***Research Article***

**ISSN:2230-7346**
*Journal of Global Trends in Pharmaceutical Sciences*
*Vol.2, Issue 4, pp -367-379, October–December 2011*

# DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RELATED SUBSTANCES METHOD FOR CLOPIDOGREL BYSULPHATE DRUG SUBSTANCE BY NORMAL PHASE HPLC

**G. Radha Krishna Reddy *[1], Prof. V. Surya Narayana Rao[1], Jayam Naga Sateesh [2]**

1. Department of Chemistry, Sri Krishna Devaraya University, Anantapur, India.
2. Jawaharlal Nehru Technological University, Anantapur, Andhra Pradesh, India.

***Corresponding Author E-mail: rkreddy5555@gmail.com**

## ABSTRACT

Clopidogrel is an oral antiplatelet agent from thienopyridine class. It is used to inhibit the blood clots in coronary artery disease, peripheral vascular disease, and cerebrovascular disease. It works by irreversibly inhibiting a receptor called P2Y12. A simple, precise cost effective and stability indicating Normal Phase-HPLC method has been developed and validated for the determination of Related Substances of Clopidogrel Bisulfate Drug Substance. Separation of all known impurities from each other and also from Clopidogrel were achieved with in shorter run time with required resolution, accuracy and precision thus enabling the utility of the method for routine analysis. Chromatographic separation was achieved on a Chiral Cel OD-H Column (250 × 4.6 mm, 5µ) using a mobile phase consisting of 920ml n-Hexane, 50ml Ethanol and 30ml of Isopropyl alcohol and 0.3ml of Diethylamine at a flow rate of 0.9 ml per minute. The detection was made at 240nm. The retention time of Clopidogre peak is 20.8 minutes. The method was found linear over the range of Limit of Quantification to 150% of Specification level. The proposed method was validated aqs per the ICH and USP guidelines.

**Key words:** Clopidogrel Bisulfate, HPLC Method development and validation

**INTRODUCTION:**

Clopidogrel Bisulfate ( Fig 1) is chemically Methyl (2S)-2-(2-chlorophenyl)-2-(6,7-dihydro-4H-thieno[3,2-c]pyridin-5 yl)acetate; sulfuric acid. Clopidogrel is an oral antiplatelet agent from thienopyridine class [1–2]. It is used to inhibit the blood clots in coronary artery disease, peripheral vascular disease, and cerebrovascular disease. It works by irreversibly inhibiting a receptor called $P2Y_{12}$ [3–5].

Clopidogrel is a pro-drug whose action may be related to adenosine diphosphate (ADP) receptor on platelet cell membranes. The specific subtype of ADP receptor that clopidogrel irreversibly inhibits is $P2Y_{12}$ and is important in platelet aggregation and the cross-linking of platelets by fibrin. The blockade of this receptor inhibits platelet aggregation by blocking activation of the glycoprotein IIb/IIIa pathway. The IIb/IIIa complex functions as a receptor mainly for fibrinogen and vitronectin but also for fibronectin and von Willebrand factor. Activation of this receptor complex is the "final common pathway" for platelet aggregation, and is important in the cross-linking of platelets by 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 300-600 mg is usually administered.[6-7]

Literature survey reveals that Characterization of degradation of Clopidogrel bisulfate under solid stress conditions [8], Determination of Clopidogrel Bisulfate In Active Pharmaceutical ingredient by Chromatography [9]. Identification and Characterization of a Principle Oxidation Impurity in Clopidogrel Drug Substances and Drug Product [10], Quantitative Determination of Clopidogrel bisulfate active metabolite in human plasma by LC-MS" [11], A validated LC method for the determination of clopidogrl in pharmaceutical preparations[12], RP-HPLC analysis of aspirin and clopidogrel bisulfate in combination [13] Simultaneous determination of Clopidogrel and Aspirin in pharmaceutical dosage form [14]. The present study illustrates development and validation of a stability indicating normal phase method for the determination of Related Substances of Clopidogrel Bisulfate drug substance.

