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Stability Indicating RP-HPLC Method Development and Validation for the estimation of Clopidogrel bisulphate.

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ABSTRACT

A simple, sensitive, accurate and specific stability-indicating high-performance liquid chromatographic method was developed and validated for the estimation of clopidogrel bisulphate in bulk. In the present study, extensive testing of clopidogrel bisulphate in different stress conditions were carried out as per the ICH guidelines Q1A (R2). The system consisted of a pump (Shimadzu, prominence, LC20AD), with 20µl sample injector, along with a PDA (Shimadzu, prominence, SPDM20A) detector at a wavelength of 254nm. Data was compiled using Shimadzu LC Solution software. The degraded products formed under various stress conditions were separated successfully from the drug by using a PHENOMENEX C8 Column (150 x 4.6mm, 5µm) with binary gradient conditions. Acetonitrile: phosphate buffer of pH 2.0 was used as mobile phase at flow rate of 1.2ml/min. Clopidogrel bisulphate was exposed to various stress conditions like oxidation, hydrolysis, photolysis and neutral decomposition. Clopidogrel bisulphate, which was found to degrade considerably in acidic, photo and oxidative conditions, was found to be stable in alkaline and neutral conditions. Apart from the formation of minor degraded products under accelerated conditions, the drug was reasonably stable in solid state. A good linear relationship over the concentration range of 150-500µg/ml was shown. Validation of the method was carried out as per the ICH guidelines. The method developed was found to be precise, accurate, specific and selective. Clopidogrel bisulphate showed degradation in 5M Hydrochloric acid at 80^{o}C, in 3% hydrogen peroxide for 5min the drug showed around 35% of degradation, when exposed to sunlight for 15 min, formed around 25-30% of degradation products. Statistical analysis shows that the method is reproducible and selective for the estimation of clopidogrel bisulphate in dosage form.

Keywords: Clopidogrel bisulphate, HPLC, stability parameters, stress conditions.

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INTRODUCTION

To Develop and Validate an RP-HPLC method for the determination of stability indicating parameters for clopidogrel bisulphate. International Conference of Harmonization (ICH) suggests that the stress studies should be carried out on a drug to determine its constitutional stability features leading to the identification of its degradation products[1, 2]. The drug stability test guideline Q1A (R2) issued by the International Conference of Harmonization (ICH) suggests that the analytical test procedures should be stability indicating and validated. Clopidogrel bisulphate, (CPG) (+)-(S)-methyl 2-(2-chlorophenyl)-2-(6, 7-dihydrothieno [3, 2-c] pyridin-5(4H)-yl) acetate (Figure 1A) is a prodrug that is converted in the liver to an active thiol metabolite, which irreversibly inhibits the platelet P2Y12 adenosine diphosphate receptor. This bioactivation is mediated by hepatic cytochrome P450 isoenzymes, with cytochrome P450 2C19 playing a major role. It is used in the Prevention of vascular ischemic events in patients with symptomatic atherosclerosis, acute coronary syndrome without ST-segment elevation (NSTEMI), ST elevation MI (STEMI).

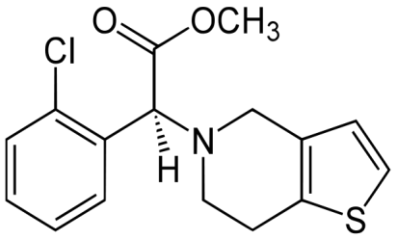

Figure 1: Structure of Clopidogrel bisulphate

Literature survey reveals that few analytical methods have been reported for clopidogrel include RP-HPLC methods[1-4], HPTLC method[5,6] , UV method[7], normal phase HPLC[8], GC method[9], LC-MS method[10], capillary electrophoresis method[11]. In the current study, a new stability indicating, simple and reliable RP-LC method for determination of clopidogrel bisulphate has been proposed.

