: 3 Blank chromatogram obtained from system

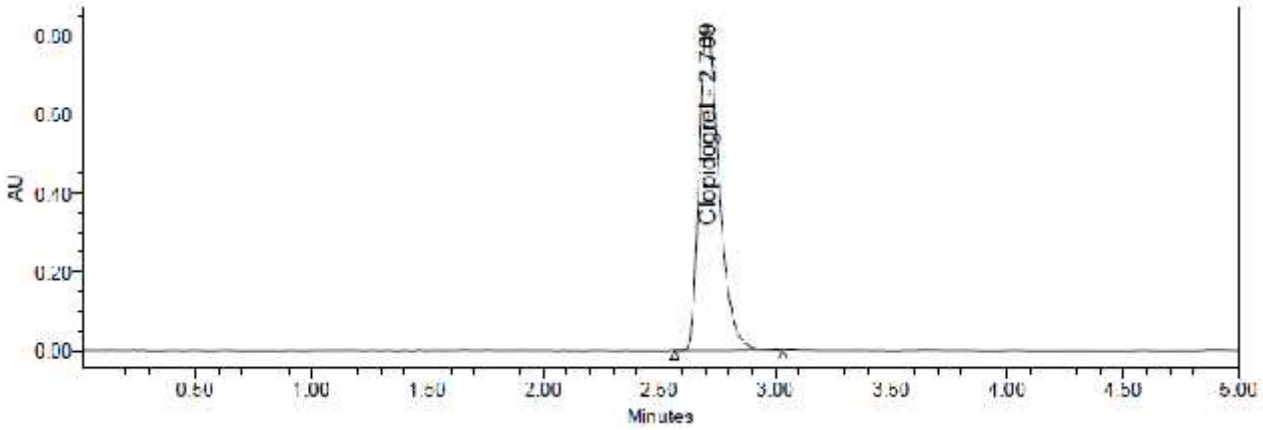

Fig no: 4 Standard peak of Clopodogrel

Linearity

To demonstrate the linearity of assay method, inject 6 standard solutions with concentrations of about 18.75 ppm to 112.5ppm of Clopidogrel. Plot a graph to concentration versus peak area. Slope obtained was 22553 Y-Intercept was 2641and Correlation Co-efficient was found to be 0.999 and Linearity plot was shown in Figure 5.

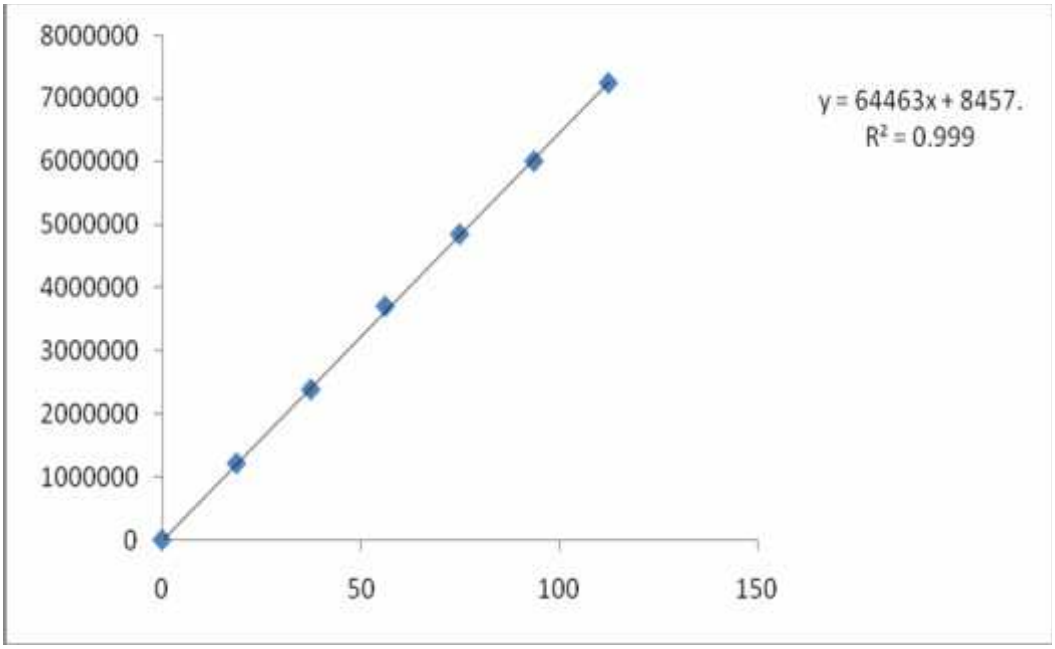

Fig no: 5 Linearity curve of different concentration of Clopodogrel

Table no:3 Linearity Concentrations and Response

Linearity Level (%)	Concentration (ppm)	Area
25	18.75	1214359
50	37.5	2388063
75	56.25	3710365
100	75	4855601
125	93.75	6015327
150	112.5	7257923

