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A Simple and Effective Stability Indicating Normal Phase HPLC Method for Development and Validation of Clopidogrel Bysulfate Drug Substance

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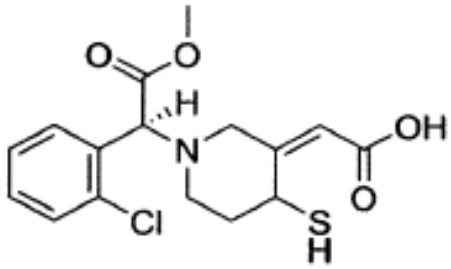
ABSTRACT

Clopidogrel is a thienopyridine antiplatelet agent that is taken orally. In coronary artery disease, peripheral vascular disease, and cerebrovascular disease, it is used to prevent blood clots. It operates by blocking the P2Y12 receptor in an irreversible manner. In this paper, A Simple and Effective Stability Indicating Normal Phase HPLC Method has been developed and validated for clopidogrel bysulfate drug substance. Chromatographic separation was performed on a Chiral Cel OD-H Column (250 × 4.6 mm, 5p) with a mobile phase of 920 mL n-Hexane, 50 mL Ethanol, 30 mL Isopropyl alcohol, and 0.3 mL diethylamine flowing at 0.9 mL per minute. At 240nm, the detection was made. Clopidogre peak has a retention time of 20.8 minutes. According to the ICH and USP requirements, the proposed approach was validated.

1. Introduction

Clopidogrel is a thienopyridine antiplatelet agent that is taken orally. In coronary artery disease, peripheral vascular disease, and cerebrovascular disease, it is used to prevent blood clots. It operates by blocking the P2Y12 receptor in an irreversible manner.



IUPAC Name: Methyl (2S)-2-(2-chlorophenyl)-2-(6,7-dihydro-4H-thieno[3,2-c]pyridin-5-yl)acetate; sulfuric acid and Molecular Weight: $C_{16}H_{18}ClNO_6S_2$

Clopidogrel is a prodrug that works by binding to an ADP receptor on platelet cell membranes. The medicine blocks the P2Y12 subtype of the ADP receptor, which is involved in platelet aggregation and cross-linking by the protein fibrin, in a particular and irreversible manner. [1] Platelet aggregation is inhibited by preventing the activation of the glycoprotein IIb/IIIa pathway, which is blocked by blocking this receptor. The IIb/IIIa complex serves as a receptor for fibrinogen, vitronectin, fibronectin, and von Willebrand factor, among other things. This receptor complex's activation is the "ultimate common pathway" for platelet aggregation, and it's crucial for platelet cross-linking by fibrin.

Clopidogrel is indicated for: [2] In patients with symptomatic atherosclerosis, preventing vascular ischemic episodes is a priority. Acute coronary syndrome without ST-segment elevation (NSTEMD, ST elevation MI) is a type of acute coronary syndrome in which there is no ST-segment elevation (STEMI) It's also used in conjunction with aspirin to avoid thrombosis when an intracoronary stent is placed, or as an alternate antiplatelet medicine for individuals who can't take aspirin. [3]

International recommendations gave clopidogrel in addition to aspirin the highest recommendation for NSTE-ACS, PCI, and stents,[clarification needed]. Patients who need antiplatelet medication but have a history of stomach ulceration should take clopidogrel instead of aspirin, according to consensus-based clinical guidelines, because aspirin (acetylsalicylic acid) inhibits the formation of prostaglandins, which might aggravate the condition. Patients taking aspirin plus the proton pump inhibitor esomeprazole had a reduced risk of recurrent ulcer bleeding than patients receiving clopidogrel, according to a research. [4] However, a recent study found that prophylaxis with proton pump inhibitors and clopidogrel after an acute coronary syndrome may enhance unfavourable cardiac outcomes, possibly due to suppression of CYP2C19, which is required for clopidogrel's conversion to its active form.[5][6][7] Clopidogrel and proton pump inhibitors may interact, according to a public statement by the European Medicines Agency.[8] However, several cardiologists have expressed concern that the research on which these warnings are based have numerous flaws and that it is unclear if clopidogrel and proton pump inhibitors interact.[9] Clopidogrel is being triggered (upper left). The first stage is oxidation, which is primarily mediated by CYP2C19. The bottom two structures are tautomers of one other, and hydrolysis is the final step. The Z configuration at the double bond C3—C16 in the active metabolite (top right) and perhaps R configuration at the newly asymmetric C4 are both present.[10]