The aim of the current study was to develop and validate an accurate, specific, linear, repeatable and robust stability indicating RP-HPLC method for clopidogrel bisulphate in presence of its degraded products for assessing the purity of the drug as per ICH.

MATERIALS AND METHODS

Materials

Pure sample of Clopidogrel bisulphate BP was received from Wintac Limited, Bangalore, Karnataka, India, of Batch No.: PNQ5039. The crude sample was used as such, which was certified to contain 99.69% (w/w) of the drug on dry weight basis. Analytical and HPLC grade reagents were used for the study.

Instrumentation

A High performance liquid chromatograph LC 10AT SHIMADZU, PROMINENCE – SPD M20A detector with Rheodyne injector, PHENOMENEX C8 (150 x 4.6mm, 5 µm) column with a flow rate of 1.2ml/min was used. The detector used was PDA. Wavelength selected was 254nm. Acetonitrile: phosphate buffer (pH 2.0) in the ratio of 40:60 was used as the mobile phase. An injection volume was 20 µl. Shimadzu LC solution was the software used for the interpretation of the peaks. The chromatogram showing peak with Acetonitrile: Phosphate buffer (3:2) mobile phase and the UV Spectrum of Clopidogrel bisulphate is shown in Figure 2 & 3.

Forced Degradation Studies

Clopidogrel bisulphate (150mg) was weighed and dissolved in 25ml of distilled water. The solution was sonicated for 20min and made upto 100ml in a volumetric flask. The resulting concentration of the stock solution was 1.5mg/ml. To assess the stability parameters and specificity of the proposed method, the clopidogrel bisulphate solution was subjected to various stress conditions. An average peak area of seven replicates was obtained for the degradation studies.

Acid-Base Degradation

To determine the degradation of the drug, the stock solution was exposed to 2M Hydrochloric acid and 2M sodium hydroxide at 80°C for 8hrs separately. The solutions obtained after degradation was diluted to obtain 150 µg/mL solutions. 20µL of this solution was injected into the HPLC system. The chromatogram recorded is presented in Figure 4.

Oxidative Degradation

To study the effect of oxidative degradation, the stock solution was exposed to 3% hydrogen peroxide at room temperature for 5min, and then heated in boiling water bath for 10min to remove the excess of hydrogen peroxide. The solutions obtained where diluted to obtain 150µg/mL solutions and 20µL was injected into the system. The chromatogram recorded is presented in Figure 5.

Photochemical Degradation

The stock solution was exposed to direct sunlight for 15min to study the photochemical degradation. The solution obtained was further diluted to obtain a concentration of 150µg/ml and 20µL was injected into the HPLC system. The chromatogram recorded is presented in Figure 6.

Method Validation

Since the HPLC method was developed, validation of the method by using various parameters was performed to ensure that the accomplishment of the method meets the requirements of the described analytical applications.

Following parameters were performed for method validation:

1. System suitability
2. Specificity
3. Detection Limit (LOD)
4. Quantification Limit (LOQ)
5. Linearity and Range
6. Precision

a) System precision

b) Method precision

c) Intermediate precision

7. Accuracy
8. Robustness

Specificity

Specificity is the capability to evaluate the analyte distinctly in the presence of expected impurities and degraded products.

Preparation of Mobile Phase

Preparation of 0.1M Phosphate Buffer

Weigh accurately about 0.136gm of Potassium dihydrogen phosphate in a beaker, add 800ml of HPLC grade water and adjust the pH to 2.5 with 10% v/v Orthophosphoric acid, make up to 1000ml with water. To 650 ml of 0.1M phosphate buffer add 350 ml of acetonitrile mix filter thorough 0.45-micron membrane filters and sonicate for 2 minutes.

Standard solution of Clopidogrel bisulphate Solution

Weigh accurately and transfer about 100mg of Clopidogrel bisulphate Working Reference Standard (WRS), and transfer into a 100ml volumetric flask. 20ml of Mobile phase was used to dissolve. Dilute to volume with mobile phase and mix well. (1000 mcg/ml of Clopidogrel bisulphate).

