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A New Reverse Phase-Hplc Method Devevelopment and Validation in Analysis of Clopidogrel Bisulfate

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Abstract:

It can be seen from the above results, the retention time of Clopidogrel Bisulfate sample can be obtained as 6.806 minutes, which near to the standard retention time of Clopidogrel Bisulfate 3.959 mins. From this results, the authors have concluded that the formulation of Clopidogrel Bisulfate (Plagril) has 98% purity when compared to Clopidogrel Bisulfate in pure form. Statistical Analysis of results shows that the proposed procedure has good linearity, accuracy and precision. Results of the analysis of pharmaceutical formulation reveal that the proposed method is suitable for their analysis with virtually no interference of usual additives present in the pharmaceutical formulation.

The proposed method obviate the need for any preliminary treatment and is simple, sensitive, reliable, more economical and can be used for the routine determination of Clopidogrel Bisulfate in bulk samples and pharmaceutical formulations.

The authors have selected REVERSE PHASE-HPLC method overall other conventional chromatographic & spectophotometric methods, because of the following reasons:

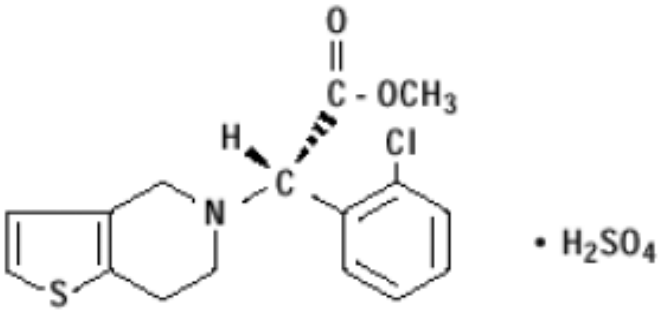
HPLC is a high resolution and high speed liquid chromatography. It has several times more resolving power than open column liquid chromatography. Hence it is used for speedy resolution of complex mixture.

Keywords: Clopidogrel bisulfate, RP-HPLC, Method Development, Method Validation, Accuracy, Precision, Quantitative estimation, Linearity

1. DRUG PROFILE

Drug Profile OF CLOPIDOGREL BISULFATE

Structure:



Chemical Name : methyl(+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2c] pyridine-5(4H)acetate sulfate(1:1)

Molecular Formula : $C_{16}H_{16}ClNO_2S.H_2SO_4$

Molecular weight : 419.9.g/Mol

Description : It is a white to off-white powder.

Category : Anti- platelate.

Solubility : Clopidogrel Bisulfate is freely soluble in Water, ACN, and Methanol

Mechanism of action

Clopidogrel is a prodrug, the action of which may be related to an ADP receptor on platelet cell membranes. The drug specifically and irreversibly inhibits the $P2Y_{12}$subtype of ADP receptor, which is important in activation of platelets and eventual cross-linking by the protein fibrin. Platelet inhibition can be demonstrated two hours after a single dose of oral clopidogrel, but the onset of action is slow, so that a loading-dose of 300 mg is usually administered.

Pharmacokinetics

Clopidogrel is a prodrug and is metabolized to a pharmacologically active metabolite and inactive metabolites.

Absorption

After single and repeated oral doses of 75 mg per day, clopidogrel is rapidly absorbed. Absorption is at least 50%, based on urinary excretion of clopidogrel metabolites.

Effect of Food

Plavix can be administered with or without food. In a study in healthy male subjects when Plavix 75 mg per day was given with a standard breakfast, mean inhibition of ADP-induced platelet aggregation was reduced by less than 9%. The active metabolite AUC 0-24 was unchanged in the presence of food, while there was a 57% decrease in active metabolite C_{max}. Similar results were observed when a Plavix 300 mg loading dose was administered with a high-fat breakfast.

Metabolism

Clopidogrel is extensively metabolized by two main metabolic pathways: one mediated by esterases and leading to hydrolysis into an inactive carboxylic acid derivative (85% of circulating metabolites) and one mediated by multiple cytochrome P450 enzymes. Cytochromes first oxidize clopidogrel to a 2-oxo-clopidogrel intermediate metabolite. Subsequent metabolism of the 2-oxo-clopidogrel intermediate metabolite results in formation of the active metabolite, a thiol derivative of clopidogrel. This metabolic pathway is mediated by CYP2C19, CYP3A, CYP2B6 and CYP1A2. The active thiol metabolite binds rapidly and irreversibly to platelet receptors, thus inhibiting platelet aggregation for the lifespan of the platelet.