## MATERIALS AND METHODS:

### I. Chemicals and Reagents:

Clopidogrel Bisulfate working standards and impurities (Impurity-A, Impurity-B1, Impurity-B2, Impurity-C, Impurity-D and Impurity-E) were procured from LGC Promochem, and the tested pharmaceutical were procured from commercial pharmacy. n-Hexane, Ethanol, Isopropyl alcohol are HPLC grade and Diethylamine AR grade were of suitable for analysis.

### II. Apparatus and Chromatographic Conditions:

HPLC analysis was performed on Waters HPLC system with diode array detector. Separations were carried on a Chiral Cel OD-H (250 × 4.6 mm, i.d., 5 µm particle size) using isocratic elution. The flow rate was 0.9 mL min-1. UV detection was performed at 240 nm. HPLC Column temperature was 30°C. Peak identity was confirmed by retention time comparison and the HPLC was operated at room temperature.

### III. Preparation of Mobile Phase and Diluent:

Mobile Phase: Mix 920 ml of n-Hexane, 50ml of Ethanol and 30ml of Isopropyl alcohol, shake well and add 0.3ml of Diethylamine, sonicate it for 2 minutes.

Diluent: Use mobile phase as diluent.

### VI. Preparation of Resolution Solution:

Mixed solutions of Clopidogrel (1000ppm), Impurity-D (2 ppm) and Impurity-E (5ppm) using 5ml of ethanol then mobile phase,

### V. Preparation of Standard Solution:

The sample solution of Clopidogrel 5ppm solution was prepared using 10ml ethanol then reaming with mobile phase

### VI. Preparation of Sample Solution:

The sample solution of Clopidogrel 1000ppm solution was prepared using 10ml ethanol then reaming with mobile phase

## RESULTS AND DISCUSSION:

### Method Development:

Chromatographic parameters were preliminary optimized to develop a stability indicating Related Substances method for Clopidogrel Bisulfate with short analyses time (<55 min). Since Clopidogrel Bisulfate is having five impurities. So these impurities need to separate from each other and also from main analyte to show the stability indicating Related Substances method.

Clopidogrel Bisulfate Impurity-B1 and Impurity-B2 both impurities are isomers. Separarion of isomers is very difficult in reverse phase method. Hence, normal phase method was opted for method development.

The development trials were initiated with the selection of mobile phase. Since the opted development method is normal phase, various non polar solvents and its different logical proportions (Solvents such as n-Hexane, Butylenechloride, Isopropyl alcohol, Ethanol etc.) were used in the initial developmental trials and concluded with the efficient mobile phase i.e. mixture of n-Hexane, Ethanol, Isopropyl alcohol. Various compositions of the selected solvents were tried on different Chiral columns available such as Chiralpack AD, Chiralpack OD, Chiral Cel OJ-H, Chirosil, Chiral-AGP etc. With the better resolution and peak shape the method was optimized by the mobile phase composition of n-Hexane, Ethanol, Isopropyl alcohol and Diethylamine in the ratio of 92:5:3:0.03 on Chiral Cel OD-H column.

System Suitability parameters were evaluated and limits fixed. Resolution between Impurity-D and Cloidogrel performed and found that within the limits.

USP Tailing factor, USP Plate count and Area ratio performed for Clopidogrel performed and found that within the limits

**Method Validation**

The above method was validated according to ICH and USP guidelines to establish the performance characteristics of a method (expressed in terms of analytical parameters) to meet the requirements for the intended application of the method [15].

**System Suitability**

In order to determine the adequate resolution and reproducibility of the proposed methodology, suitability parameters including Retention Time, USP Resolution, USP Tailing factor, USP Plate count and Area Ratio of Clopidogrel peak areas were investigated. The results are summarized in Table 1.