Blank: Mobile phase.

Procedure

20 µl of the blank was injected in duplicate to the HPLC system and chromatographed. 20 µl of Clopidogrel bisulphate standard solution was injected in duplicate to the HPLC system. Standard chromatograms obtained are presented in Figures 7 & 8.

Determination of detection limit for clopidogrel bisulphate (LOD)

The detection limit is the least amount of analyte present in a sample which can be detected, but not necessarily quantified as an exact value.

Working standard solution

From the standard stock solution volumes of 1, 1.5, 2, 2.5 and 3ml were transferred into four different volumetric flasks of 10ml and diluted with the mobile phase to obtain the concentration of 150, 200, 250, 300, 350, 400, 450 and 500mcg/ml.

Procedure

20 µl of different working standard solution were injected to the HPLC system till the solution gave response. Chromatogram recorded is presented in Figure 8.

Determination of quantitation limit for clopidogrel bisulphate (LOQ)

Quantitation limit is the least amount of analyte present in a sample which can be quantitatively determined with suitable accuracy and precision.

Procedure

20 µl of each solution of Clopidogrel bisulphate were injected in to the chromatograph till the smallest concentration that gives response area.

Linearity

The linearity of an analytical procedure is its ability to elicit a direct proportionality between the concentration (amount) of analyte in the sample and the test result within a given range.

Preparation of Linear Solutions

Pipette out 8.0 ml, 9.0 ml, 10.0 ml, 11.0 ml to 12.0 ml, 13.0 ml, 14.0 ml and 15.0 ml from the standard stock solution to different volumetric flasks labeled as L_1, L_2, L_3, L_4 and L_5 and make up the volume to 100 ml using mobile phase to obtain a concentration of 150, 200, 250, 300, 350, 400, 450 and 500 mcg/ml of clopidogrel bisulphate.

Procedure

20 µl of the mobile phase was injected to the HPLC system and chromatographed. 20 µl of Linearity level-1 (L_1) solution was injected six times to the HPLC system and chromatographed. 20 µl Linearity level solutions L_2, L_3, L_4, were injected one time each, into the HPLC system and chromatographed. 20 µl of Linearity level-5 (L_5) solution was injected six times and chromatographed. The data obtained is presented in Table 1.

The standard calibration curve for Clopidogrel bisulphate obtained is presented in Figure 9.

Accuracy

The accuracy of an analytical method is the percentage of relativeness between the conventional true value and the value obtained by that method. Recovery study for Clopidogrel bisulphate at

80%, 100 and 120% levels:

Solutions for Accuracy study

Accuracy level-1 solution: A_1 (80% of target concentration)

A solution of Clopidogrel bisulphate of concentration 150mcg/ml was prepared from the stock solution. Prepare in triplicate.

Accuracy level-2 solution: A_2 (100% of target concentration)

A solution of Clopidogrel bisulphate of concentration 300mcg/ml was prepared from the stock solution. Prepare in triplicate.

Accuracy level-3 solution: A_3 (120% of target concentration)

A solution of Clopidogrel bisulphate of concentration 500mcg/ml was prepared from the stock solution. Prepare in triplicate.

Procedure

20 µl of mobile phase was injected to the HPLC system and chromatographed. 20 µl of standard solution was injected to the HPLC system and chromatographed six times. 20 µl of each of the accuracy solution A_1, A_2 & A_3 injected individually, to the HPLC system and chromatographed. The concentration in the sample solutions was calculated by comparing with the standard solutions. Percentage recovery was concentrated. The average value for each level was reported. The peak area obtained at 3 different levels is presented in Table 2.

RESULTS AND DISCUSSION

A peak of Clopidogrel bisulphate was well detected with the solvent system of Acetonitrile: Phosphate Buffer in the ratio of 3:2. The chromatogram obtained is shown in figure 2.

