BIOANALYTICAL RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF CLOPIDOGREL BISULFATE IN WISTAR RAT PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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ABSTRACT

Objective: A novel, simple, precise, accurate, sensitive, and reproducible HPLC method for determining clopidogrel bisulfate in Wistar rat plasma was developed and validated.

Methods: The chromatographic separation was performed using Xterra C18 (250 x 4.6 mm, 5μ) column. Mobile phase composed of Acetonitrile ACN: 0.05M potassium dihydrogen orthophosphate buffer pH 4.2 and in the ratio of 75:25% v/v at a flow rate of 1.2 ml/min. Detection was carried out using a PDA detector at 220 nm. The bioanalytical clopidogrel method was validated as per ICH guidelines.

Results: The selected chromatographic condition was found to efficiently separate clopidogrel bisulfate (RT-2.838 min). The calibration curve was linear over the concentration range 40-200 ng/ml in Wistar rat plasma with a correlation coefficient of 0.999, respectively. The precision study revealed that the cumulative percentage variation was within the acceptable limit, and accuracy research showed the value of mean percent recovery between 99.72-99.83 %.

Conclusion: A simple, rapid, specific, accurate, and precise analytical method was developed and validated using Wistar rat plasma. The technique was strictly validated according to the ICH guidelines. Acquired results demonstrate that the proposed strategy can be effortlessly and advantageously applied for routine analysis of clopidogrel in the Wistar rat plasma.

Keywords: RP-HPLC, Bioanalytical method, Clopidogrel bisulfate, Wistar rat plasma

INTRODUCTION

Bioanalysis is the approach used to determine the concentration of drugs and metabolites in the biological matrices like plasma, serum, cerebrospinal fluid, urine, saliva, etc. [1]. Bioanalytical methods are essential for bioavailability and bioequivalence studies [2]. Bioanalytical method and validation are utilized to build up that a quantitative analytical approach can be connected for the biochemical process [1, 3]. Validation involves laboratory investigations that the method is suitable and reliable for the intended applications [4]. It is utilized to assess bioavailability and bioequivalence studies, quantitative evaluation of drug and metabolites, new drug development, clinical pharmacokinetics, research process, and therapeutic drug monitoring. Bioanalytical techniques are persistently undergoing changes and improvements that are the cutting edge of technology [5].

Platelets play a vital role in the hemostasis and wound-healing processes [6]. However, their hyperactivity is involved in the development and complications of several cardiovascular diseases, including thrombosis, atherosclerosis, myocardial infarction, peripheral artery disease, and ischemic stroke. Furthermore, excessive platelet activation is endorsed by the high mechanical shear forces in the circulation and vascular damage [7]. Indeed, following blood vessel injury and atherosclerotic plaque erosion, platelets adhere by their surface receptors to the subendothelial matrix, which triggers their activation and subsequent aggregation [8, 9]. Activated platelets release prothrombotic mediators retained within their granules like adenosine diphosphate (ADP), serotonin, P-selectin, fibrinogen, Ca2+, and thromboxane A2 (TXA2), which further amplify platelet activation and thrombus formation [9, 10]. The presence of a thrombus in an artery providing blood to a vital organ such as the heart or brain is the most common cause of acute coronary disorders, including myocardial infarction and angina [11]. Thus, the prevention of arterial thrombotic diseases has a high priority in developed countries. In this sense, the inhibition of platelet aggregation can protect against cardiovascular diseases that affect millions of people worldwide. A series of antiplatelet drugs have been used clinically to treat and prevent coronary syndromes and stroke, such as aspirin, clopidogrel [12].