Plasma concentrations of the parent drug, which has no platelet inhibitory action, are very low following repeated 75 mg oral doses of clopidogrel (base) and are generally below the quantitation limit (0.258 g/L) beyond two hours after treatment. Clopidogrel is a prodrug that cytochrome P450 enzymes, particularly CYP2C19, activate in the liver. The chemical structure of the active has three stereochemically significant sites due to the opening of the thiophene ring, resulting in a

total of eight potential isomers. The initial stereocentre at C7, a double bond at C3—-C16, and a stereocentre at C4 (connected to the —SH thiol group). Only one of the eight structures is an antiplatelet medication in use. The following is how it's set up: Work with the active metabolite of the related drug to determine the Z configuration at the C3—C16 double bond, the original S configuration at C7, and, while the stereocentre at C4 cannot be determined directly due to the thiol group's reactivity, work with the related drug's active metabolite to determine the stereocentre at C4. Prasugrel suggests that the C4 group's R-configuration is important for P2Y12 and platelet-inhibitory function. [requires citation]

The active metabolite operates by creating a disulfide bond with the platelet ADP receptor and has an elimination half-life of roughly eight hours. Patients with a CYP2C19 variant allele are 1.5 to 3.5 times more likely than patients with the high-functioning allele to die or develop problems.[11][12][13] In humans, nearly 50 percent of 14C-labeled clopidogrel was eliminated in the urine and roughly 46 percent was excreted in the faeces five days after dosage.

Clopidogrel bisulfate administration with meals had no effect on clopidogrel bioavailability as measured by the pharmacokinetics of the primary circulating metabolite. Clopidogrel is readily absorbed after oral administration of repeated doses of 75 mg clopidogrel (base), with peak plasma levels of the principal circulating metabolite (about 3 mg/L) reaching one hour after treatment. In the dose range of 50 to 150 mg of clopidogrel, the pharmacokinetics of the primary circulating metabolite are linear (plasma concentrations increased in proportion to dose). Based on urine excretion of clopidogrel-related metabolites, absorption is at least 50%. Clopidogrel and its primary circulating metabolite bind to human plasma proteins reversibly in vitro (98 percent and 94 percent, respectively). In vitro, up to a concentration of 110 g/mL, the binding is nonsaturable.

Clopidogrel exhibits fast hydrolysis in vitro and in vivo, converting it to its carboxylic acid derivative. The carboxylic acid derivative's glucuronide can also be found in plasma and urine. Plavix received a boxed warning from the US Food and Medicine Administration (FDA) in March 2010, indicating that it may be less effective in patients who are unable to metabolise the drug and convert it to its active form.[14][15]

Pharmacogenetics

Antidepressants, barbiturates, proton pump inhibitors, antimalarials, and anticancer medicines are among the numerous clinically beneficial pharmaceuticals that CYP2C19 catalyses the biotransformation of. One of the medicines processed by this enzyme is clopidogrel.