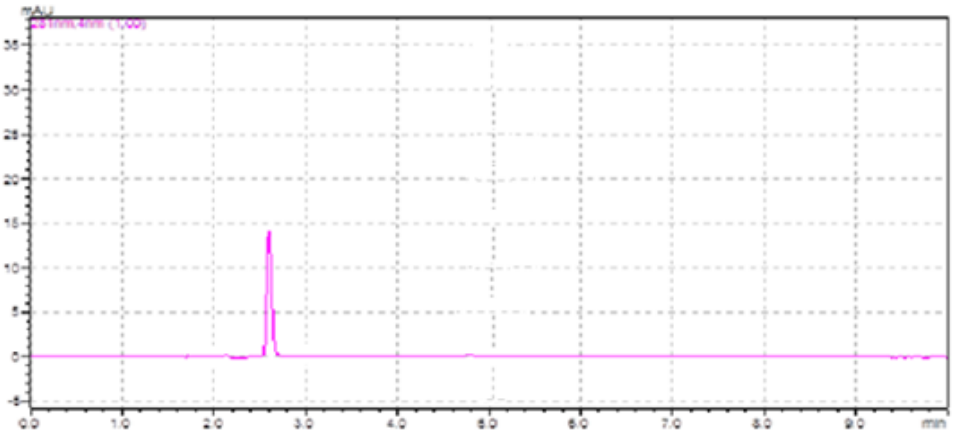

Figure 2: HPLC Chromatogram for Clopidogrel bisulphate

Selection of wavelength for detection:

Standard solutions of Clopidogrel bisulphate (100µg/ml) were scrutinized in the wavelength range of 200-400 nm using acetonitrile: phosphate buffer in the ratio of 3:2 as diluent and UV spectrum obtained is presented in figure 3. The wavelength selected was 254nm.

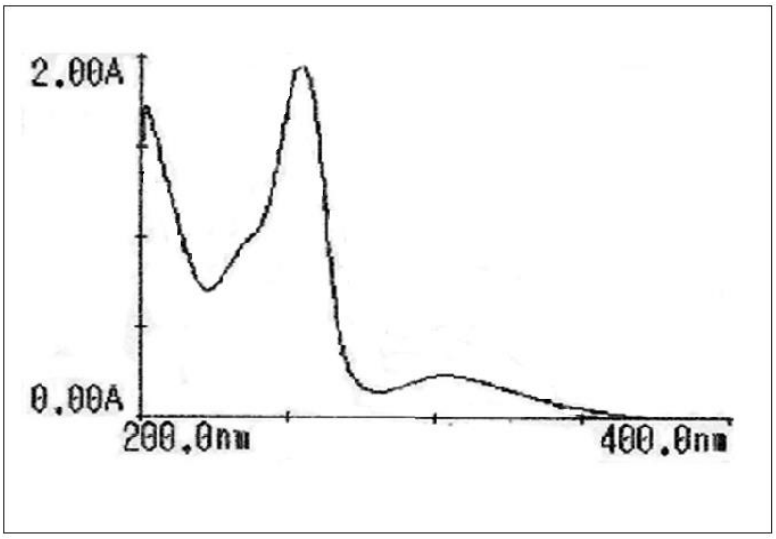

Figure 3: UV Spectrum of Clopidogrel bisulphate at 254nm.

Stability indicating method

Clopidogrel bisulphate was subjected to various stress conditions such as acid-base degradation, oxidation, photochemical degradation and neutral degradation. The samples obtained were studied by HPLC adopting acetonitrile: phosphate buffer of pH 2.0 as mobile phase. The degradation behaviors are compiled below.