Accuracy: Three Concentrations of 50%, 100%, 150% are Injected in a triplicate manner and %Recovery was calculated as 99.41. And chromatograms were shown in fig 6.11-6.13.

Table no: 4 Accuracy data of Clopodogrel with range of 50% to 150%

Name of drug	% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
Clopidogrel	50%	37.5	37.40	99.72	99.41%
		37.5	37.32	99.51	
		37.5	37.22	99.26	
	100%	75	74.44	99.26	
		75	74.35	99.13	
		75	74.60	99.46	
	150%	112.5	111.82	99.40	
		112.5	112.04	99.59	
		112.5	111.75	99.33	

Degradation Studies

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation studies.

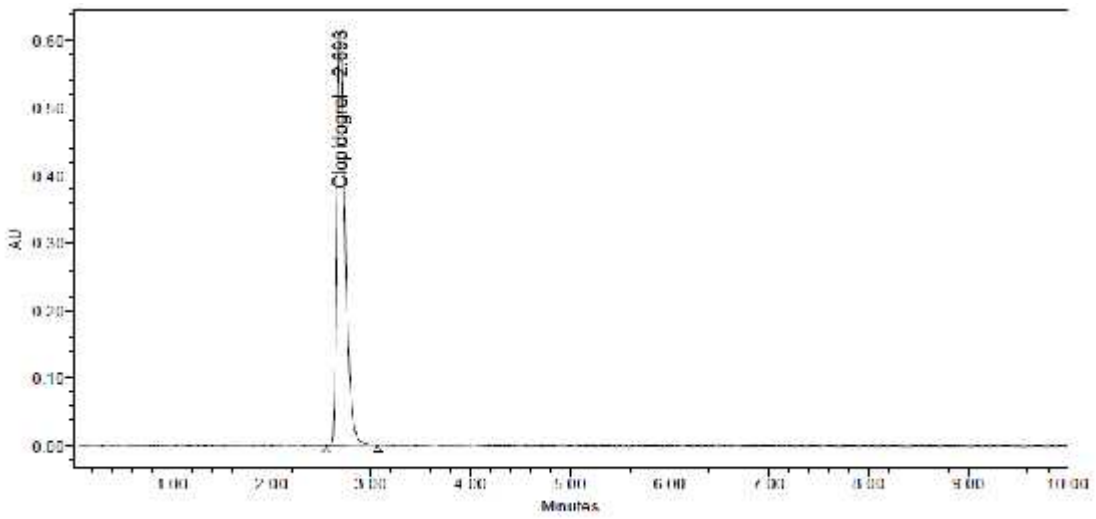

Fig no: 6 Acid degradation chromatogram

Precision

Table no: 5 Precision study of Clopidogrel

S.NO	Peak area(S.P)	Peak area(I .P)
1	4775540	4820642
2	4773641	4823262
3	4777015	4808323
4	4745620	4821318
5	4792242	4811875
6	4775672	4817458
AVG	4773288	4817146
Std dev	15162.7	5878.1
%rsd	0.3	0.1

*S.P – System

Precision; I .P – Intermediate precision

LOD: Detection limit of the Clopidogrel in this method was found to be 0.052μg/ml

LOQ: Quantification limit of the Clopidogrel in this method was found to be 0.158μg/ml.

Table no: 6 Results of LOD and LOQ of clopodogrel

S.No	Parameters	Clopidogrel
1.	LOD (μg/ml)	0.318
2.	LOQ (μg/ml)	0.964

Robustness

Small Deliberate change in the method is made like Flow minus, flow plus, Mobile phase minus, Mobile phase plus, Temperature minus, Temperature Plus. %RSD of the above conditions is calculated.