**Specificity**

**Interference from Blank:**

The specificity of an analytical method may be defined as the ability to unequivocally determine the analyte in the presence of additional components such as impurities, degradation products and matrix. Specificity was evaluated by injecting the blank solution to observe for interference at the retention times of all known impurities and principle peak. It was observed that there was no interference from the blank solution. The Blank chromatogram was shown in figure- 6.

**Interference from Impurities:**

All known impurities are injected individually and spiked into test at specification level and injected in to the system. All the impurities were well

separated from each other and from main analyte. The Spiked chromatogram was shown in Figure- 7.

**Forced degradation Studies:**

Drug Substance subjected to forced degradation at various stressed conditions like acid, base, hydrolysis, peroxide, heat, photo light, U.V light and Humidity. All the samples were analyzed for peak purity of all known impurities and Clopidogrel peaks using Empower software. For all stressed samples the peak purity of Clopidogrel and its all known impurities were found within the limits. The results are summarized in Table 2 and degradation chromatograms are shown in figure-(8-10).

**Precision:**

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Six replicate samples were prepared by spiking with all known impurities at specification level and analyzed as per the test procedure. The % Relative standard deviations for content of all individual known impurities were calculated and the results are found to be within the acceptance criterion.

**Accuracy:**

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Recovery study was performed at 50%, 75%, 100%, and 150% of the specification level of all the known impurities by spiking them with the drug substance. Three replicates each at 50%, 75%, 100% and 150% levels. Spiked samples were extracted and analyzed. The amount spiked, amount recovered, percent recovery and its mean were calculated. The results are shown in Table 3.

**Limit of Detection and Limit of Quantification:**

The limit of detection and (LOD) and limit of quantitation (LOQ) is determined by signal to noise ratio method by using the formula. Signal to noise ratio (S/N) = 2H/h. H - Height of the analyte peak, h - Height of the noise. LOD and LOQ value was verified by giving six replicate injections of solution containing known impurities and Clopidogrel at this level. The percentage relative standard deviation

(%RSD) calculated for the peak areas and found to be within the limit. The results are shown in Table 4.

**Linearity of Detector Response:**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity of detector response for Clopidogrel and its known impurities were established by analyzing A series of solutions of Clopidogrel and its impurities at the concentration ranging from Limit of Quantification level to 150% level of specification level were prepared and injected into the HPLC system. The final concentration of each solution in µg per mL was plotted against peak area response. Slope, correlation coefficient (R) and intercept were found to be within the limit. The results are shown in Table 4.

**Robustness:**

Robustness of the method was verified by deliberately varying the following conditions.

By changing the flow rate by ± 10%.

By changing the column oven temperature by ± 5°C.

By changing the organic content in mobile phase by ± 2% absolute.

System suitability solutions and test solutions by spiking with all known impurities at specification level were prepared as per the test procedure and analysed in each varied condition

System suitability parameters and RRT of all known impurities were evaluated with each varied condition and compared with test method conditions was found to be within the limit.

**Ruggedness:**

**Bench Top Stability of Test Solution:**

Bench top stability of test solution of Clopidogrel Bisulfate drug substance was conducted over a period of 2 days and found that test solution is stable on Bench top for 2 days.

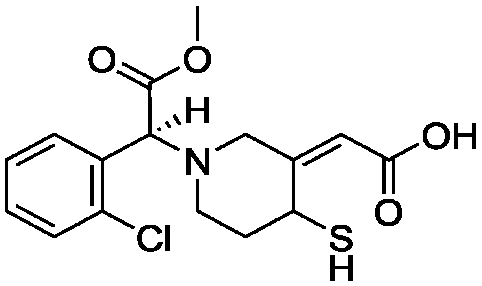
**Refrigerator Stability of test solution:**

Refrigerator stability of test solution of Clopidogrel Bisulfate drug substance was conducted over a period of 2 days and found that test solution is stable in refrigerator for 2 days.

**Bench Top Stability of Mobile Phase:**

Bench top stability of mobile phase was conducted over a period of 2 days and found that mobile phase is stable on Bench top for 2 days.