Acid-base degradation

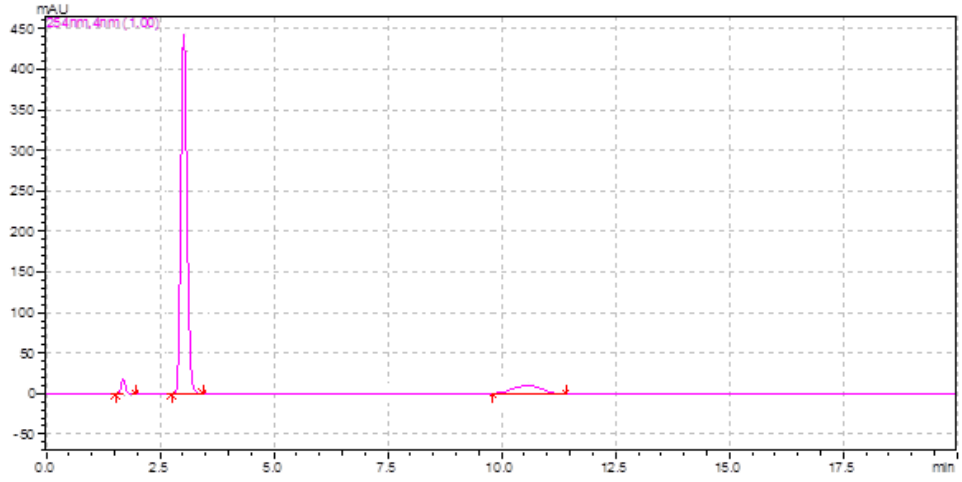

Figure 4: HPLC Chromatogram for acid degradation of clopidogrel bisulphate.

The drug when exposed to 1M Hydrochloric acid at 80^{o}C did not show any degradation until 12 hrs. Thus the acid strength was increased subsequently to 5M. Studies performed using 5M

Hydrochloric acid at 80^{o}C showed slight degradation at 12 hrs. which was detected at 1.62 and 10.70 min. Thus the drug was found to be unstable to acid degradation as shown in figure 4. Similarly, when drug was exposed to 1M NaOH at 80°C for 12hrs, no degraded products were observed. Hence the strength of the base was increased to 5 M. The studies performed in 5M NaOH did not show any degradation until 12hrs at 80°C. Thus the drug was found to be stable to base degradation.

Oxidation

The clopidogrel bisulphate, when exposed to 3% hydrogen peroxide for 30min at room temperature, was degraded completely. Thus the exposure time was reduced to 5 min, where the drug showed around 35% of degradation. The major degradation products formed were detected at 5.58 min, as shown in Figure 5.

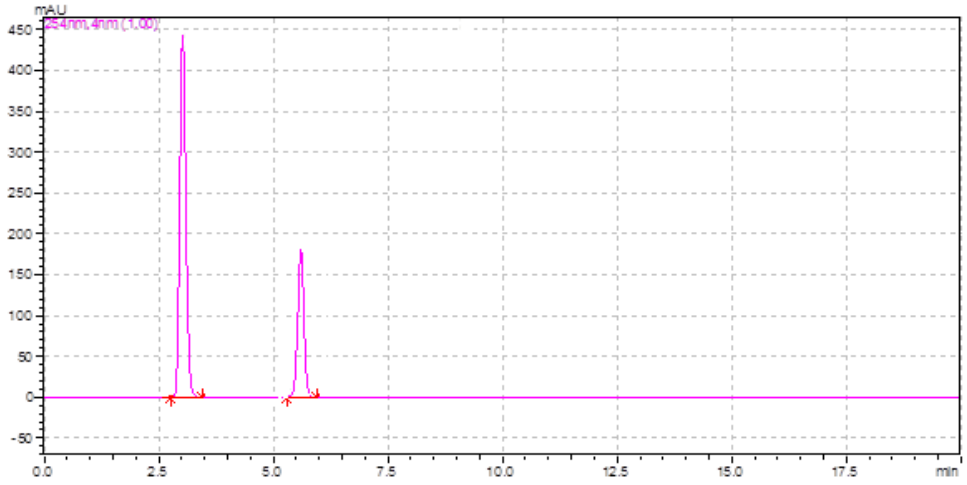

Figure 5: HPLC Chromatogram for oxidative degradation of clopidogrel bisulphate.