Several recent landmark studies have demonstrated the relevance of 2C19 genotyping in clopidogrel or Plavix treatment. Plavix received a black box warning from the FDA in March 2010 to inform patients and healthcare professionals that CYP2C19 poor metabolizers, who account for up to 14% of patients, are at high risk of treatment failure and that testing is available.[16] Researchers discovered that patients with cytochrome P-450 2C19 (CYP2C19) variants have lower levels of the active clopidogrel metabolite, less platelet inhibition, and a 3.58 times higher risk of major adverse cardiovascular events like death, heart attack, and stroke; the risk was greatest in CYP2C19 poor metabolizers.[17]

2. Development of analytical methods

Chromatographic parameters were tuned in advance to establish a Clopidogrel Bisulfate stability indicating Related Substances technique with a quick analysis time (55 minutes). Due to the presence of five contaminants in Clopidogrel Bisulfate. To demonstrate the stability of the Related Substances technique, these contaminants must separate from each other as well as from the primary analyte.

Clopidogrel Bisulfate Impurity-B1 and Impurity-B2 are both isomers of Clopidogrel Bisulfate. In the reverse phase approach, isomer separation is extremely challenging. As a result, for method development, the normal phase approach was chosen.

With the selection of the mobile phase, the development trials began. Because the chosen development method is normal phase, various non-polar solvents and their various logical proportions (Solvents such as n-Hexane, Butylenechloride, Isopropyl alcohol, Ethanol, etc.) were used in the initial developmental trials, which were eventually concluded with the efficient mobile phase, which was a mixture of n-Hexane, Ethanol, and Isopropyl alcohol. Several solvent compositions were tested on various Chiral columns, including Chiralpack AD, Chiralpack OD, Chiral Cel OJ-H, Chirosil, Chiral-AGP, and others.

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 was tuned for higher resolution and peak form.

The parameters of system suitability were assessed, and limits were set. Impurity-D and Cloidogrel were resolved and confirmed to be within acceptable limits.

Clopidogrel was tested using the USP Tailing factor, USP Plate count, and Area ratio and confirmed to be within the limits.

3. Analytical Method:

Chemicals and Reagents:

LGC Promochem provided the Clopidogrel Bisulfate working standards and impurities (Impurity-A, Impurity-B1, Impurity-B2, Impurity-C, Impurity-D, and Impurity-E), while commercial pharmacy provided the tested pharmaceutical. HPLC grade n-hexane, ethanol, and isopropyl alcohol were suitable for analysis, as was Diethylamine AR grade.

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 0.3m1 of Diethylamine, sonicate it for 2 minutes.

Diluent: Use mobile phase as diluent.

Preparation of Resolution Solution:

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

Preparation of Standard Solution:

Clopidogrel Sppm solution sample solution was made with 10ml ethanol and then reaming with mobile phase.

Preparation of Sample Solution:

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

4. Procedure:

Inject the following solutions and record the responses.

Solution Name	No. of injections
Blank (diluent)	1
Resolution solution	1
Standard solution	6
Sample solution	1

Evaluation of system suitability:

- USP Resolution from resolution solution between Impurity-D and Clopidogrel peaks is not less than 2.0.
- Clopidogrel peak from standard solution has a USP tailing factor of no greater than 1.5.
- Clopidogrel peak from standard solution must have a USP plate count of at least 4000.
- Clopidogrel peak relative standard deviation from six duplicate injections of standard solution is less than 10%.

5. Method Validation Results

The above method was verified in accordance with ICH and USP criteria to determine a method's performance characteristics (expressed in terms of analytical parameters) in order to meet the requirements for the method's intended application. [15].

System Suitability:

Suitability criteria such as Retention Time, USP Resolution, USP Tailing factor, USP Plate count, and Area Ratio of Clopidogrel peak regions were evaluated in order to determine the suggested methodology's acceptable resolution and repeatability. Table 1 summarizes the results.

System Suitability

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	0.6	Not more than 2.0

6. Specificity:

Interference from Blank:

The capacity of an analytical method to determine the analyte definitively in the presence of other components such as contaminants, degradation products, and matrix can be characterised as specificity. By injecting the blank solution and looking for interference at the retention periods of all known contaminants and the principle peak, specificity was determined. There was no interference from the blank solution, according to the results. Figure 6 depicted the blank chromatogram.