The Cmax of the active metabolite is twice as high following a single 300 mg clopidogrel loading dose as it is after four days of 75 mg maintenance dose. Cmax occurs approximately 30 to 60 minutes after dosing. In the 75 to 300 mg dose range, the pharmacokinetics of the active metabolite deviates from dose proportionality: increasing the dose by a factor of four results in 2.0- and 2.7-fold increases in Cmax and AUC, respectively.

Elimination

Following an oral dose of 14C-labeled clopidogrel in humans, approximately 50% of total radioactivity was excreted in urine and approximately 46% in feces over the 5 days post-dosing. After a single, oral dose of 75 mg, clopidogrel has a half-life of approximately 6 hours. The half-life of the active metabolite is about 30 minutes.

Contra indication

Active Bleeding

Plavix is contraindicated in patients with active pathological bleeding such as peptic ulcer or intracranial hemorrhage.

Hypersensitivity

Plavix is contraindicated in patients with hypersensitivity (e.g., anaphylaxis) to clopidogrel or any component of the product

Side effects

Serious adverse drug reactions associated with clopidogrel therapy include:

- Severe neutropenia (low white blood cells),
- Thrombotic thrombocytopenic purpura (TTP),
- Hemorrhage,
- Bleeding in the post operative period.

Other side effects may include:

- ✓ Other gastrointestinal side effects
  - Upper GI discomfort (27% Vs 29% in patients taking aspirin alone),
  - Gastric or duodenal ulcer, gastritis,
  - Diarrhea.
- ✓ Rash (6% overall, 0.33% severe)
- ✓ Respiratory (infrequent)
  - Upper respiratory infections, rhinitis, shortness of breath, cough.
- ✓ Cardiovascular
  - Chest pain,
  - Edema (generalized swelling).
- ✓ Thrombocytopenia

Drug Interactions

Clopidogrel interacts with the following drugs: proton pump inhibitors (except possibly pantoprazole), phenytoin (Dilantin); tamoxifen(Nolvadex); tolbutamide(Orinase); torsemide (Demadex); fluvastatin (Lescol);a blood thinner such as warfarin(Coumadin), heparin,ardeparin (Normiflo), dalteparin (Fragmin), danaparoid (Orgaran), enoxaparin (Lovenox),or tinzaparin (Innohep);(Activase),anistreplase (Eminase), dipyridamole (Persantine), streptokinase (Kabikinase,Streptase), ticlopidine (Ticlid),and urokinase.

In November 2009, the FDA announced that clopidogrel should be used with caution in patients on proton pump inhibitors such as omeprazole and esomeprazole.

Dosage and Administration

Usual Adult Dose for Ischemic Stroke:

75mg orally once a day with or without food. Aspirin therapy should be initiated and continued in combination with clopidogrel.

Usual Adult Dose for Myocardial Infarction:

75mg orally once a day with or with out food. Aspirin therapy should be initiated and continued in combination with clopidogrel.

Usual Adult Dose for Acute Coronary Syndrome - Prophylaxis:

75mg orally once a day with or with out food. Aspirin therapy should be initiated and continued in combination with clopidogrel.

Usual Adult Dose for Peripheral Arterial Disease:

75mg orally once a day with or with out food. Aspirin therapy should be initiated and continued in combination with clopidogrel.

Usual Adult Dose for Acute Coronary Syndrome:

Unstable angina, non ST segment elevation myocardial infarction (UA/NSTEMI): Initial: 300 mg loading dose, followed by 75 mg once daily for at least 1 month and ideally up to 12 months (in combination with asprin 75 to 162mg once daliy indefinitely).ST segment elevation acute myocardial infarction (STEMI): 75 mg once daily (in combination with aspirin 162 to 325 mg initially, followed by 81 to 162 mg/day); Note: The CLARITY TIMI 28 study used a 300 mg loading dose of clopidogrel (with thrombolysis) demonstrating an improvement in the patency rate of the infarct related artery and reduction in ischemic complications. The duration of therapy was less than 28 days (usually until hospital discharge) unless non primary percutaneous coronary intervention (PCI) was performed.

