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RP-HPLC METHOD FOR ESTIMATION OF CLOPIDOGREL BISULPHATE IN RAT PLASMA

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ABSTRACT

A simple, sensitive, and specific reversed phase liquid chromatographic method was described for the determination of clopidogrel bisulphate. The separation was achieved on a Phenomenex ODS C18 (250 mm x 4.6 mm, 5 µ) column at ambient temperature using isocratic water alliance 2695 HPLC system equipped with empower version 2.0 software and with UV-visible detector. The mobile phase consisted of potassium dihydrogen orthophosphate buffer and acetonitrile in a ratio of 30:70 v/v. The detection was carried out at wavelength of 220 nm. The method was validated for system suitability, linearity, accuracy, precision, robustness and stability tests. The results indicated that the reported method is highly specific and reproducible.

Keywords: Clopidogrel bisulphate, HPLC, UV-visible detector, potassium dihydrogen orthophosphate buffer and acetonitrile.

INTRODUCTION

Clopidogrel bisulphate is, chemically methyl (+)-(S)-α-(2-chlorophenyl)-6, 7-dihydrothieno [3, 2-c] pyridine-5(4H) acetate sulphate, is a selective inhibitor of platelets aggregation[1]. It has successfully been used prophylactically in myocardial infarction, stroke, unstable angina, and other cardiovascular diseases [2]. It undergoes hepatic oxidation by cytochrome P450 (CYP3A4 and CYP3A5) and forms a thiol compound that inhibits ADP-induced platelet aggregation directly by inhibiting binding of (ADP) to its receptor on the platelets and thus preventing the formation of GPIIb-Ia complex[3].

A few among the numerous analytical methods available for the estimation of clopidogrel bisulphate (CBS) are reported, Pravin B C*etal*[4] reported on the development and validation of spectrophotometric method for clopidogrel bisulfate in bulk and formulations. **AnandakumarK *et al*[5] developed a** reverse phase high performance liquid chromatography method was developed for the simultaneous estimation of aspirin and clopidogrel bisulphate in formulation. Lakshmi Prasanna I *et al*[6] reported on the spectrophotometric determination of clopidogrel in the presence of asprin (Clopin-A) and its assay by charge-transfer complex method using 2, 3-Dichloro-5, 6-Dicyano-1, 4-Benzoquinone (DDQ). Patel RB *et al*[7] carried out studies on simultaneous estimation of acetylsalicylic acid and clopidogrelbisulfate in pure powder and tablet formulations by high-performance column liquid chromatography and high-performance thin-layer chromatography.

EXPERIMENT

Development of a RP-HPLC method for estimation of CBS in rat plasma

Instrumentation:

Liquid chromatography method was developed for determination of clopidogrel by using isocratic

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water alliance 2695 HPLC system equipped with empower version 2.0 software and with UV-visible detector. The separation was achieved on a Phenomenex ODS C18 (250 mm x 4.6 mm, 5 µ) column at ambient temperature.

Preparation of standard solution:

Accurately weighed 20.0 mg of clopidogrel was transferred in 10.0mLvolumetric flask and added 2.0mL of methanol to dissolve and diluted up to the mark with methanol. The resultant solution was sonicated to dissolve the drug and filtered through 0.22 µm filter membrane. From the filtered solution 5mL was pipetted out and diluted to 10mL with methanol.

Calibration curve dilutions:

Different solutions of CBS were prepared from the stock solution (**Table 1**) to get a concentration range of 1.047 – 86.093 µg/mL using the diluents (1:1 mixture of potassium dihydrogen phosphate buffer and acetonitrile. These solutions were further used for spiking the screened blank plasma. Young, healthy male Wistar rats were used for the studies and all experiments were conducted in accordance with the guidelines laid by local ethical committee of the institute for animal experiments.