Interference from Impurities:

All known contaminants are injected one by one, spiked to specification level in the test, then injected into the system. All of the contaminants were kept apart from one another and from the main analyte. Figure 7 depicts the spiked chromatogram.

Forced degradation Studies:

Drug substance was treated to stress conditions such as acid, base, hydrolysis, peroxide, heat, photo light, ultraviolet light, and humidity. Using Empower software, all of the samples were examined for peak purity of all known contaminants as well as Clopidogrel peaks. Clopidogrel's peak purity and all known contaminants were detected within the limits in all stressed samples.

Table 2 summarises the results, and Figure 1 depicts degradation chromatograms (8-10).

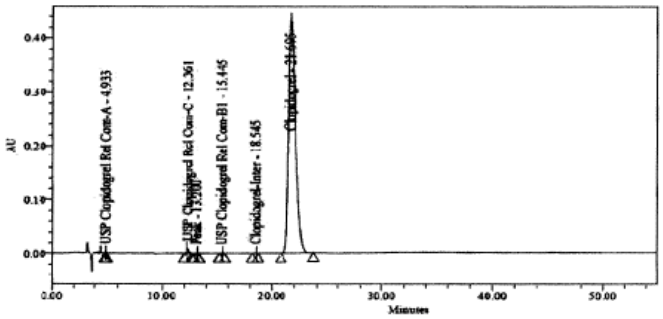
Table 2: Forced Degradation Data:

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

Acceptance Criteria:

1. Purity angle should be less than Purity Threshold.
2. Clopidogrel and each related substance peak should not have any flag in purity results table (For Waters Empower software)

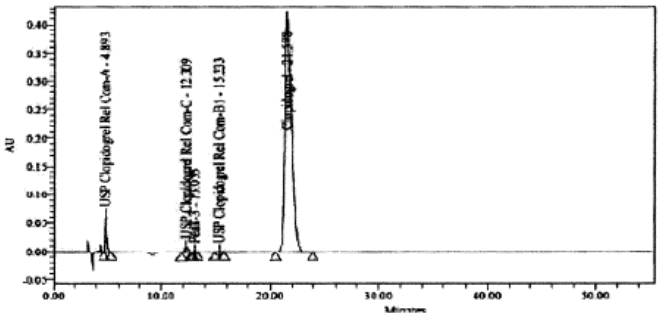
Chromatogram of Themal Degradation:



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

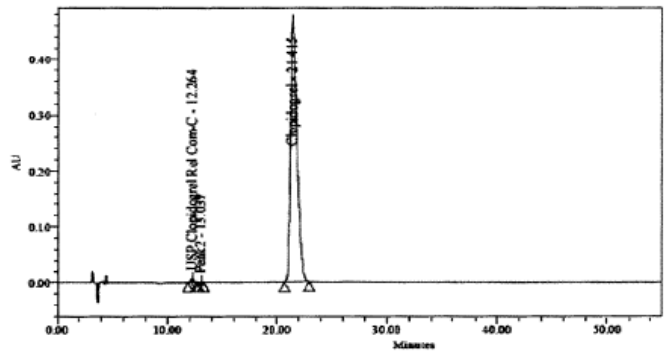
Chromatogram of Humidity Degradation:



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

Chromatogram of Photolytic Degradation:



Peak Table:

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

7. Method Precision:

The degree of dispersion (closeness of agreement) between a set of measurements acquired from multiple sampling of the same homogeneous sample under the stipulated conditions is expressed by the precision of an analytical method. There are three levels of accuracy: repeatability, intermediate precision, and reproducibility.

Six replicate samples were made by spiking all known contaminants at the specification level and analysing them according to the test procedure. The percent relative standard deviations for all known contaminants were determined, and the findings were found to be within the acceptable range.

Accuracy:

The closeness of agreement between the value acknowledged as a conventional true value or an approved reference value and the value found is expressed by the accuracy of an analytical method.