Clopidogrel bisulfate is a weak base, a second-generation thienopyridine that is known chemically as Methyl (S)-α-(2chlorophenyl)-6,7di-hydrothieno[3,2-c] pyridine-5(4H)-acetate sulfate. Clopidogrel is a potent antiplatelet agent widely used to prevent coronary artery diseases and treat vascular thrombosis [13, 14]. Its antiplatelet activity results from selective inhibition of ADP binding to the platelet receptor preventing ADP-mediated platelet responses [15, 16]. Therefore, Clopidogrel should be used with caution in patients receiving other drugs that increase bleeding risk include anticoagulants, other antiplatelet, and NSAIDs [17].
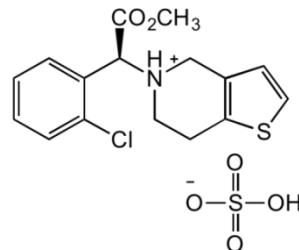

Fig. 1: Chemical structure of clopidogrel bisulfate [17]

Drug literature review discloses that only a few analytical quantification methods for the clopidogrel bisulfate in bulk, formulations, and biological matrices either individually or in combination with other drugs. The reported analytical techniques were UV [18, 19], HPLC [17], LC-MS/MS [20] and Liquid chromatography-tandem mass spectrometry [21]. The goal of the research was to develop a fast and empathetic bioanalytical HPLC technique for the quantitation of clopidogrel bisulfate

in plasma samples and the application of ICH regulatory guidelines for method validation.

MATERIALS AND METHODS

Chemicals and reagents

Clopidogrel bisulfate was kindly provided as a gift sample by Lantech Pharmaceuticals Ltd, Hyderabad. Analytical grade of potassium dihydrogen orthophosphate purchased from SD Fine-Chem Limited, Mumbai, and HPLC grade of methanol and acetonitrile purchased from Merck Specialties Private Limited, India. Analytical grade orthophosphoric acid purchased from Rankem Ltd., India.

Instrumentation

Chromatographic separation of the clopidogrel bisulfate was performed on Waters HPLC Alliance 2695 separation module with autosampler equipped with Empower 2 software with PDA detector 996 model. Separation was attained using the Xterra C18 column. Electronic balance was used for weighing. The ultracentrifuge (Remi C24BL), cyclomixer (Remi CM 101) were used for sample preparation.

Liquid chromatographic conditions

Chromatographic separation was carried out using Xterra C18 (250 mm × 4.6 mm), 5 μm with a mobile phase composed of Acetonitrile: 0.05M potassium dihydrogen orthophosphate buffer in 75:25 ratio, pH 4.2. The analyte was monitored with a PDA detector at 220 nm, and samples of 10 μl were injected into the HPLC system. The mobile phase was acetonitrile and 0.05M potassium dihydrogen orthophosphate buffer pH 4.2 in the ratio of 75:25 (v/v) at a flow rate of 1.2 ml/min. The mobile phase wasfiltered through 0.45 μm filter (Sartorius, Germany) and degassed for 10 min by sonication.

Preparation of stock and standard working solutions

All standard stock solutions and working standard solutions were prepared in a volumetric flask. First, 100 mg clopidogrel was dissolved in the mobile phase to give 100 mg/ml. standard stock solution. Next, the working standard solutions were prepared by further serial dilution from the stock solutions with mobile phase mixture. Calibration standards of 40, 80, 120, 160, and 200 ng/ml in control Wistar rat plasma samples were prepared by mixing with 10 μl of working stock solutions of clopidogrel. All solutions were stored in amber-colored bottles at-4 °C prior to use.

Sample preparation

Frozen rat plasma samples were obtained from the National Institute of Nutrition, Hyderabad, thawed and allowed to reach room temperature. An aliquot of 200 μl of Wistar rat plasma sample. Subsequently, the sample was extracted with 1.5 ml of tertiary-butyl methyl ether (TBME). The mixture was vortexed for 15 min and centrifuged at 3,000 rpm for 10 min. After centrifugation, the organic phase was transferred to glass tubes and evaporated to dryness using TurboVap LV Evaporator at 50 °C under a stream of nitrogen for 5 min. The residue that remained after drying was reconstituted with 150 μl of mobile phase mixture and vortexed for 1 min. Then, a volume of 10 μl was injected into the HPLC system [22].

Method validation

Validation of the developed method was carried out as per ICH guidelines for specificity, linearity, accuracy, precision, LOD, LOQ, and ruggedness [23].

Specificity

Specificity evaluation was carried out by screening six different sources of blank Wistar rat plasma. Samples were processed and injected into the HPLC to assess the extent to which endogenous constituents might interfere with the retention time of clopidogrel. If there is any interfering peak present at the retention time of the drug, then its response should be less than 20% of the mean response of six extracted Wistar rat plasma samples [24].