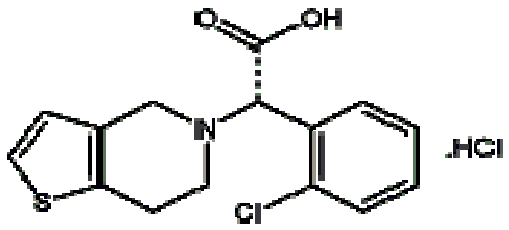
**Figure- 1**: Chemical Structures of Clopidogrel Bisulfate




**IUPAC NAME:** Methyl (2S)-2-(2-chlorophenyl)-2-(6,7-dihydro-4H-thieno[3,2-c]pyridin-5-yl)acetate; sulfuric acid

**Molecular Weight:** $C_{16}H_{18}ClNO_6S_2$

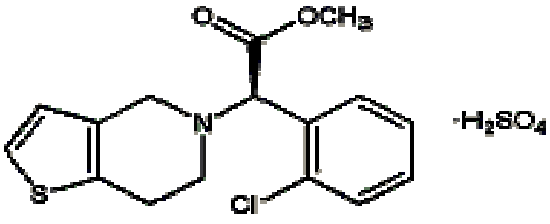
**Figure- 2**: Impurity-A**:**




Clopidogrel Related Compound A (USP)

**Figure- 3**: Impurity-B**:**

**Figure -4:** Impurity-C:




Clopidogrel Related Compound C (USP)

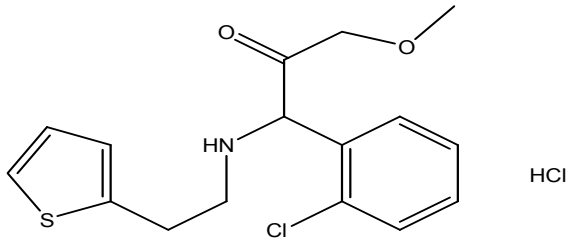



**Figure- 5:** Impurity-D:

**Figure- 6:** Chromatogram of Blank

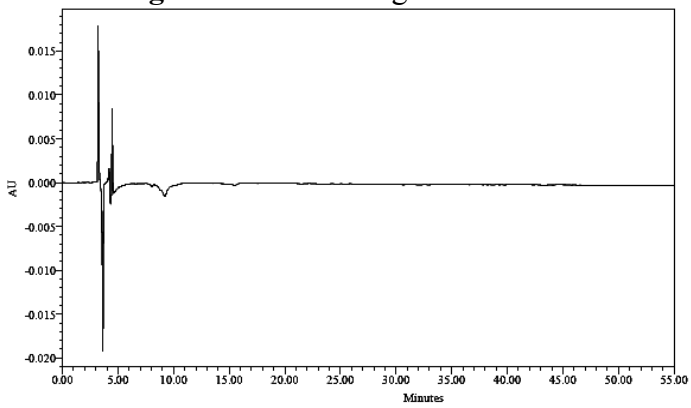
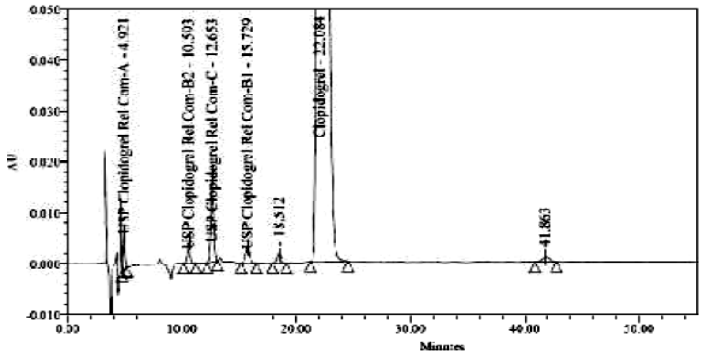