By spiking all known impurities with the drug ingredient, recovery studies were conducted at 50 percent, 75 percent, 100 percent, and 150 percent of the specification level of all known impurities. At 50 percent, 75 percent, 100 percent, and 150 percent, three duplicates were performed. Extracted and examined spiked samples. We calculated the amount spiked, the amount recovered, the percent recovery, and the mean.

Table 3 : Ssummarizes the results .

Clopidogrel Bisulfate related substances - Method Precision				
Sample Preparation	Name of the Impurity			
	Impurity-A	Impurity-B1	Impurity-C	Impurity-D
	% w/w, on as is basis			
1	0.245	0.352	1.452	0.156
2	0.232	0.325	1.478	0.154
3	0.229	0.361	1.335	0.147
4	0.214	0.359	1.506	0.147
5	0.253	0.322	1.479	0.156
6	0.236	0.358	1.401	0.157
Average	0.235	0.346	1.442	0.153
%RSD	5.7	5.2	4.4	3.0

Intermediate Precision:

On two distinct days, a research was done on two different analysts using different columns and HPLC apparatus, injecting six test solutions according to the proposed approach, spiking all known contaminants at specification level. Both sets of researchers examined the system suitability characteristics using the test method, and the percent relative standard deviation for each clopidogrel-related impurity was found to be within acceptable limits.

When the results of both sets are compared, it is clear that the related compounds test procedure is reliable in both cases.

Limit of Detection and Limit of Quantification:

The signal to noise ratio approach is used to calculate the limit of detection (LOD) and limit of quantitation (LOQ). S/N (signal-to-noise ratio) = 2H/h

H stands for the height of the analyte peak, while h stands for the height of the noise.

Six replicate injections of solution containing known contaminants and Clopidogrel at this level were used to verify the LOD and LOQ values. For the peak locations, the percentage relative standard deviation (percent RSD) was calculated and determined to be within the acceptable range. Table 4 displays the results.

Table 4: LOD and LOQ

Clopidogrel Bisulfate related substances - Limit of Detection and Limit of Quantitation				
Parameter	Name of the Component			
	Impurity-A	Impurity-B1	Impurity-C	Impurity-D
LOD(%w/w)	0.0035	0.010	0.0085	0.0085
LOD (S/N)	4	3	4	3
LOQ(%w/w)	0.013	0.037	0.027	0.027
LOQ (S/N)	10	11	11	10
%RSD for Precision at LOQ level	3.48	7.34	9.12	9.66

Acceptance Criteria:

1) LOD: Signal to noise ratio should be about **3**

2) LOQ: Signal to noise ratio should be about **10**

3) The **% Relative standard deviation** of area counts of each known impurity from six replicate injections should **be not more than 10.0**

Detector Response Linearity:

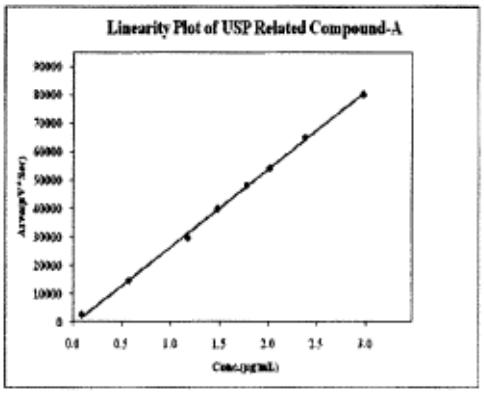
The capacity of an analytical process to produce test results that are directly proportional to the concentration (quantity) of analyte in the sample (within a specific range) is known as linearity. Linearity of detector response for Clopidogrel and its known impurities was established by analysing a series of Clopidogrel and its known impurities solutions prepared and injected into the HPLC system at concentrations ranging from the Limit of Quantification level to 150 percent of the specification level. The peak area response was plotted versus the final concentration of each solution in pg per mL. The slope, correlation coefficient (R), and intercept were all found to be within the acceptable ranges. Table 5 displays the results.