Table 1: Calibration curve dissolution data for CBS in spiking solution

Stock/ SSID	Stock/SS Concentrations (µg/mL)	Stock/SS Volume (mL)	Diluent Volume (mL)	Total Volume (mL)	Final Conc (µg/mL)	Spiking solution ID
Stock solution	999.0	0.25	9.75	10	361.182	SS-7
SS-6	361.182	0.74	4.26	5	86.093	SS-6
SS-5	86.093	1.60	3.40	5	60.456	SS-5
SS-4	60.456	1.20	3.80	5	37.281	SS-4
SS-3	37.281	0.83	4.17	5	22.524	SS-3
SS-2	22.524	1.00	4.00	5	9.923	SS-2
SS-1	9.923	0.81	4.19	5	1.047	SS-1

Spiked calibration curve plasma standards:

The above calibration curve dilutions were used to spike the screened blank rat plasma matrix to prepare the plasma calibration curve standards in the range 52.35 – 4304.456ng/mL as given in the **Table 2**. Aliquots containing 0.50 mL of the above plasma calibration curve standards were taken in polypropylene vials, labelled properly, tightly closed and stored in a freezer at -70^0C for further use.

Table 2: Calibration curve dissolution data for CBS in spiking plasma standards

Stock/SSID	Stock/ SS Concentrations (µg/mL)	Stock/ SS volume (mL)	Plasma volume(mL)	Total Volume (mL)	Final Conc (µg/mL)	Spiking solution ID

SS-6	86.093	0.50	9.50	10	4.304	SS-6
SS-5	60.456	0.50	9.50	10	3.022	SS-5
SS-4	37.281	0.50	9.50	10	2.217	SS-4
SS-3	22.524	0.50	9.50	10	1.126	SS-3
SS-2	9.923	0.50	9.50	10	0.496	SS-2
SS-1	1.047	0.50	9.50	10	0.052	SS-1

Preparation of solutions:

Preparation of 0.01M potassium dihydrogen phosphate buffer:

Accurately weighed 1.36 gm of potaasiumdihydrogen orthophosphate transferred in 1000 mL volumetric flask. To this 900 mL of milli Q water was added. The solution was sonicated for 2 minutes and final volume was made up to 1000mLwith water. 1 mL of triethylamine was added and pH was adjusted to 3.5 by using diluteorthophosphoric acid solution. The solution was stored at room temperature and used up to three days of preparation.

Preparation of mobile phase:

The mobile phase was prepared by mixing 30 parts of buffer solution and 70 parts of acetonitrile in a reagent bottle, sonicated for 5 minutes and filtered through 0.45µ nylon filter. The mobile phase was stored at room temperature and used up to three days of preparation.

Preparation of diluent:

A volume of 500 mL of acetonitrile was transferred into 1000 mL reagent bottle and 500 mL of buffer was added to it, mixed and sonicated for 5 minutes. The solution was stored at room temperature and used within seven days from the date of preparation.

Rinsing solution:

A volume of 500 mL of acetonitrile was transferred into a 1000 mL reagent bottle; 500 mL of buffer solution was added, mixed and sonicated for 5 minutes. The solution was stored at room temperature and used within seven days from the date of preparation.This solution was used for rinsing the injection needle of the HPLC instrument.

Method development and optimization of the chromatography conditions:

For the development of RP-HPLC method for the assay of clopidogrel, different parameters were studied by altering one parameter at a time, keeping all the remaining parameter constant. A non polarPhenomenex ODS C18 (250 mm x 4.6 mm, 5 µ) column was chosen as the stationary phase for this study.

Detection of wavelength:

The UV absorption spectrum was taken and the λ_{max} was found to be at 220 nm. Hence further analysis and detection of drug was carried out at 220 nm.

The mobile phase and the flow rate:

In order to get sharp peak and baseline separation of the components, various trials has been taken by using different mobile phases (single solvent or combination of solvents like acetonitrile, water, methanol with or without buffer) on C18 column as a stationary phase. A binary mixture of potassium dihydrogen orthophosphate buffer (pH 3.5) and acetonitrile in a ratio of 30:70 v/v was

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found to be suitable mobile phase with well defined and well resolved peaks without tailing. A mobile flow rate 0.8 mL/min was found to be suitable when tried in the range of 0.5 to 1.5 mL/min.