Linearity

Plasma sample's linearity was quantified using the peak-area ratio versus analyte nominal concentration [25]. For the establishment of the linearity range of the proposed method, calibration curves in plasma were prepared in the concentration range of 40–200 ng/ml.

Accuracy

Acquisition studies were conducted using the standard addition method wherein a known amount of clopidogrel was added to the pre-analyzed samples according to 80, 100, and 120% levels of labeled claim further subjected to the contemplated analytical process. The percent recovery and relative standard deviation (% RSD) were calculated for each concentration. Accuracy was calculated using the formula $\%RE = (E - T)(100/T)$, where E is the experimentally determined concentration, and T is the theoretical concentration [26].

Recovery

The recovery studies were carried out in Wistar rat plasma. The percentage recovery was determined using accuracy samples at 80%, 100%, and 120% concentrations of 96, 120, and 144 ng/ml and at one concentration injection volume (10 μg/ml). Recovery was calculated by comparing the mean peak area of three replicates ($n=3$) of extracted samples with the mean peak area obtained for the quality control samples of unextracted standards.

Precision

The precision was established using quality control samples ($n = 3$) at low, medium, and high clopidogrel concentrations of 40, 120, and 200 ng/ml in Wistar rat plasma [27]. The assay precision was assessed by calculating inter-and intra-day variability of quality control samples. Inter-day data was obtained by analyzing the quality control samples on three consecutive days of an assay, while intra-day precision data were obtained by analyzing three sets of quality control samples in a single day. The assay precision was calculated using the formula

$\% RSD = \left(\frac{SD}{M}\right) * 100$, where M is the mean of the experimentally determined concentrations and SD is the standard deviation of M.

Robustness

The robustness of the developed method was studied by evaluating the effect of slight but deliberate variations in chromatographic conditions. The parameters studied were flow rate and mobile phase composition [28].

Ruggedness

The ruggedness of the method was studied by changing the experimental condition with precision and accurate batch [25]. Ruggedness was performed by using different analysts [29] in the same laboratory.

Limits of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ of the developed method were estimated on the based on standard deviation and slope of the calibration curve as 3.3 δ/m and 10 δ/m, respectively. Here, δ was the regression standard deviation of intercept, and m was the slope of the calibration curve [30].

Pharmacokinetic studies

The applicability of the developed HPLC method for clopidogrel in rat plasma was demonstrated by the results obtained from pharmacokinetic studies conducted on male Wistar rats (n=6). Male Wistar rats, weighing approximately 160-180 g, were purchased from Sainath Agencies, Musheerabad, Hyderabad, India, and Rats housed in cages were kept in a room under controlled temperature (20–22 °C) and 12 h day-night cycle. After 1 w of acclimatization with free access to water and food, animals were used for the study. The animal study protocol was reviewed and approved by the Institutional Animal Ethics Committee (1757/PO/RcBiBt/S/14/CPCSEA). Each rat was treated with oral pure clopidogrel suspension at a dose of 7.70 mg/kg in a single dose and was administered by oral route with a gastric catheter or oral gavages, feeding needle [31]. Serial blood samples (2 ml) were collected from retro-orbital venous plexus with a hematocrit over a period of 24 h (four biological half-lives). Rats were further divided into two subgroups (n=3) for convenient blood sampling over entire study periods.

Blood samples from each group were collected at predetermined time intervals alternatively from each subgroup into heparinized plastic tubes. All these whole blood samples were kept in cold refrigerated conditions (2-8 °C) until the separation of plasma. Each sample was processed further by the method mentioned under sample preparation and subjected to HPLC analysis to estimate drug content by a previously validated bioanalytical method [30]. The pharmacokinetic calculations were performed on the basis of plasma concentration-time data using PK solver add-in in MS-Excel 2007 [32].