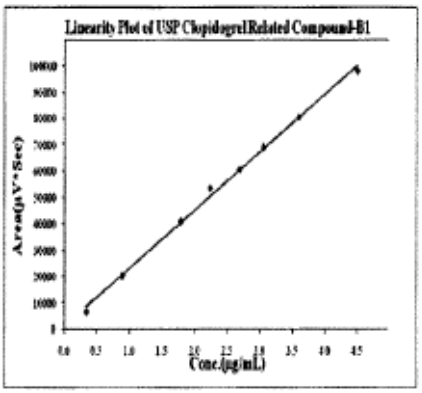
TABLE 5 : LINEARITY

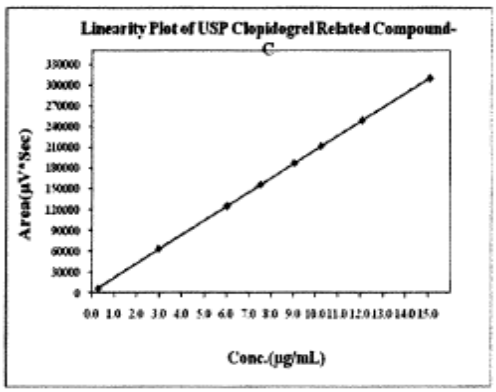
Name	**Impurity-A**		**Impurity-B1**		**Impurity-C**	
Weight taken in mg	2.005		4.706		8.107	
Wt in mg	2.005		2.258		7.544	
Purity	100.00		47.98		93.05	
Level	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area
L1	3.008	80059	4.516	98049	15.088	309754
L2	2.406	65017	3.613	80370	12.070	248801
L3	2.045	54120	3.071	68832	10.260	212065
L4	1.805	48151	2.710	60688	9.053	187096
L5	1.504	39834	2.258	53519	7.544	156306
L6	1.203	29671	1.806	40911	6.035	125014
L7	0.602	14644	0.903	20277	3.018	63419
L8	0.120	2491	0.361	6758	0.302	6398
SLOPE		**27257**		**22002**		**20515**
STEYX		**876**		**1742**		613
INTERCEPT		**-1498**		**767**		**1102**
RRF		**0.8**		**0.9**		1.0
CC		**0.9995**		**0.9985**		**0.9999**

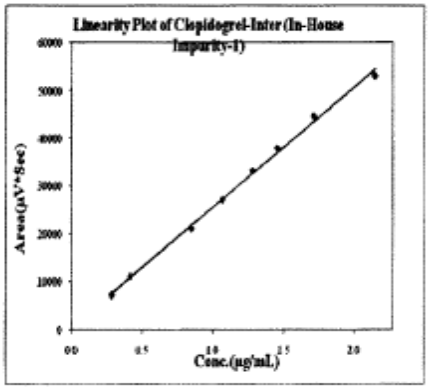
Name	**Impurity-D**		**Clopidogrel**	
Weight taken in mg	5.351		7.552	
Wt in mg	5,423		7.876	
Purity	99.85		98.9	
	Conc. (µg/mL)	Area	Conc. (µg/mL)	Area
Ll	2.169	53030	2,169	53030
L2	1.735	44615	1.735	44615
L3	1.475	38026	1.475	38026
L4	1.302	33249	1.302	33249
L5	1.085	27070	1,085	27070
L6	0.868	21052	0.868	21052
L7	0.434	11047	0.434	11047
L8	0.304	7239	0.304	7239
SLOPE		**25112**		20734
STEYX		**931**		2367
INTERCEPT		**-3**		2124
RRF		**0.8**		**1.0**
CC		**0.9985**		**0.9997**