Retention time of CBS:

A model chromatogram, showing the separation of CBS under the above optimized conditions at a retention time of 4.12 min was obtained as shown in **Figure 2.**

Data acquisition and processing:

The chromatograms were obtained and the data was processed by the peak area ratio method using the Empower software. The concentration of the unknown samples was calculated from the following equation of the regression analysis of the spiked plasma calibration graph using $1/X^2$ as the weighting factor.

Y= mX+ C

Where, X= Analyte concentration

Y= Analyte area ratio

m= Slope of the calibration curve

C= Intercept value

Extraction process of plasma samples and their dying:

A volume of 400 µL of spiked plasma calibration curve standards was transferred to a set of prelabelled polypropylene tubes. To this 25 µL of CBS (approximately 500 µg/mL) was added and vortexed for 10 seconds. To this 1.2 mL of HPLC grade methanol is added to precipitate the plasma proteins. The samples are then centrifuged for 15 minutes at 4000 rpm in a refrigerated centrifuge. The supernatant is transferred to another set of pre labelled polypropylene tubes and evaporated to dryness under nitrogen at 40ºC. The dried sample is reconstituted with 300 µL of mobile phase, vortex thoroughly and transferred to auto sampler vials for analysis. 20 µL was taken as an injection volume during final analysis.