Usual Adult Dose for Percutaneous Coronary Intervention:

Percutaneous coronary intervention (PCI) for UA/NSTEMI or STEMI: Loading dose: 300 to 600 mg (600 mg may be preferred for early invasive strategy with UA/NSTEMI) given as early as possible before or at the time of PCI followed by 75 mg once daily. Note: If an initial loading dose of 300 mg was given prior to PCI, a supplemental loading dose of 300 mg (total loading dose: 600 mg) may be

administered. For patients with UA/NSTEMI, it has been recommended that the loading dose be given at least 2 hours (or 24 hours in patients unable to take aspirin) prior to PCI.

Higher vs standard maintenance dosing: May consider a maintenance dose of 150 mg once daily for 6 days, then 75 mg once daily thereafter in patients not at high risk for bleeding; however, in another study, in patients with high on treatment platelet reactivity, the use of 150 mg once daily for 6 months did not demonstrate a difference in 6 month incidence of death from cardiovascular causes, nonfatal MI, or stent thrombosis compared to standard dose therapy (Price,2011). Duration of clopidogrel (in combination with aspirin) after stent placement: Premature interruption of therapy may result in stent thrombosis with subsequent fatal and nonfatal MI. With STEMI, clopidogrel for at least 12 months regardless of stent type (either bare metal or drug eluting stent) is recommended. With UA/NSTEMI, at least 12 months of clopidogrel is recommended in patients receiving a drug eluting stent (DES) unless the risk of bleeding outweighs the benefits. For bare metal stent (BMS) placement, at least 1 month and ideally up to 12 months duration is recommended unless the risk of bleeding outweighs the benefits; then, a minimum of 2 weeks is recommended. In either setting, a duration greater than 15 months may be considered in patients with DES placement. For patients without ongoing ACS, clopidogrel should be continued for at least 1 month (for BMS) or at least 12 months (for DES).

Usual Geriatric Dose for Acute Coronary Syndrome:

The American College of Chest Physicians recommends: Patients over 75 years: 75 mg once daily for up to 28 days (with or without thrombolysis).

Usual Paediatric Dose for Platelet Aggregation Inhibition:

Note: Safety and efficacy have not been established in paediatric patients; optimal dose is not known; limited do singin formation is available; further paediatric studies are needed. Neonates and Infants up to 2 years: 0.2 mg/kg once daily was found to achieve a mean inhibition of platelet aggregation similar to adults receiving the recommended dose. This dose comes from the PICOLO study which included paediatric patients with a systemic to pulmonary artery shunt, intracardiac or intravascular stent, Kawasaki disease, or arterial graft; 79% of patients received concomitant aspirin; patients less than 2 kg and those born at less than 35 weeks gestational age were excluded.

Children over 2 years of age: Optimal dose is not established; some centres use the following: Initial dose: 1 mg/kg once daily; titrate to response; in general, do not exceed adult dose.

Over Dose

Platelet inhibition by Plavix is irreversible and will last for the life of the platelet. Overdose following clopidogrel administration may result in bleeding complications. A single oral dose of clopidogrel at 1500 or 2000 mg/kg was lethal to mice and to rats and at 3000 mg/kg to baboons. Symptoms of acute toxicity were vomiting, prostration, difficult breathing, and gastrointestinal haemorrhage in animals.

Based on biological plausibility, platelet transfusion may restore clotting ability.

2. MATERIALS AND METHODS

MATERIALS

Drug Sample

Clopidogrel Bisulfate was obtained as a gift sample from **AUROBINDO** Pharmaceuticals Pvt.Ltd., Hyderabad.

Formulation Used

Plavix vial containing 5.35mg/vial of Clopidogrel Bisulfate purchased from local Pharmacy.

Chemicals and Solvents

All the following chemicals used were of analytical and HPLC grade.

- Clopidogrel Bisulfate
- Acetonitrile
- Methanol
- Purified water (for HPLC)

Instruments

Instruments employed for the study were,

- SHIMADZU AX -200 Digital Balance.
- HPLC

  - LC – 2010 A SHIMADZU solvent deliver module.