Photochemical Degradation

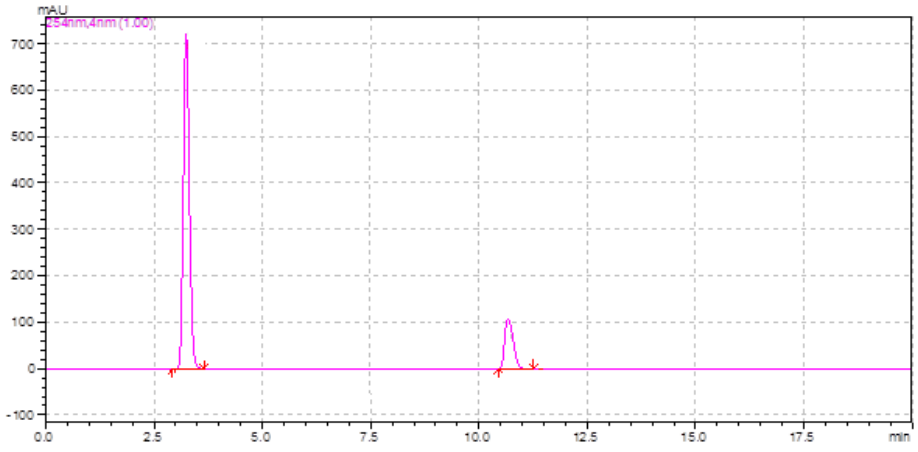

Figure 6: HPLC Chromatogram for photochemical degradation of clopidogrel bisulphate.

Clopidogrel bisulphate in methanol solution when exposed to sunlight for 15 min, formed around 25-30% of degradation products, which was detected at 10.61 min, as shown in figure 6.

Neutral Degradation

The drug was found to be stable to neutral degradation, even upon boiling at 80^{o} C in water for 12 hrs. The drug peak area was found to be unaffected.

Specificity

This parameter was performed to assess and ensure that the impurities, degraded products and diluent does not affect the samples analyzed.20 µl of diluent, Clopidogrel bisulphate (150µg/ml) were injected into the HPLC system and chromatograms were recorded as in figure 7 and 8.

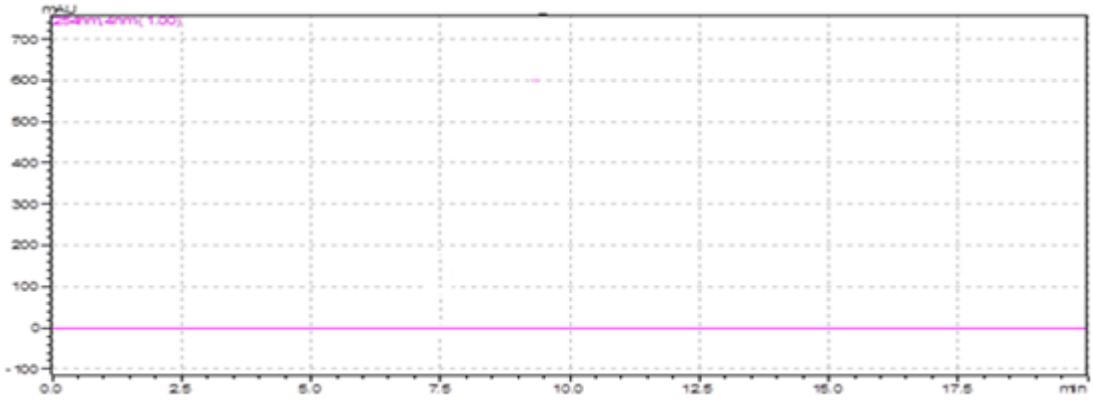

Figure 7: HPLC Chromatogram for blank.