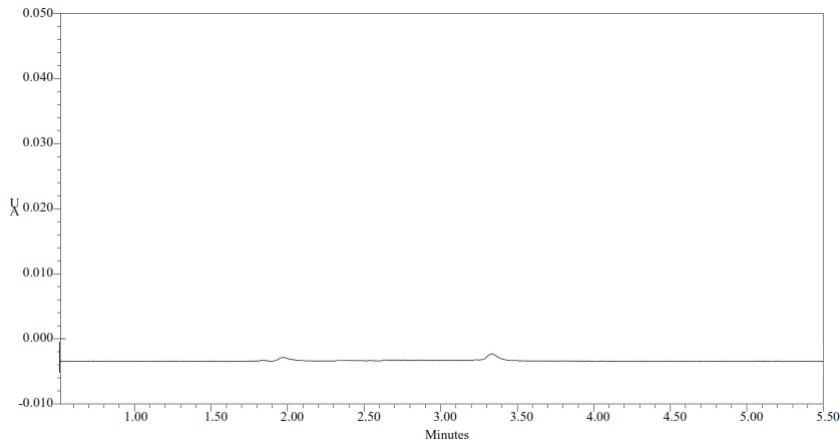## RESULTS AND DISCUSSION

Method development

Fig. 2: Representative chromatogram of blank Wistar rat plasma
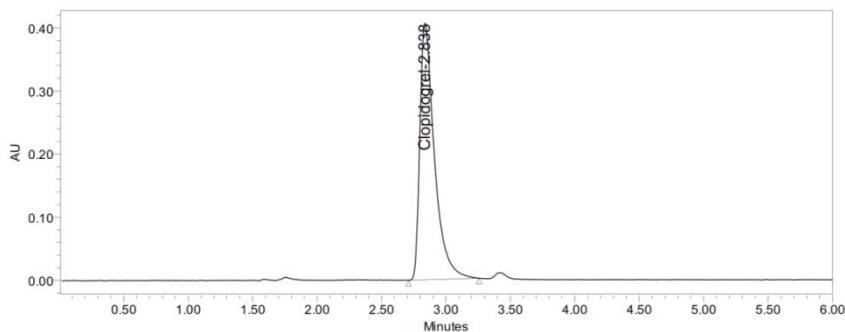

Fig. 3: Typical chromatogram of clopidogrel

Since clopidogrel is a poorly water-soluble and highly lipophilic compound, it was necessary for the method development to optimize parameters such as choice of organic solvent, pH, the composition of mobile phase,flow rate, and oven temperature. The optimum wavelength for detecting Clopidogrel with adequate sensitivity was found to be 220 nm. Potassium dihydrogen orthophosphate buffer and acetonitrile at 0.05 M were again selected for the separation studies. At pH 4.2, clopidogrel was resolved with good peak shape, but at pH>4.2, peak tailing of clopidogrel was observed. Hence Potassium dihydrogen orthophosphate buffer (0.05 M; pH 4.2) and acetonitrile were selected for the initial separation studies.

Experiments were carried out with different compositions of acetonitrile in the mobile phase for better resolution of clopidogrel. The amount of organic content in the mobile phase signtly altered the retention time of clopidogrel because of the poor aqueous solubility. The best peak shape was observed with the mobile phase consisting of ACN: 0.05M potassium dihydrogen orthophosphate buffer pH 4.2 and in the ratio of 75:25 (v/v); slight changes in the mobile phase composition were observed to cause subtle differences in the retention time of clopidogrel without altering its peak response.

The effect of flow rate with optimized mobile phase composition was studied at 1.0, 1.2, and 1.4 ml/min. When the flow rate increased, there was a decrease in the peak width of clopidogrel, but at flow rates of 1.2 and 1.4 ml/min, there was no significant decrease in the peak width. Hence a flow rate of 1.2 ml/min was selected as the optimum flow rate since it yielded good peak shapes without endogenous peak interference at the retention time of analyte. Experiments were also performed with C_4, C_8, and C_{18} columns. C_4 and C_8 columns were unable to resolve clopidogrel from endogenous compounds. However, the C_{18} column analyte was determined from endogenous compounds with good peak symmetry and reproducibility.

The effect of column temperature was studied at room temperature and elevated temperatures. There was no significant effect on the peak width clopidogrel, but an increase in oven temperature caused a decrease in the peak width of clopidogrel. Thus Ambient 25 °C (Room Temperature) was selected as the optimized oven temperature with the optimized chromatographic condition. The average retention time for clopidogrel was 2.838 min.