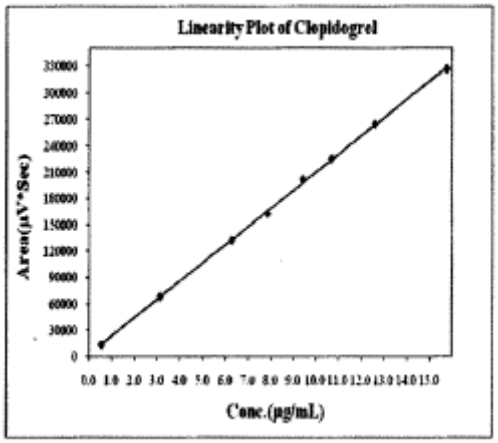
Acceptance Criterion:
The correlation coefficient should be not less than 0.98
Linearity graphs for Clopidogrel and its known impurities:











Robustness:

The method's robustness was tested by purposefully changing the following conditions.

By increasing the flow rate by a factor of ±10%.

By increasing the temperature of the column oven by ±5° Celsius.

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

System suitability solutions and test solutions were made according to the test process, spiked with all known contaminants at specification level, and analysed under each condition.

When the system suitability characteristics and RRT of all known contaminants were examined under various settings and compared to test method conditions, they were found to be within the acceptable range.

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.

TABLE 6: RUGGEDNESS- BENCH TOP STABILITY OF TEST SOLUTIONS

Name of the Impurity		Initial	Day - 1	Day - 2
Impurity-A	Content in % w/w	0.208	0.205	0.202
	% Difference w.r.t initial	NA	1.4	2.9
Impurity-BI	Content in % w/w	0.312	0.315	0.320
	% Difference w.r.t initial	NA	1.0	2,6
Impurity-C	Content in % w/w	1.021	1.026	1.035
	% Difference W t initial	NA	0.5	1.4
Impurity-D	Content in % w/w	0.158	0.150	0.147
	% Difference w.r.t initial	NA	5.1	7.0
Highest Unknown Impurity	Content in % w/w	0.054	0.054	0.056
	% Difference w.r.t initial	NA	0.0	3.7
Total Unknown Impurities	Content in % w/w	0.122	0.120	0.128
	% Difference w.r.t initial	NA	1.6	4.9

Where, NA = Not Applicable

Acceptance Criteria

The **% Difference with respect to initial** of each known impurities, highest unknown impurity and total unknown impurities should be **not more than 10.0.**

TABLE 7 : RUGGEDNESS — BENCH TOP STABILITY OF MOBILE PHASE

System suitability parameters	Observed value			Acceptance limit
	Initial	Day-1	Day-2	
1,USP Resolution between Impurity-D and Clopidogrel from Resolution Solution	4.1	3.9	4.2	Not less than 2.0
2.USP Tailing Factor Clopidogre Peak from Standard Solution	1.1	0.0	1.2	Not more than 1.5
3.USP Plate count of Clopidogrel from Standard Solution peak	6820	6645	6135	Not be less than 4000

S.No	Impurity Name	Relative Retention Time		
		Initial	Day-1	Day-2
1	Impurity-A	023	0.23	0.24
2	Impurity-B2	0.47	0.48	0.49
3	Impurity-C	0.56	0.58	0.55
4	Impurity-BI	0.72	0.73	0.75
5	Impurity-D	0.85	0.88	0.87

Acceptance Criteria

1) The system suitability parameters should all be in order.
2) The relative retention time should be similar to the initial retention time.

8. Conclusion

For the analysis of Clopidogre Bisulphate Drug Substance related chemicals, a simple, precise, cost-effective, and stability-indicating Normal Phase HPLC technique has been designed and validated. The approach was determined to be Specific, Precise, Rugged, Accurate, Linear, Robust, and Stable, indicating that it is appropriate for its intended application. Chromatographic separation was performed on a Chiral Cel OD-H Column (250 x 4.6 mm, 5p) with a mobile phase of 920 mL n-Hexane, 50 mL Ethanol, 30 mL Isopropyl alcohol, and 0.3 mL diethylamine flowing at 0.9 mL per minute. At 240nm, the detection was made.

Clopidogre peak has a retention time of 20.8 minutes. According to the ICH and USP requirements, the proposed approach was validated.

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