Detection limit (LOD) for clopidogrel bisulphate

The least detectable amount of Clopidogrel bisulphate by the developed HPLC method was examined by injecting 20µl of Clopidogrel bisulphate solution. The flow rate was adjusted to 1ml/min and detected at 254nm by UV detector. The LOD for Clopidogrel bisulphate was found to be 150mcg/ml.

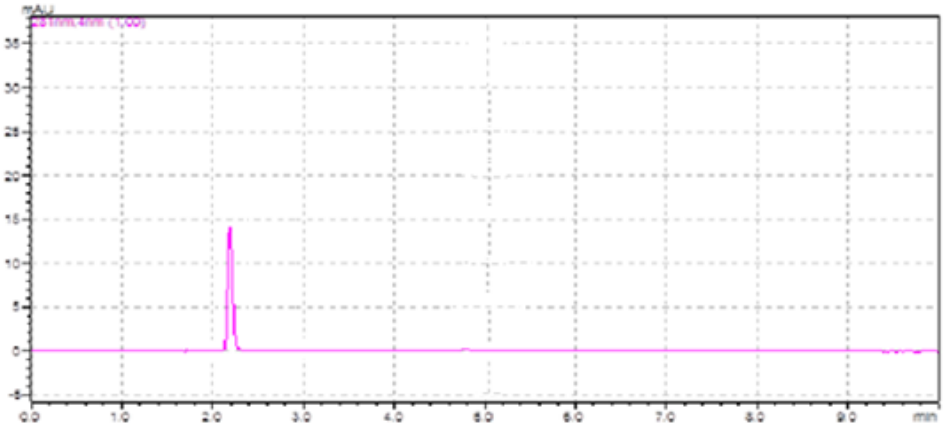

Figure 8: HPLC Chromatogram for clopidogrel bisulphate.

Quantification limit (LOQ) for clopidogrel bisulphate

LOQ parameter was performed to determine the least amount of analyte in the sample and quantify with accuracy and precision. It was determined by injecting 20µl of solution of Clopidogrel bisulphate at a flow rate of 1ml/min. The LOQ of Clopidogrel bisulphate was determined to be 175mcg/ml.

Linearity

The linearity parameter was performed to ensure that the test results and the concentration of analyte sample are directly proportional. 20µl of working standard solution of Clopidogrel bisulphate was injected in to HPLC system. The peak area and concentration were plotted to get a standard calibration curve. The correlation coefficient, regression coefficient and % curve fitting was calculated. The results obtained are tabulated in table 1. The standard calibration curve is presented in figure 9.

Table 1: Linearity data for Clopidogrel bisulphate

Sl. No	Concentration(mcg/ml)	Peak Area*
1	150	1039.03
2	200	1051.35
3	250	1064.45
4	300	1092.11
5	350	1149.21
6	400	1180.44
7	450	1208.15
8	500	1293.02