Specificity

Specificity was evaluated using six different blank Wistar rat plasma samples. Chromatograms obtained from Wistar rat plasma samples are shown in fig. 2,3. There were no interfering peaks of endogenous compounds observed at the retention time of clopidogrel. The analyte were eluted at 2.838 min for clopidogrel. However, sample runtime was optimized to 6 min.

Table 1: Summary of chromatographic conditions

Parameters	Description
Equipment	High-performance liquid chromatography WATERS Alliance 2695 separation module equipped with 996 PDA detector, with Empower 2 software
Column	Xterra C18, 250 mm × 4.6 mm, 5 μm (particle size)
Mobile phase	ACN: 0.05M potassium dihydrogen orthophosphate buffer pH 4.2 and in the ratio of 75:25
pH	4.2
Flow rate	1.2 ml/min
Column and sample temperature	Ambient 25 °C/Room Temperature
Detection wavelength	220 nm
Injection volume	10 μl
Run time	6 min
Retention time	2.838 min

Linearity

A five-point linearity curve was constructed for clopidogrel Samples were quantfied using the peak area of clopidogrel. Standard calibration curves were constructed by plotting the peak area of clopidogrel concentration in plasma samples. Least squares linear regression analysis y = mx + c was performed to determine slope, intercept, and coefficient of correlation.

The correlation coeficient of the calibration curves for clopidogrel was 0.999. The mean (±SD) slope and intercept values for the calibration curve of clopidogrel were 1039 and 1873, respectively.
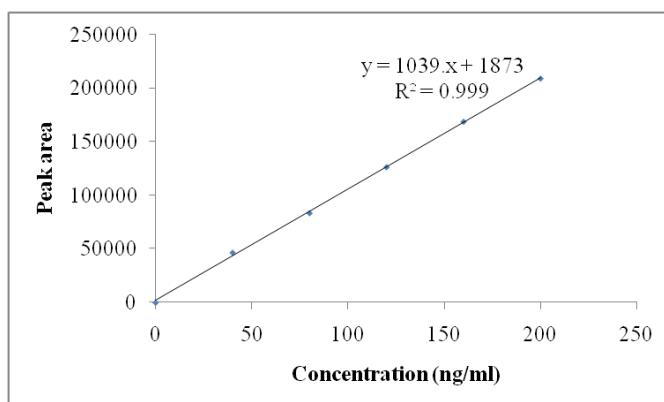
Fig. 4: Linearity curve of clopidogrel in plasma at 220 nm HPLC method

Accuracy and precision

The accuracy of an analytical method describes the closeness of test results to the accurate concentration of the analyte. In contrast, precision measures the degree of reproducibility of the analytical method [28, 33]. The results of intra-day and inter-day precisions are summarized in table 3, which revealed that the developed method was accurate and precise for the quantification of clopidogrel in plasma samples.

Table 2: Accuracy of clopidogrel in plasma by HPLC method

(%) drug added level	Theoretical content (ng/ml)	Amount found (ng/ml) AM±SD n=3	% Recovery	%RSD
80	96	95.8±0.25	99.79	0.26
100	120	119.8±0.40	99.83	0.33
120	144	143.6±0.55	99.72	0.38

Each value represents the mean±SD (n=3)

Table 3: Precision of clopidogrel in Wistar rat plasma by RP HPLC method

Concentration, ng/ml	Intra-day		Inter-day	
	Concentration found (AM±SD) (n=3)	%RSD	Concentration found (AM±SD) (n=3)	%RSD
40	39.47±0.21	0.55	39.61±0.44	1.13
120	120.36±0.22	0.18	119.71±0.55	0.46
200	199.57±0.28	0.14	200.03±0.72	0.36

Each value represents the mean±SD (n=3)

Recovery

For recovery determination, three replicates at 80, 100, and 120% quality control concentration were prepared for clopidogrel in Wistar rat plasma. The mean recovery for clopidogrel in Wistar rat plasma was 99.79%, 99.83%, 99.72%, respectively.

Robustness

The low values of %RSD for drug proposed that during all deliberate variations, assay value of test preparation was not affected, and it was by actual (table 4). Hence, the newly developed analytical method was considered to be robust.