Shimadzu High Performance Liquid Chromatography:

(instruction manual)

Detector Specifications	
Light source	Deuterium Arc lamp
Measurement waveleng	190 to 700 nm
Spectral Band Width	5 nm
Wavelength Accuracy	± 1nm

Cell path length	10 nm
Cell volume	20µlop
Operating temperature range	4 to 4°C (39 to 104°F)
Recording range	0.0001 to 4.000AUFS
Operating temperature	40 to 75%
Pump Specifications	
Pump type	515
Pumping methods	Double reciprocating plunger pump
Suction filter	45µm
Line filter	5µm mesh
Operating temperature	4 to 4°C (39 to 104°F)

3. METHODS

High Performance Liquid Chromatographic Method

Selection of chromatographic method

Proper selection of the method depends upon the nature of the sample, molecular weight, and solubility. The drug selected for the present study was polar. So reversed phase chromatography can be used, this reverse phase HPLC was selected for the initial separation from the knowledge of properties, C_{18} column was chosen as stationary phase with different composition such as ACN, HPLC – Methanol were used.

Selection of mobile phase and λ_{max}

A 12.5mg of Clopidogrel Bisulfate was dissolved in 25ml of mobile phase (i.e., ACN: Methanol 60:40v/v). The λ_{max} was found at 230 nm. This wavelength was followed for the estimation of Clopidogrel Bisulfate by RP- HPLC method, and the stability was observed for up to 4 hours

Optimized Chromatographic Condition

Following Optimized Chromatographic parameters were used for the estimation of Clopidogrel Bisulfate.

Mode of operation - Isocratic

Stationary phase - ODS C_{18} column (5µm, 4.6X250mm)

Mobile phase - ACN: Methanol

Ratio - 60:40v/v

Detection wavelength - 230 nm

Flow rate - 0.8 ml/min
Temperature - Ambient
Load - 10 µl

Preparation of standard solution

Weigh accurately about 12.5mg bulk drug and dissolve in 25ml of mobile phase and sonicated for 10min. Make up the volume with mobile phase to get the final concentration with 1mg/ml.

Preparation of working standard solution

From above standard solution take 1ml of solution dilute to 10ml with mobile phase to get the final concentration 1µg/ml.

Assay

Separately inject equal volumes (about 10 ml) of diluent, standard preparation and sample preparation into chromatograph, record the chromatograms, and measures the peak area response for the major peaks, calculate the amount of Clopidogrel Bisulfate in the portion of Clopidogrel Bisulfate injection by the formula.

$$\frac{\text{Sample peak area} \times \text{Dilution factor of standard} \times \text{Average weight of tablets}}{\text{Standard peak area} \times \text{Dilution factor of sample}}$$

Linearity:-

Linearity of an analytical procedure is to validate the ability to elicit test results that are directly proportional to the concentration of analyte in sample with in a given range.

- Linearity shall be established directly on Clopidogrel Bisulfate working standard across the specified ranges with minimum 5 concentrations that are with in the specified range.
- For assay 8µg/mL to 12µg/mL (i.e., 0.8µg/mL, 0.9µg/mL, 1.0µg/mL, 1.1µg/mL, 1.2 µg/mL,) of the test concentration (10 mcg/mL of Clopidogrel Bisulfate) shall be considered as a minimum specified range of drug substance. The values are tabulated in Table-7.3
- Inject each linearity solution in duplicate. Estimate the degree of linearity by calculating the correlation co-efficient, y-intercept, residual sum of squares and slope of the regression line should be calculated and reported.
- Establish a plot of data for analyte response Vs its concentration is shown in Fig.7.1

Accuracy:-

- Accuracy is to validate the closeness of test results obtained by the analytical procedure to the true value. The accuracy should be established across the specified range of the analytical method.
- Accuracy for the assay of drug products in which there is an extraction stage in the sample preparation is determined by applying the method in triplicate to samples (or) mixtures of excipients to which known amounts of analyte have been added at about 80%, 100%, and 120% of the target concentration (10mcg/mL). The accuracy as then calculated from the test results as the percentage of analyte recovered by the assay.

Acceptance criteria:-

- The accuracy (recovery) for the average of triplicate in each concentration. Level should be within 98.0%- 102.0%

Precision:

The Precision of an analytical procedure expresses the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous sample under prescribed conditions. Precision shall be carried out at following levels.

- Method Repeatability

The Precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Method Repeatability:

Repeatability expresses the precision under the same operating conditions over a short interval of time by conducting the study as per test procedure in the same laboratory, by the same analyst and using the same equipment.