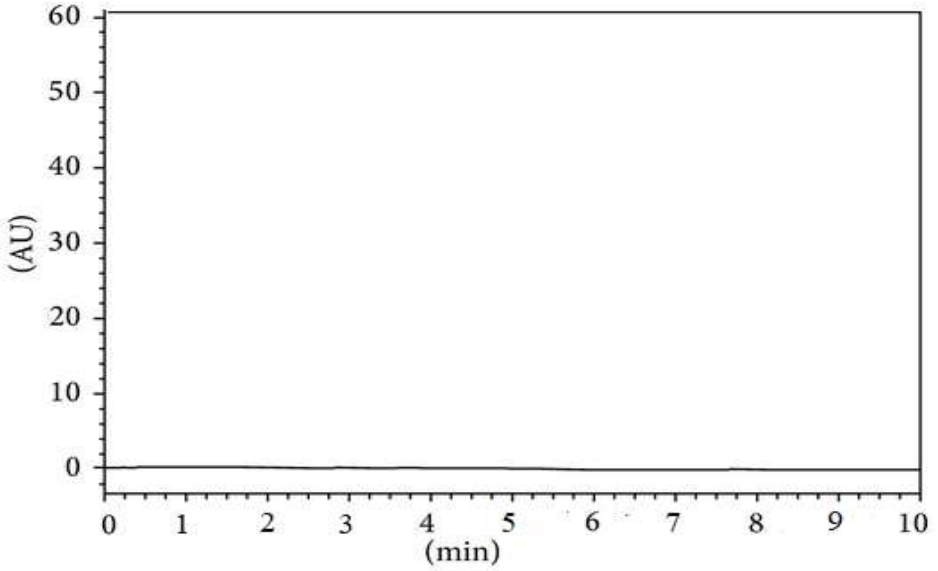

Figure 1 : Chromatogram of extracted blank plasma sampleB

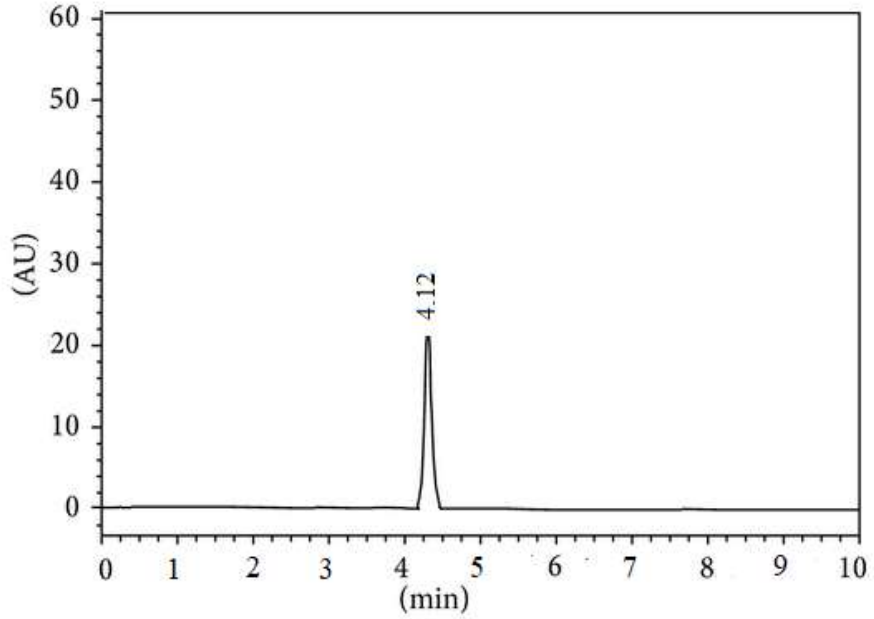

Figure 2 : Chromatogram of clopidogrel with blank plasma

Validation of HPLC method:

Specificity:

Specificity of the developed method was validated to check the interference of any compound from formulation matrix. To determine specificity the standard, sample and placebo were injectedand the recorded chromatograms are depicted in **Figures 3 to 4**. No peaks were observed at the retention times of CBS under optimized conditions which confirm that the selected drug is evidently allotted and hence the proposed HPLC method is selected.