Table 4: Robustness of clopidogrel in plasma by HPLC method

Flow rate, ml/min	Retention time		Theoretical plates	
	(AM±SD) (n=3)	%RSD	(AM±SD) (n=3)	%RSD
1.0	3.272±0.010	0.309	6412±10.44	0.16
1.2	2.838±0.055	1.938	6830±59.02	0.86
1.4	2.336±0.010	0.439	6315±9.16	0.14

Each value represents the mean±SD (n=3)

Table 5: Ruggedness of clopidogrel in plasma by RP-HPLC method

Analyst	Clopidogrel concentration, ng/ml	%RSD
1	90.20±0.04	0.049
2	89.32±0.22	0.251

Each value represents the mean±SD (n=3)

Ruggedness

The ruggedness of the method was carried out by changing by a different analyst. The results are summarised in the table. The %RSD values were less than 2, suggesting the ruggedness of the proposed analytical method.

Limits of detection (LOD) and quantitation (LOQ)

The LOD and LOQ were observed as 1.617 ng/ml and 5.391 ng/ml, respectively. These low values were indicative of the high sensitivity of the developed method.

Pharmacokinetic studies

The proposed HPLC method was successfully applied to monitor quantitatively the time course of plasma clopidogrel bisulfate concentrations after oral administration of a single 7.70 mg/kg body weight dose to adult male Wistar rats. The mean plasma drug concentration-time profile observed in these clopidogrel pharmacokinetics studies is shown in fig. 5. The values of all major pharmacokinetic parameters like maximum plasma concentration (C_{max}), time required for maximum plasma concentration (T_{max}), area under the curve (AUC_{0-24}), terminal half-life ($t_{1/2}$), elimination rate constant, mean residence time (MRT), clearance and volume of distribution (V_d) have been summarized in table 5.

Table 5: Pharmacokinetic parameters of clopidogrel

Pharmacokinetic parameter	Clopidogrel pure drug
C_{max} (ng/ml)	85.09±2.98
t_{max}(h)	2
AUC_{0-24} (ng-h/ml)	938.666±19.11
Half-life $t_{1/2}$(h)	7.179±0.13
Elimination rate constant (h^{-1})	0.0965±0.001
MRT $_{0-24}$ (h)	7.847±0.004
Clearance(CL) (mL/min)	0.0711±0.001
V_d (L)	0.737±0.005

Each value represents the mean±SD (n=6)
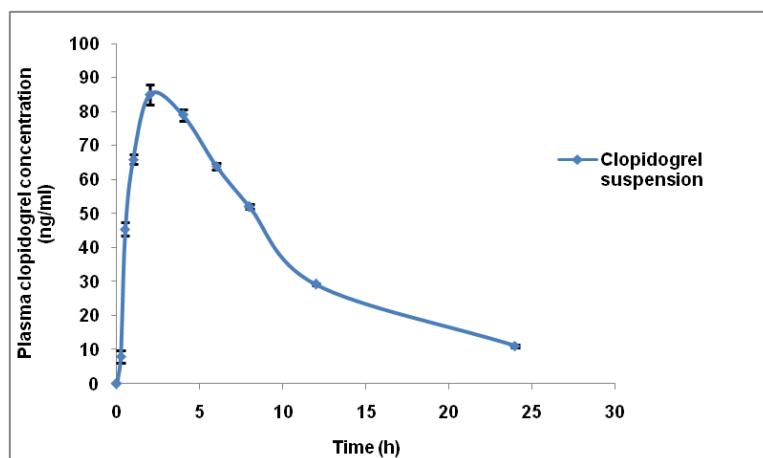
Fig. 5: Mean plasma concentration vs time profile in rats (*n* = 6)

CONCLUSION

A simple, rapid, specific, sensitive, and reproducible bioanalytical HPLC method has been developed and validated for the quantitative determination of clopidogrel in small volumes of rat plasma. The method was successfully applied for studying the pharmacokinetic parameters of clopidogrel. This method can also be used for quality control tests of clopidogrel in plasma samples.

AUTHORS CONTRIBUTIONS

Ramya Sri Sura has generated the research plan, prepared and revised the manuscript. Subrahmanyam CVS and Shyam Sunder Rachamalla have given guidance and supervision to carry out this study.

CONFLICT OF INTERESTS

None

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