Table no: 7 Robustness data of Clopodogrel

S.No	Parameter	%RSD
1	Flow rate (-) 0.7ml/min	1.1
2.	Flow rate (+) 0.9ml/min	0.7
3.	Mobile phase (-) 60B:40A	1.3
4.	Mobile phase (+) 50B:50A	0.4
5.	Temperature (-) 25°C	1.3
6.	Temperature (+) 35°C	0.1

Table no: 8 Stability studies of Clopidogrel

Time period (hours)	Clopidogrel				
	Retention time	Peak area	Tailing factor	Plate count	%area
Control	2.710	3916020	1.44	4882	100
4	2.703	4775540	1.40	5174	100
8	2.704	4773641	1.40	5247	100
12	2.704	4777015	1.40	5216	100
16	2.704	4745620	1.40	5263	100
20	2.704	4792242	1.40	5251	100
24	2.709	4775672	1.39	5216	100
Mean		4773288			
Std. Dev		15162.7			
%Rsd		0.3			

Assay of marketed formulation

Standard solution and sample solution were injected separately into the system and chromatograms were recorded and drug present in sample was calculated using before mentioned formula.

Table no: 9 Assay of Formulation

Sample No	%Assay
1	99.37
2	99.42
3.	99.11
4.	99.38
5.	99.19
6.	99.30
AVG	99.29
STDEV	0.1212
%RSD	0.12