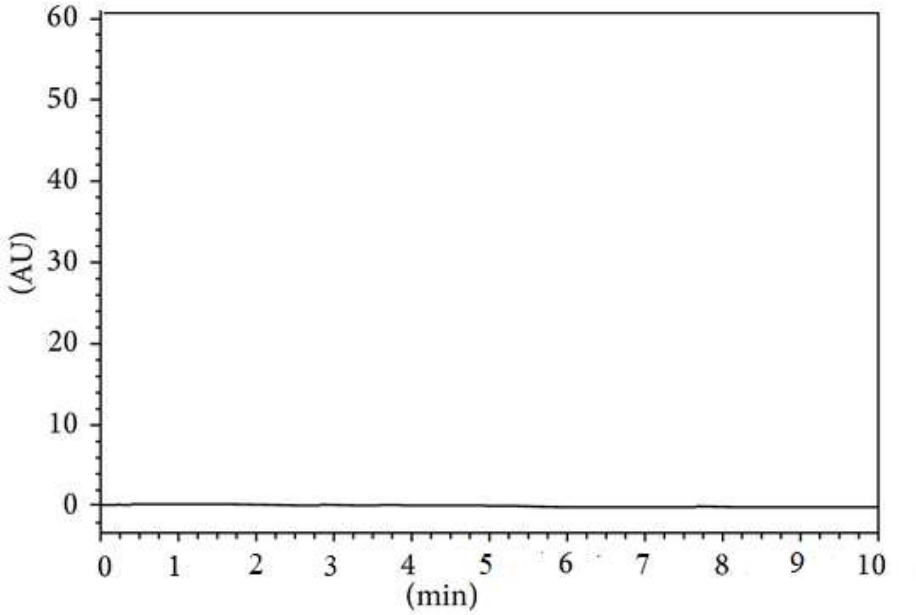

Figure 3: Chromatogram of placebo

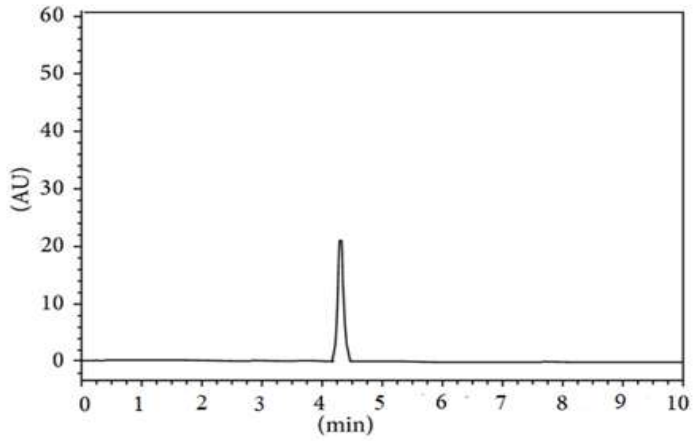

Figure 4 : Chromatogram of standard CBS solution

Linearity range:

The standard concentrations were injected in given range and analyzed according to the developed method. Acceptance of linearity data is judged by examining the calibration curve equation and correlation coefficient (r) and shown in **Table 3 and Figure 5**. The standard solution containing 1000 µg/mL of clopidogrel was prepared and diluted to appropriate concentration from 10-50 µg/mL as a working concentration for each standard 20µL of each solution was injected and analyzed using developed chromatographic method. The chromatograms were recorded and the peak areas of the drug were calculated. The data obtained was subjected to least square regression analysis within microsoft excel to calculate calibration curve equation and correlation coefficient (r). Limit of detection (LOD) and Limit of quantitation (LOQ) were determined from the slope of calibration curve using following formula,

$LOD = 3.3\sigma / S$

$LOQ = 10\ \sigma / S$

Where, σ is the standard deviation of the response

and S is the slope of the calibration curve.

Table 3 : Linearity study of clopidogrel bisulphate

Conc(µg/mL)	Peak area (µ Volts × min)
10	216291
20	451421
30	606813
40	817032
50	984753
Calibration curve equation	Y=18225X + 7560
Correlation	0.998

coefficient (r)	
LOD (µg /mL)	0.467
LOQ (µg /mL)	1.752

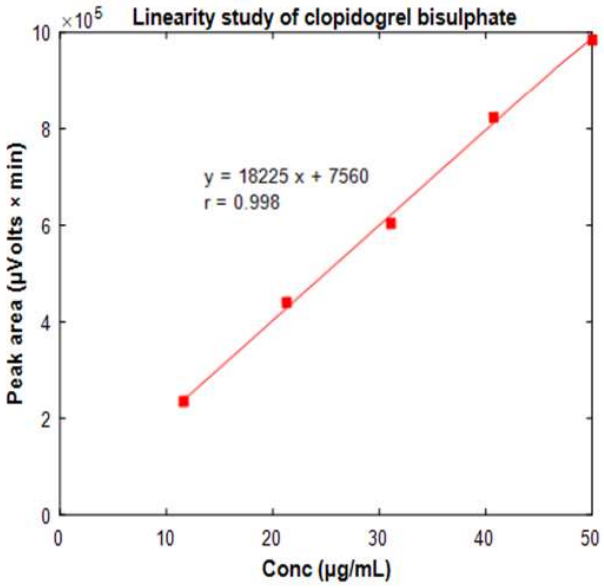

Figure 5: Linearity study of clopidogrel bisulphate

Accuracy:

The accuracy of method was tested by calculating recoveries of clopidogrel by standard addition method. Correct amount of standard solution each 80%, 100% and 120% were spiked to pre-quantified solution, and the amount of compound recovered and estimated. The results are tabulated in **Table 4**.

Table 4: Accuracy study of clopidogrel bisulphate

S.No	%Concentration (at specification level)	% Recovery	Mean Recovery%
1	80	93.31	**93.90**
2	80	94.87	
3	80	93.53	
4	100	98.82	**97.66**
5	100	97.64	
6	100	96.58	
7	120	99.14	**100.29**

8	120	100.19	
9	120	100.02	

Precision:

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Repeatability

Six replicates of standard mixture were injected and analyzed using optimized method. The average peak area along with the % RSD is shown in the **Table 5**. The % RSD for CBS was found to be 0.89.

