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Original Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF CLOPIDOGREL BISULFATE IN HUMAN PLASMA BY RP-HPLC

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ABSTRACT

Objective: A simple, sensitive, rapid and precise bioanalytical RP-HPLC method was developed for estimation of clopidogrel bisulfate in human plasma.

Methods: The work was carried out on Shimadzu LC-2010 CHT HPLC system equipped with Waters C18 (250×4.6 mm, 5µ) column with a mobile phase containing acetonitrile: methanol: water (75:20:05 v/v/v). The flow rate of mobile phase was 1 ml/min and the detection was carried out at 225 nm. The retention time of clopidogrel bisulfate was found to be 4.6 min.

Results: The developed bioanalytical method was found to be linear in the concentration range of 30.76-69.23 µg/ml. The simple regression analysis of chromatographic response showed the value of R^2= 0.9917. The precision study revealed that the cumulative percentage variation was within acceptable limit and accuracy study showed the value of mean percent recovery between 103.60 to 109.80 %. The clopidogrel was stable in human plasma at different storage conditions.

Conclusion: The validation parameters of the method met the acceptance criteria. Sufficient stability of both LQC and HQC was shown to allow for completion of sample analysis in clinical trials. From the results, we can conclude that developed bioanalytical method is simple, rapid, accurate, and precise and can be used for routine analysis of clopidogrel bisulfate.

Keywords: Bioanalytical Method, Clopidogrel Bisulfate, RP-HPLC, Validation

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INTRODUCTION

Clopidogrel bisulfate is an oral thienopyridine class of antiplatelet agent that used to inhibit blood clots in coronary artery disease and to prevent myocardial infraction and stroke [1]. It is chemically methyl (S)-α-(o-chlorophenyl) 6, 7-dihydrothieno [3, 2-c] pyridin-5-(4H)-acetate sulphate [2] (fig. 1). It is a prodrug that is converted in the liver to an active thiol metabolite, which inhibits adenosine diphosphate (ADP) binding to its platelet receptor and subsequent ADP-mediated activation of the glycoprotein IIb/IIIa complex, thus inhibiting platelet aggregation. Clopidogrel irreversibly modifies the ADP receptor, therefore platelets are affected for the remainder of their lifespan [3]. Clopidogrel should be used with caution in patients receiving another drug that increase the risk of bleeding include anticoagulants, other antiplatelet and NSAIDs.

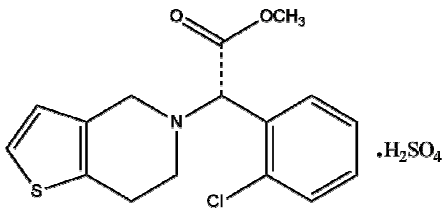

Fig. 1: Structure of clopidogrel bisulfate

The literature survey revealed that various UV [4, 5] and HPLC [6, 7] methods had been reported for the estimation of clopidogrel bisulfate either individually or in combination with other drugs. Few bioanalytical methods also have been reported using hyphenated and UPLC techniques [8-10], which required sophisticated and costly instruments. Therefore, an economical RP-HPLC system was used to develop a simple bioanalytical method for estimation of clopidogrel bisulfate and validate as per USFDA guideline [11].

MATERIALS AND METHODS

Chemicals and reagents

A sample of clopidogrel bisulfate was supplied by Ipca Laboratory Ltd. Mumbai. Methanol and acetonitrile of HPLC grade were obtained from Merck India Ltd. Mumbai. Water is purified by purification system for HPLC, ELGA Purelab UHQ. The human plasma was procured from Smt. Kashibai Navle General Hospital Blood Bank, Pune. (Approval no. 4444, Seq. no. 40SV8605).

Instrumentation

Chromatographic separation of the drug was performed on Shimadzu LC-2010 CHT equipped with LC solution software with UV detector. Separation was attained using Waters C18 column. Shimadzu ATY 224 Electronic balance was used for weighing. The Remi C24BL ultracentrifuge and Remi CM 101 cyclomixer were used for sample preparation.

Preparation of stock solution

Clopidogrel bisulfate 100 mg API powder was accurately weighed and transferred in 100 ml volumetric flask. The drug was dissolved and diluted up to mark with methanol. This solution was further diluted to get 100 µg/ml of clopidogrel bisulfate.

Preparation of mobile phase

The mobile phase was prepared by mixing of acetonitrile, methanol and water in the ratio of 75:20:05 v/v/v and filter through 0.45µ membrane filter. The mobile phase was sonicated in an ultrasonic bath for 10 min.