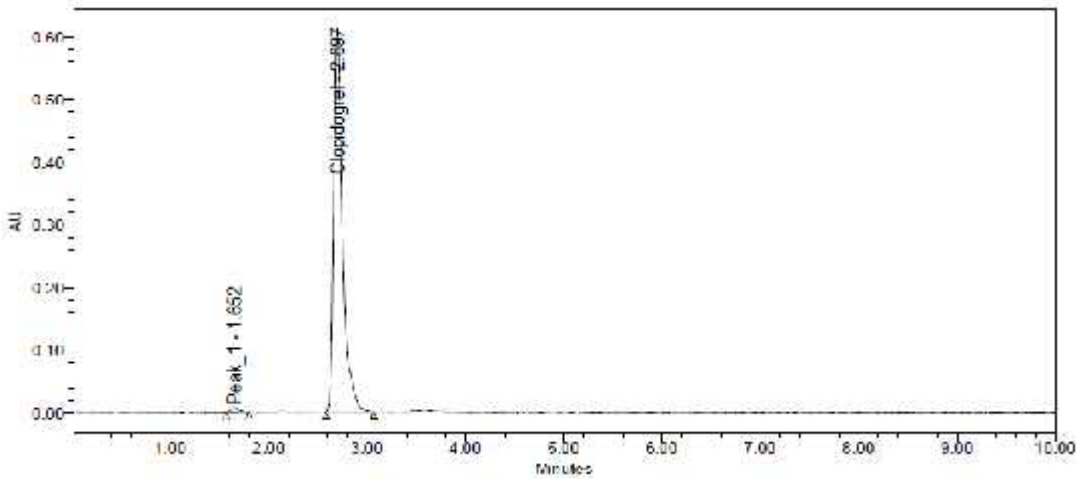

Fig no: 7 Base degradation chromatogram

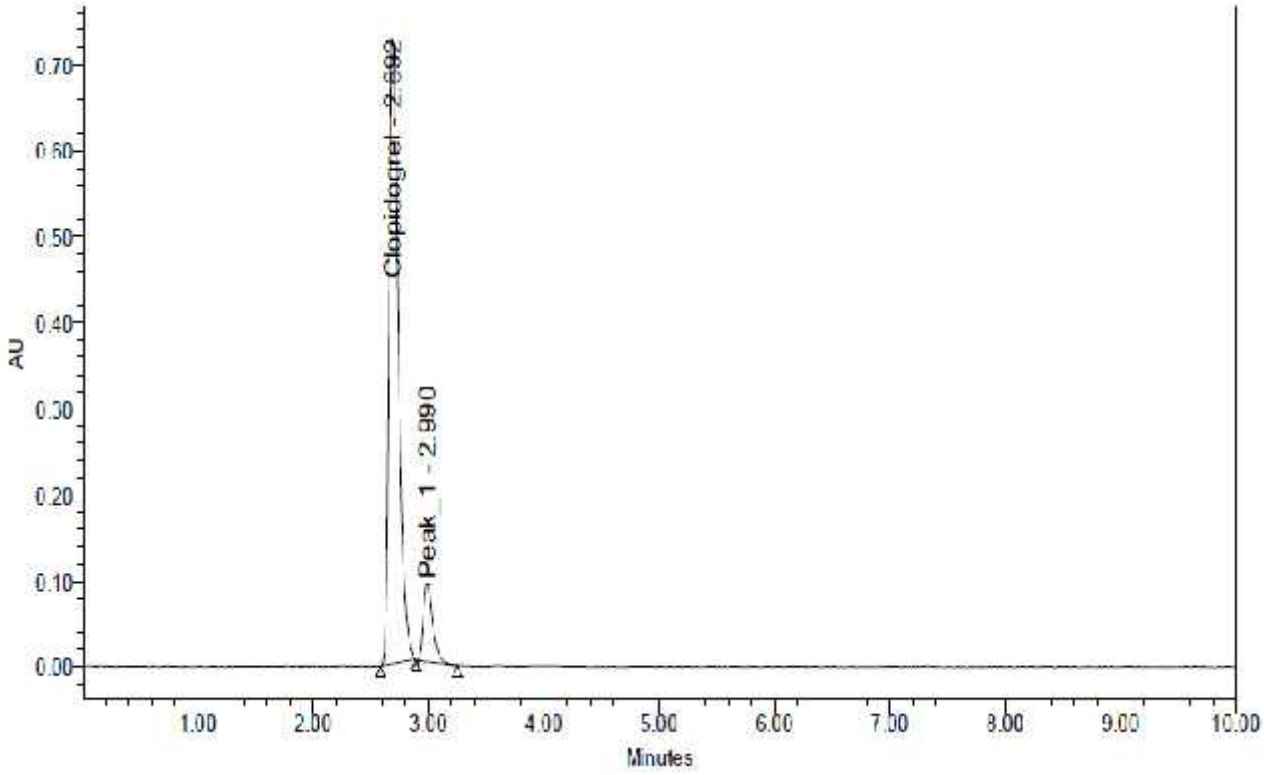

Fig no: 8 Peroxide degradation chromatogram

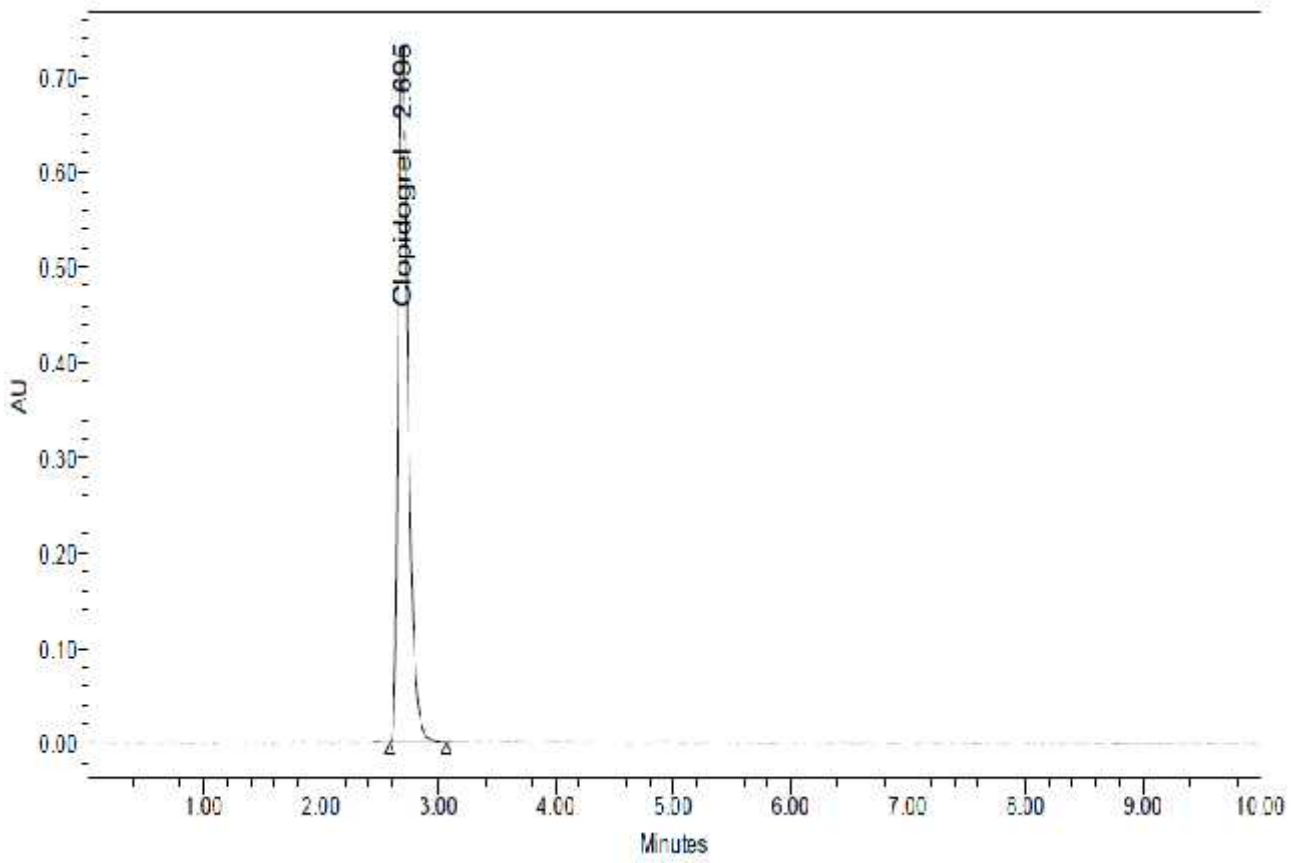

Fig no: 9 Thermal degradation chromatogram

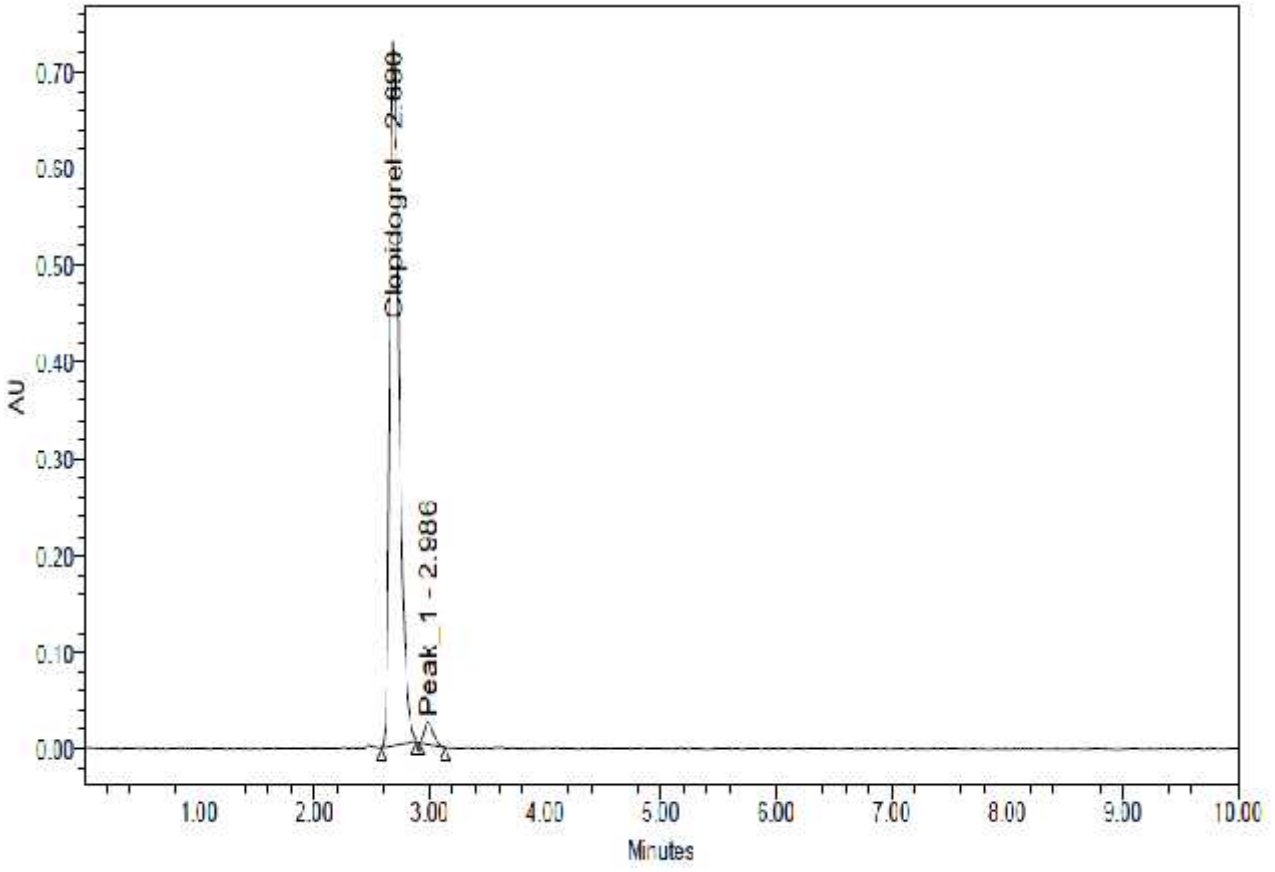

Fig no: 10 UV degradation chromatogram

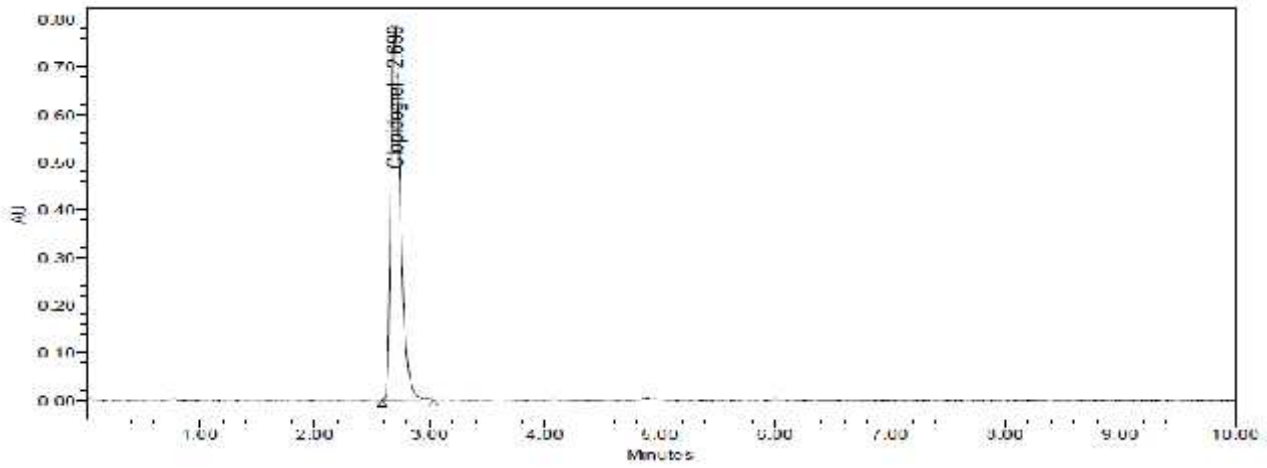

Fig no: 11 Water degradation chromatogram

Table no: 10 Degradation Data of Clopidogrel

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.74	0.268	0.442
2	Alkali	3.20	0.312	0.489
3	Oxidation	3.03	0.235	0.369
4	Thermal	2.65	0.254	0.406
5	UV	1.24	0.238	0.366