1) Repeatability should be assessed using a minimum of 6 determinations at 100% of the test concentration.

2) Determine the precision of test method by Assay six replicate sample preparations of Clopidogrel Bissulfate 1mg/ml. Calculate % of relative standard deviation for six replicate samples.

Acceptance Criteria:

- The relative standard deviation of the six sample preparations should be not more than 2.0%.

System suitability:

Inject standard preparations six times into the chromatograph and evaluate
the system suitability parameters as per test procedures.

Acceptance criteria:

- % RSD for 6 replicate injection of peak area response for Clopidogrel Bisulfate peak from the standard preparation should be not more than 2.0
- The tailing factor for Clopidogrel Bisulfate peak from standard preparation should be not more than 2.0
- Theoretical plate count for Clopidogrel Bisulfate should not be less than 2000.

TABLE- SYSTEM SUITABILITY PARAMETERS FOR RP-HPLC METHOD

Sl.no	Parameters	Results
1	Retention time(min)	3.959
2	Column length in cm.(L)	15
3	Theoretical plates	4618
4	HETP	0.0051821
5	Tailing factor	1.01

TABLE – VALIDATION PARAMETERS RESULTS

Validation parameters	Results
Linearity	Correlation coefficient – 0.990 Regression - $Y=1X10^{-6}x-0.015$ Slope - 1X10-6 Intercept- -0.015

Accuracy	Spiked concentration 80% - 99.94 100% - 99.92 120% - 99.90
Precision	a) Method precision-0.117 b) System precision- 0.59

Table LINEARITY OF CLOPIDOGREL BISULFATE

Concentration (µg/ml)	**Peak area**	**Height**	**Statistical Analysis**	
80	634223	27050	Slope	$1X10^{-6}$
90	692528	30080	Intercept	0.015
100	786897	34779	Correlation coefficient	0.990
110	839775	37156		
120	940390	40753		

TABLE – METHOD PRECISION

Concentration 100%	**Injection**	**Peak Areas of Clopidogrel Bisulfate**
	1	16546806
	2	15862490

	3	15811596
	4	15875015
	5	15860369
	6	15861305
Statistical Analysis		
	Mean	2661599.5
	SD	283623
	% RSD	0.177

TABLE – SYSTEM PRECISION

	Injection	**Peak Areas of Clopidogrel Bisulfate**
Concentration 100%	1	16032274
	2	15943557
	3	15757441
	4	15943523
	5	15965672
	6	15985337
Statistical Analysis		