Sample preparation and extraction

Clopidogrel from the plasma was extracted using protein precipitation technique [12-14]. Frozen human plasma was thawed to ambient temperature. Aliquots of 400 µl plasma were taken into eppindorf tube and 200 µl of stock solution was added and the plasma proteins were precipitated by using methanol. The tube was vortexed for 1 min and the solution was centrifuged at 5 °C, 8000 rpm for 10 min. The supernant was taken and transferred to HPLC vials.

Method validation

The method performance was evaluated for accuracy, precision, linearity and stability during various stress conditions including freeze-thaw stability, stock solution stability and short-term stability [15].

Linearity and range

The working solution of various concentrations was injected under the operating chromatographic conditions and peak area of each concentration were calculated at 225 nm. The calibration curves were constructed using simple linear regression between peak area and corresponding concentration (fig. 4). The range of solution has been decided according to a correlation coefficient of the regression equation [16, 17].

Accuracy

The accuracy of the method was performed by calculating % recovery for the different concentration levels of the drug. The samples of three concentration levels were prepared as a low-quality control, medium quality control and high-quality control by the standard addition method.

Precision

The precision of this method was evaluated by the % CV at different concentration levels corresponding to LQC, MQC and HQC. The intraday and interday precision were evaluated in 3 replicate batches of different concentrations (38.46, 53.84, 69.23 µg/ml).

Stability studies

The stability of clopidogrel in solution and plasma sample was evaluated using two concentration levels (low and high-quality control, corresponding to 38.46 and 69.23 µg/ml, respectively). The stability of clopidogrel bisulfate was also evaluated in deep freezing at-20 ˚C for 12 h. The plasma samples were kept in the freezer and after being stressed to 3 freeze-thawing cycles (for 24 h per cycle). All samples described above were compared to freshly prepared clopidogrel bisulfate sample at the same concentration levels.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase was selected on the basis of its polarity and different trials were taken. Finally, a mobile phase consisting acetonitrile, methanol, water (75:20:05 v/v/v) at flow rate 1 ml/min was selected. A simple method has been developed as mobile phase has not required any buffer and weighing, sonication, filtration, adjustment of pH steps (for the preparation of buffer) have been eliminated. The retention time of clopidogrel was found to be 4.6 min. Therefore, the developed method is time-saving and more number of samples can be estimated in less time. The chromatogram of clopidogrel bisulfate obtained by optimized conditions is shown in fig. 3. The optimized chromatographic conditions and system suitability parameters are listed in table 1.

Table 1: Optimized chromatographic conditions and system suitability parameters

S. No.	Condition/parameter	Details
1.	Column	Waters C18 5 µm (4.6 X 250 mm)
2.	Mobile Phase	ACN: Methanol: Water (75: 20: 05 v/v/v)
3.	Flow Rate	1.0 ml/min
4.	Retention time	4.6 min
5.	Column Temperature	28 ˚C
6.	Volume of Injection	20 µl
7.	Detection wavelength	225 nm
8.	Theoretical plate	3689.90
9.	Retention time	4.6
10.	Tailing factor	1.35