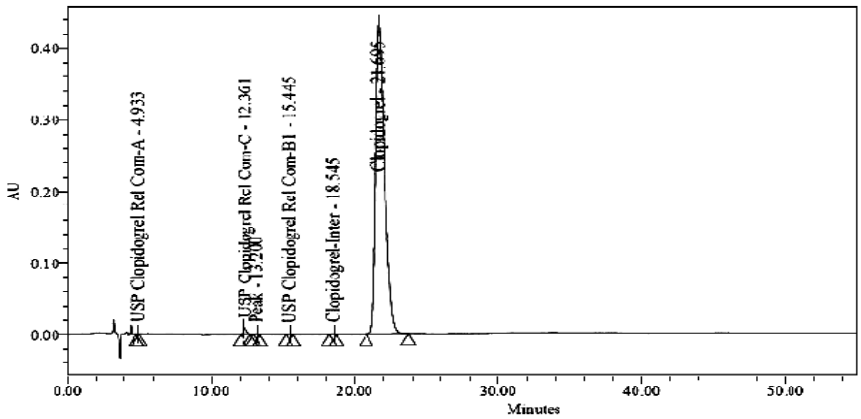

**Figure-7**: Chromatogram of Spiked Sample




**Peak Table:**

| S.No.: | Peak Name | RT |
|---|---|---|
| 1 | Impurity-A | 4.921 |
| 2 | Impurity-B2 | 10.593 |
| 3 | Impurity-C | 12.653 |
| 4 | Impurity-B1 | 15.729 |
| 5 | Impurity-D | 18.512 |
| 6 | Clopidogrel | 22.084 |
| 7 | Unknown | 41.863 |

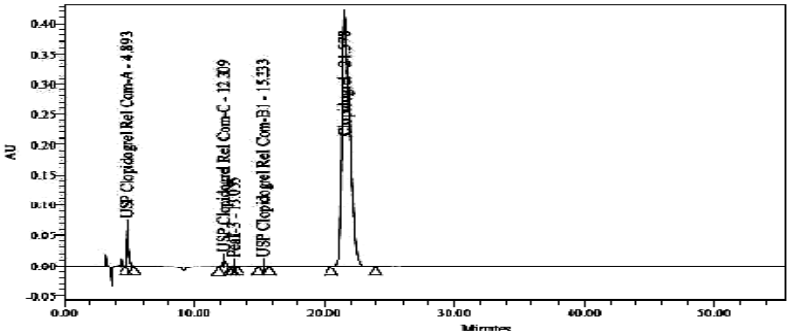
Figure 8: Chromatogram of Themal Degradation




**Peak Table:**

| S.No: | Peak Name | RT |
|---|---|---|
| 1 | Impurity-A | 4.933 |
| 2 | Impurity-C | 12.361 |
| 3 | Unknown | 13.200 |
| 4 | Impurity-B | 15.445 |
| 5 | Impurity-D | 18.545 |
| 6 | Clopidogrel | 21.695 |

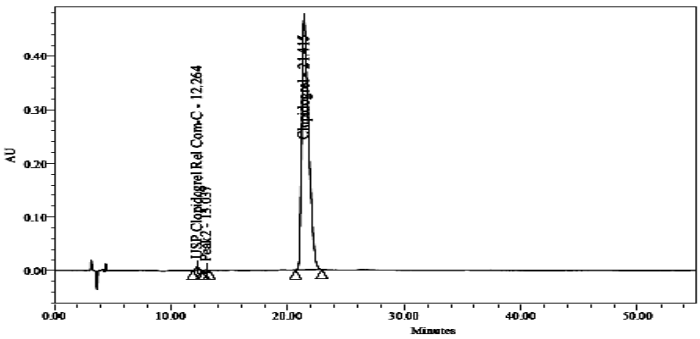
**Figure-9**: Chromatogram of Humidity Degradation