6	Water	0.94	0.343	0.531

Regarding the pH adjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time.

Table no: 11 Summary Tables

Parameters	Clopidogrel	LIMIT
Linearity : Range (µg/ml)	18.75-112.5 µg/ml	R< 1
Regression coefficient	0.999	
Slope(m)	64463	
Intercept(c)	8457	
Regression equation (Y=mx+c)	y = 64463x + 8457	
Assay(% mean assay)	99.29%	90-110%
Specificity	Specific	No interference of any peak
System precision %RSD	0.6	NMT 2.0%
Method precision %RSD	0.1	NMT 2.0%
Accuracy %recovery	99.41%	98-102%
LOD	0.318	NMT 3
LOQ	0.964	NMT 10
Robustness		%RSD NMT 2.0

Conclusion

The proposed stability indicating RP-HPLC method was found to be simple, rapid and reliable analytical method for determination of clopidogrel in pharmaceutical preparation using HPLC with PDA detector. An analytical run takes about 2.7min. Separation of compounds is very fast with good reproducibility and peak asymmetry. Validation of this method was accomplished and the results obtained meet all requirements. The method is also found to be highly reproducible with a good accuracy and precision. The proposed method allows reliably for the analysis of clopidogrel in forced degradation conditions even and bulk , different pharmaceutical dosage forms.

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