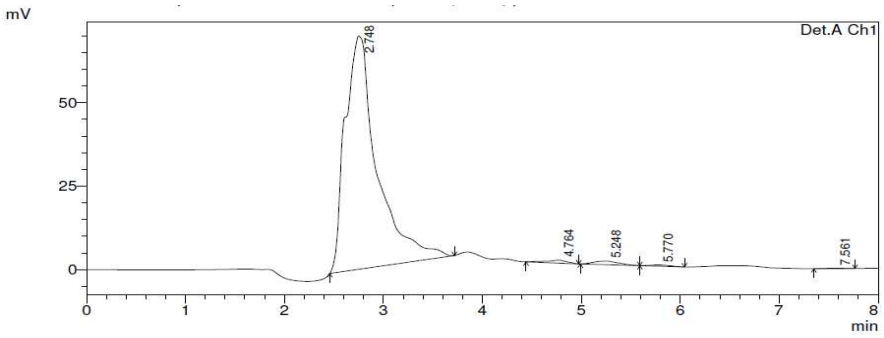

Fig. 2: Chromatogram of blank plasma

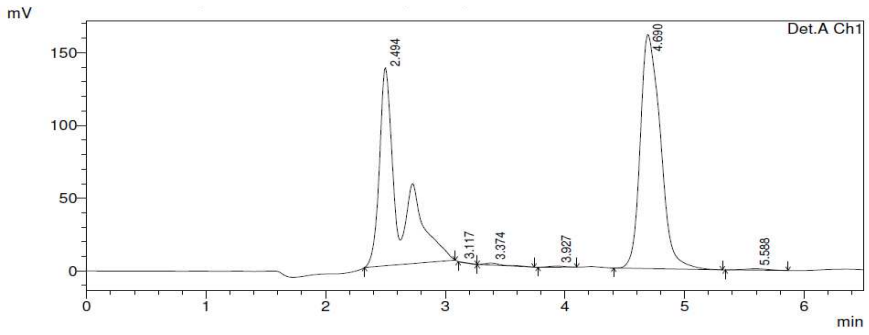

Fig. 3: Chromatogram of plasma spiked with drug

Linearity and range

The value of R^2 was found to be 0.9917, which indicated that the developed method is linear in the selected concentration range. The developed method is sensitive as the range of this method was found from 30.76 to 69.23 µg/ml concentrations of the drug.

Accuracy

The mean % recovery of calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels are ranged from 103.60 % to 109.80 %, which is within acceptance criteria 85.00-115.00 % (table 2).

Precision

The % CV of calculated concentrations for all quality control samples at LQC, MQC and HQC concentration levels are ranged from 0.196-1.774 % for intraday and 0.451-1.303 % for interday precision, which is within acceptance criteria 15.00 % (table 3).

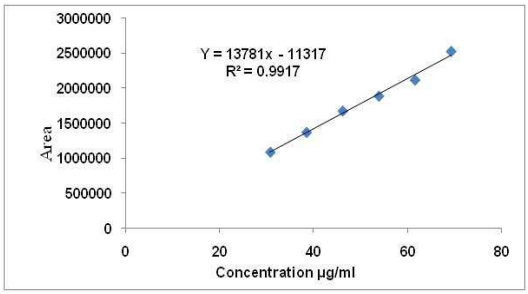

Fig. 4: Calibration curve of clopidogrel bisulfate

Table 2: Results of accuracy studies

S. No.	LQC	MQC	HQC
Mean (µg/ml)±SD*	39.87±1.148	56.36±1.406	76.06±1.625
% CV	2.879	2.474	2.137
Mean % recovery	103.60	105.53	109.80

*Each value is represented as a mean±SD of 5 observation (n=5), SD: Standard Deviation, CV: Coefficient of Variation

Table 3: Results intraday and interday precision of clopidogrel bisulfate

Parameter		LQC	MQC	HQC
Intraday	SD	0.683	0.104	0.956
	% CV	1.774	0.196	1.385
Interday	SD	0.511	0.240	0.533
	% CV	1.303	0.451	0.765

Each value is represented as a mean±SD and % CV of 5 observation (n=5), SD: Standard Deviation, CV: Coefficient of Variation)

Stability studies

The results of all stability studies are within acceptance criteria (table 4). The results of freeze-thaw stability studies suggested that clopidogrel bisulfate was stable in human plasma for at least 24 h. The results of short-term stability studies indicated that the quality control samples were stable for 12 h at-20 ˚C. Similarly, the results of stock solution studies confirmed the stability of stock solutions.

Table 4: Results of stability studies

Concentration measured µg/ml			
LOW QC	**Stock solution (5h)**	**Short term(-20 ˚c,12 h)**	**Freeze-thaw (24 h)**
Mean (µg/ml)±SD*	40.12±0.909	40.03±0.803	37.46±0.755
%CV	2.267	2.006	2.016
% Stability	102.34	103.92	95.43
HIGH-QC			
Mean (µg/ml)±SD*	74.09±2.435	69.92±2.027	65.76±1.900
%CV	3.286	2.899	2.889
% Stability	106.40	101.23	94.44

*Each value is represented as a mean±SD of 3 observation (n=3), SD: Standard Deviation, CV: Coefficient of Variation

CONCLUSION

The work described in this paper deals with the analysis of clopidogrel bisulfate using RP-HPLC method in human plasma. The precision and accuracy of the method met the criteria laid down in guidance for industry, Bioanalytical method validation, USFDA. Sufficient stability of both LQC and HQC was shown to allow for completion of sample analysis in clinical trials. From the results, we can conclude that developed method is simple, accurate, rapid and precise. Thus, it can be used for routine analysis of clopidogrel bisulfate in human plasma.

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CONFLICTS OF INTERESTS

Declare none

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