Table 5 : Repeatability of clopidogrel bisulphate

Parameter	Clopidogrel*
Average Peak area	2138455 ± 11016
% RSD	0.89

* mean± S.D (n=3)

Intermediate precision

Intermediate precision was carried out by intraday and inter day assay method and results were tabulated in **Table 6**. The results showed no significant variation in % RSD of peak area of clopidogrel bisulphate.

Table 6: Intermediate assay precision of clopidogrel bisulphate

Compound	*Intra day (%RSD)	*Inter day (% RSD)
Clopidogrel sodium	1.05	0.83

Robustness and ruggedness:

The robustness of the method was unaffected when small, deliberate changes like, flow change, mobile phase composition, column temperature were performed at 100% test concentration. The ruggedness of the proposed method studied under different columns, analyst and instrument, laboratories analysis of the same sample and method found robust at different conditions and shown in **Table 7**.

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Table 7 : Robustness study of clopidogrel bisulphate

Change in parameters	% RSD peak area
Flow rate (0.8 mL/min)	1.71
Flow rate (1.2 mL/min)	1.21
Wave length 227 nm	1.49
Wave length 223 nm	1.03

STABILITY STUDY:

Forced degradation stability indicating studies like acidic, alkali, oxidative etc. were performed and from the degradation studies it was observed that clopidogrel is most sensitive for alkali stress than remaining stress studies as shown in **Table 8.**

Table 8 : Degradation studies of Clopidogrel bisulphate

S.No	Type of Degradation	% Degradation	% Recovery
1	Acid	16.40	83.53
2	Alkali	16.85	83.15
3	Hydrolysis	15.82	84.19
4	Peroxide	12.71	87.29
5	Photo	14.82	85.18
6	Reduction	14.34	85.66
7	Thermal	14.05	85.95

RESULTS AND DISCUSSION

The RP-HPLC method developed was statistically validated in terms of selectivity, accuracy, linearity, precision, and robustness, stability of solution and mobile phase stability. The chromatograms were recorded for extracted blank sample, CBS with blank sample, placebo and standard CBS solution the peaks were well separated from each other.

The LOD and LOQ were found to be 0.467µg/mL and 1.752µg/mL, respectively. The linearity results in the specified concentration range are found satisfactory calibration curve was plotted with correlation coefficient (r) 0.998.

Accuracy studies were shown as % recovery for CBS at 80, 100 and 120%. The limit of % recovered shown is not less than 93% and the results obtained were found to be within the limits. Hence the method was found to be accurate.

For precision studies six replicate injections were performed. % RSD was determined from peak

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areas and the results were found to be within the acceptance limits. Intermediate assay precision of CBS results showed no significant variation in % RSD of peak area of clopidogrel bisulphate. The result of the robustness study shows the method was robust at different conditions. Forced degradation studies were performed and were observed that clopidogrel is most sensitive for alkali stress than remaining stress studies.

Hence, the chromatographic method developed for CBS is simple, rapid, sensitive, precise and accurate.

CONCLUSION

A RP-HPLC method reported in literature was adopted and was modified for the purpose of the present work and was validated as per ICH guidelines. A simple, specific and reliable method reported in the literature was adopted studied by validation for estimation of the CBS. The total run time was 10 minutes where clopidogrel got separated at 4.12 minutes. There was no interference of any other peak with clopidogrel peak. When the same sample containing clopidogrel was injected 6 times, it did not affect the retention time of the drug. The developed method was validated for intraday and inter day variations. The results indicated that the reported method is highly specific and reproducible. Hence, it was concluded that the reported method may be used in the formulation development of the selected drug candidate, namely clopidogrel bisulphate.

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