	Mean	2656327.83
	SD	94400.26
	% RSD	0.59

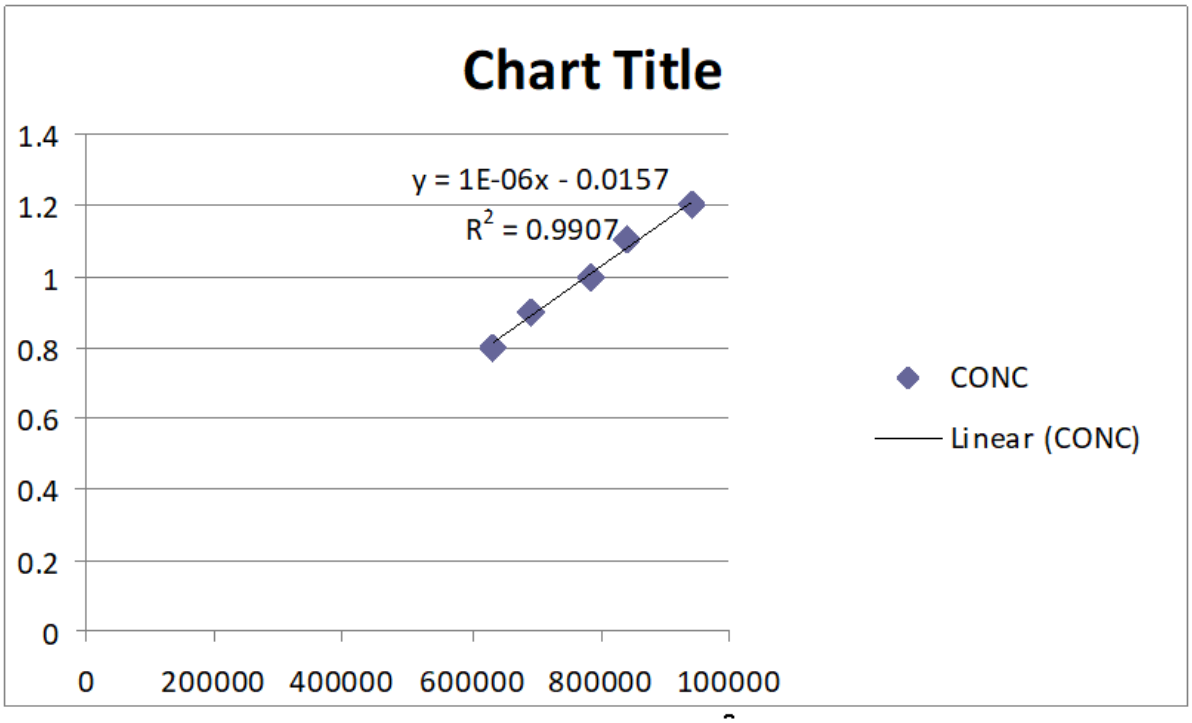
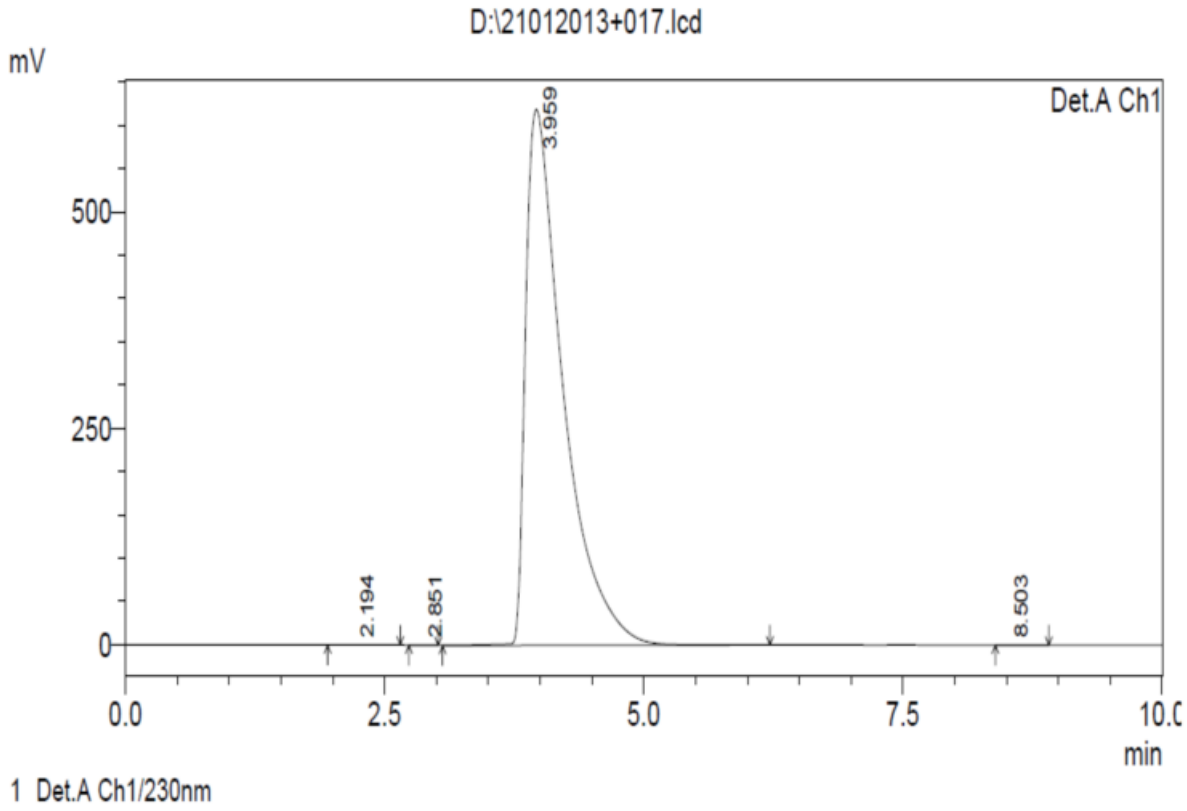