**Peak Table:**

| S.No.: | Peak Name | RT |
|---|---|---|
| 1 | Impurity-A | 4.893 |
| 2 | Impurity-C | 12.309 |
| 3 | Unknown | 13.055 |
| 4 | Impurity-B | 15.333 |
| 5 | Clopidogrel | 21.578 |

**Figure-10**: Chromatogram of Photolytic Degradation




**Peak Table:**

| S.No.: | Peak Name | RT |
|---|---|---|
| 1 | Impurity-C | 12.624 |
| 2 | Unknown | 13.039 |
| 3 | Clopidogrel | 21.578 |

**Table-1:** System Suitability

| Parameter | Result | Acceptance Criteria |
|---|---|---|
| 1. USP Resolution between Impurity-D and Clopidogrel from Resolution Solution | 3.8 | Not less than 2.0 |
| 2. USP Tailing Factor Clopidogre Peak from Standard Solution | 1.0 | Not more than 1.5 |
| 3. USP Plate count of Clopidogrel peak from Standard Solution | 7563 | Not be less than 4000 |
| 4. %RSD of Clopidogrel peak from Standard Solution | 0.6 | Not more than 2.0 |

**Table-2**: Forced Degradation Data

| **Clopidogrel Bisulfate Related Substances - Forced Degradation** | | | | |
|---|---|---|---|---|
| Condition | %Degradation | Purity Angle | Purity Threshold | Purity Flag |
| Humidity Stress-25°C/97%RH for 350 hrs | 10 | 0.165 | 1.035 | No |
| Heat Stress-105°C for 350 hrs | 2 | 0.163 | 1.082 | No |
| Photolytic Stress-UV for 350 hrs | 2 | 0.212 | 1.062 | No |
| Photolytic Stress-Light for 350 hrs | 8 | 0.186 | 1.042 | No |

**Table-3**: Recovery

| Clopidogrel Bisulfate related substance - Recovery | | | | |
|---|---|---|---|---|
| **Name of Impurity** | **Impurity-A** | **Impurity-B1** | **Impurity-C** | **Impurity-D** |
| Experiment | Average % Recovery | Average % Recovery | Average % Recovery | Average % Recovery |
| **Recovery –LOQ Level** | 106.3 | 93.1 | 104.2 | 98.2 |
| **Recovery –50% Level** | 94.6 | 101.8 | 96.6 | 95.0 |
| **Recovery –75% Level** | 95.8 | 102.2 | 97.5 | 96.3 |
| **Recovery –100% Level** | 98.2 | 105.3 | 99.9 | 98.1 |
| **Recovery –150% Level** | 103.5 | 103.4 | 96.6 | 97.3 |

**Table-4**: LOD, LOQ and Linearity

| **Clopidogrel Bisulfate related substances - Limit of Detection and Limit of Quantitation** | | | | |
|---|---|---|---|---|
| **Name of the Component** | **Impurity-A** | **Impurity-B1** | **Impurity-C** | **Impurity-D** |
| LOD(%w/w) | 0.0035 | 0.010 | 0.0085 | 0.0085 |
| LOQ(%w/w) | 0.013 | 0.037 | 0.027 | 0.027 |
| %RSD for Precision at LOQ level | 3.48 | 7.34 | 9.12 | 9.66 |
| Linearity-Correlation Coefficient | 0.9995 | 0.9985 | 0.9999 | 0.9985 |

**CONCLUSION:**

A simple, rapid, cost effective and accurate Normal Phase-HPLC method was developed for the Stability indicating Related Substances method for Clopidogrel Bisulfate drug substance. The analytical conditions and the solvent system developed provided good resolution between Impurity-D and Clopidogrel within a short run time. The HPLC method was validated and demonstrated good linearity, precision, accuracy, specificity and stability indicating. Thus, the developed HPLC method can be utilized for routine analysis and stability studies for Clopidogrel Bisulfate Drug Substance.

**Acknowledgment:**

The authors are thankful to Dr. V. Suranarayana Rao for providing the working standards of Clopidogrel Bisulfate.

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