* Average of 3 values

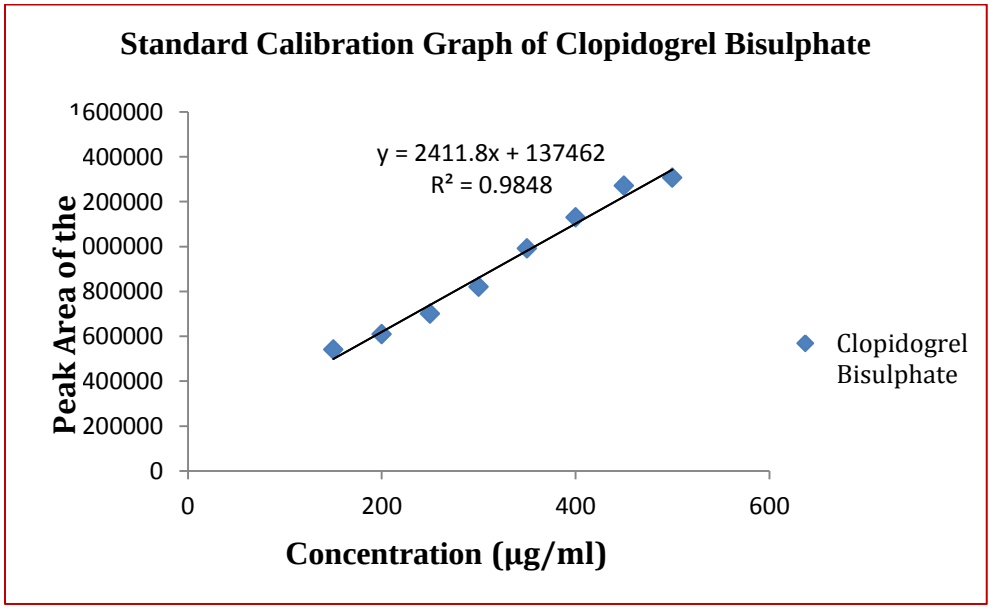

Figure 9: Standard calibration curve for clopidogrel bisulphate.

Accuracy

This parameter was performed to determine the proximity of test results with that of the true value which is expressed as % recovery. This study was carried out using 3 different percentage solutions of concentration 80, 100 and 120. The amount of Clopidogrel bisulphate recovered was calculated. The results are presented in table 2.

Table 2: Recovery studies data for Clopidogrel bisulphate

Sl. No.	Amount of standard (µg/ml)	Peak Area*	Amount of standard recovered (µg/ml)	% Recovery	Acceptance Limit
1	150	994.76	152.37	101.58	97 – 103 %
2	300	1072.24	298.70	101.13	
3	500	1266.71	471.50	102.82	

* Average of three readings

Table 3: Summary of validation parameters data for clopidogrel bisulphate

Parameters		Clopidogrel bisulphate	Acceptance criteria
Retention Time (min)		2.58	-
LOD (µg/ml)		150	-
LOQ (µg/ml)		175	-
Linearity (µg/ml)		150 - 500	-
Accuracy (% Recovery)		101.13 -102.82%	97 -103%
Precision (%RSD)	**System**	0.025	< 2%
	Method	0.0020	
	Intermediate precision	0.72	
Specificity		No peak of diluent, excipients and impurities were detected.	No peak should be detected
Robustness: Mobile phase ratio	**9:1**	0.9551	% RSD should be < 2%
	7:3	1.0581	
Robustness: pH of Sodium Perchlorate Buffer	**2.3**	1.4709	
	2.7	1.8693	
System Suitability Parameters	**N**	9772	>2000
	HETP	0.0021	-
	Asymmetry	1	~1
	Resolution	1.115	

CONCLUSION

In the current study, as per ICH recommended stress conditions, an inherent stability was established for clopidogrel bisulphate. The drug was found to be stable in solid form, whereas unknown decomposition products were found under the stress conditions. Clopidogrel bisulphate was found to be stable in alkaline and neutral condition, whereas significant degradation was found

in acidic, oxidative and photo condition. Clopidogrel bisulphate was analyzed in the presence of different degradation products by HPLC method using RESTEX Allure C8 (150 x 4.6mm, 5µm) column from USA with binary gradient conditions and mobile phase containing Acetonitrile: phosphate buffer pH adjusted to 2.5 with Orthophosphoric acid at flow rate of 1ml/min using UV detection at 254nm. The procedure has been evaluated for the linearity, accuracy, precision and robustness in order to ascertain the suitability of the analytical method. The method was found to be selective and linear between concentration range 150-500mcg/ml. LOD was found to be 150mcg.ml and LOQ was found to be 175mcg/ml. The summary of validation parameters data for clopidogrel bisulphate is given in table 3. The developed method is reproducible and selective for the estimation of clopidogrel bisulphate in marketed solid dosage forms.

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