Fig: 4 Calibration curve for linearity

Fig: 5 Chromatogram for standard

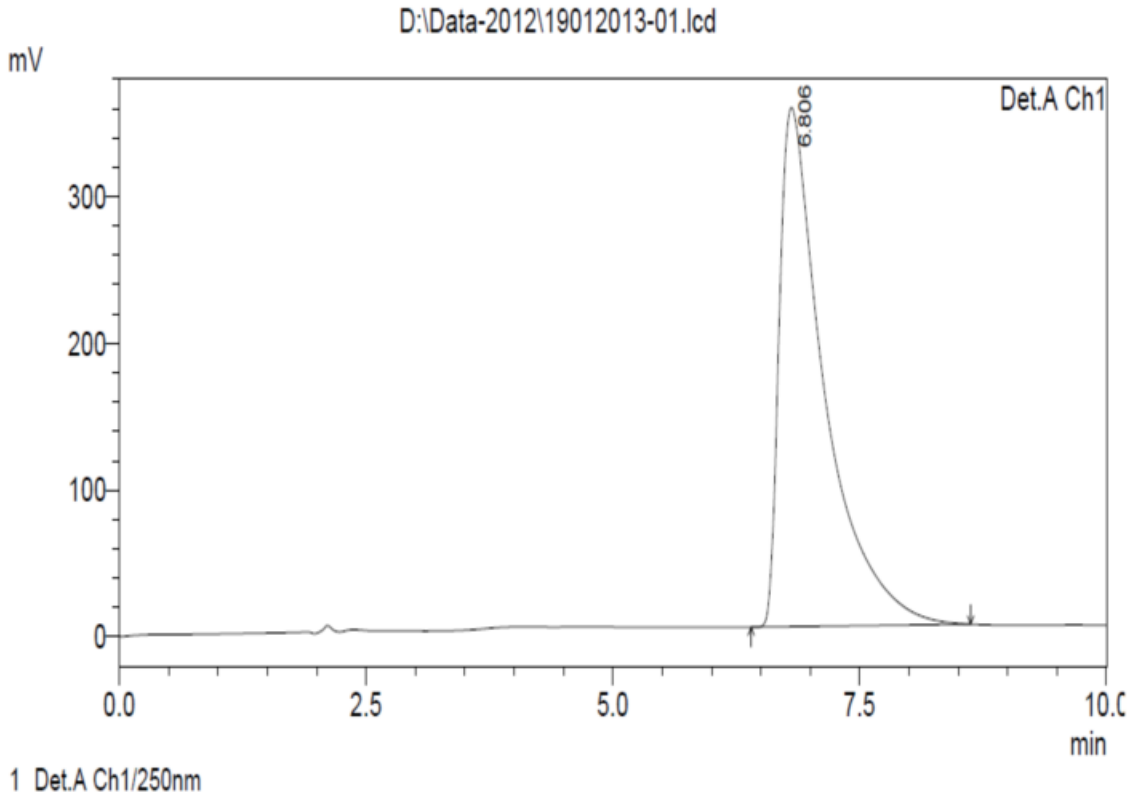


PeakTable

Detector A Ch1 230nm

Peak#	Ret. Time	Area	Height	Area %	Name
1	2.194	7225	428	0.046	
2	2.851	2235	234	0.014	
3	3.959	15597525	618957	99.932	
4	8.503	1213	67	0.008	
Total		15608199	619685	100.000	

Fig: 6 Chromatogram for sample



PeakTable

Detector A Ch1 250nm

Peak#	Ret. Time	Area	Height	Area %	Name
1	6.806	11243282	353556	100.000	RT:6.806
Total		11243282	353556	100.000	

4. CONCLUSION

It can be seen from the above results, the retention time of Clopidogrel Bisulfate sample can be obtained as 6.806 minutes, which near to the standard retention time of Clopidogrel Bisulfate 3.959 mins. From this results, the authors have concluded that the formulation of Clopidogrel Bisulfate (Plagril) has 98% purity when compared to Clopidogrel Bisulfate in pure form. Statistical Analysis of results shows that the proposed procedure has good linearity, accuracy and precision. Results of the analysis of pharmaceutical formulation reveal that the proposed method is suitable for their analysis with virtually no interference of usual additives present in the pharmaceutical formulation. The proposed method obviate the need for any preliminary treatment and is simple, sensitive, reliable, more economical and can be used for the routine determination of Clopidogrel Bisulfate in bulk samples and pharmaceutical formulations.

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