A VALIDATED REVERSE PHASE HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CLOPIDOGREL BISULFATE AND RIVAROXABAN IN PHARMACEUTICAL APPLICATION

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A simple, sensitive and precise reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Clopidogrel bisulfate and rivaroxaban in pharmaceutical dosage forms. The combination drug was analyzed on BDS hypersil C_{18}, 250mm × 4.6mm, 5µ, Thermo scientific. Mobile phase consisted of buffer (0.05M KH_2PO_4 pH 4.0) : methanol in the ratio of 30:70 v/v delivered at a flow rate of 1.0 ml / min and wavelength of detection at 220 nm. The retention times of Clopidogrel bisulfate and Rivaroxaban were 2.39 min and 4.04 min respectively. The developed method was validated according to ICH guidelines. The proposed method can be used for the determination of these drugs in combined dosage forms.

INTRODUCTION

Clopidogrel bisulfate is an adenosine diphosphate receptor inhibitor that prevents platelets in the blood from sticking together and forming clots. It is routine component of the clinical management of patients after acute coronary syndrome.It has been reported that this drug would reduce rates of major cardiovascular events [1,2]. Chemically it is methyl (+)-(*S*)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(*4H*) acetate sulfate (1:1). The empirical formula of clopidogrel bisulfate is $C_{16}H_{16}Cl\ NO_2S{\bullet}H_2SO_4$ and its molecular weight is 419.9. Clopidogrel bisulfate, USP is a white to off-white powder. Clopidogrel bisulfate tablets, contains 97.875 mg of clopidogrel bisulfate which is the molar equivalent of 75 mg of clopidogrel base [3,4]. Clopidogrel bisulfate is fairly soluble and stable in aqueous solution at low pH, however solubility drops steeply when the solution of pH is above 3. Clopidogrel bisulfate exhibits poor dissolution in the pH range of 4.5 to 6.8[5].

On July 1, 2011, the U.S. Food and Drug Administration (FDA) approved Rivaroxaban for prophylaxis of deep vein thrombosis (DVT), which may lead to pulmonary embolism (PE), in adults undergoing hip and knee replacement surgery[6]. On November 4, 2011,the U.S. FDA approved Rivaroxaban for stroke prophylaxis in patients with non – valvular atrial fibrillation[7]. The addition of very low dose anticoagulation with Rivaroxaban may represent a new treatment strategy in patients with a recent acute coronary syndrome. A recent published trial found that a low dose rivaroxaban to optimal anti platelate therapy reduces mortality, cardiovascular mortality, infaract or stroke without significantly increasing fatal bleeding.[8]

The chemical name of rivaroxaban is 5-Chloro-N-(((5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-1,3-oxzolidin-5-yl)methyl) -2-thiophenecarboxamide. It is a white to yellowish powder with a molecular weight of 435.89. Rivaroxaban is only slightly soluble in organic solvents (e.g. acetone, polyethylene glycol 400) and is practically insoluble in water and aqueous media with pH 1 – 9 (pH independent 5 – 7 mg/L are soluble at 25°C . It could be classified as a Class II substance in the Biopharmaceutics Classification System (low solubility, high permeability)[9].

Literature survey reveals colorimetric method, RPHPLC method and bio analytical method for estimation of Rivaroxaban[10-13]. Several spectophotometric and high performance liquid chromatographic (HPLC) methods have been reported for the separation and quantitation of clopidogrel or pravastatin in biological fluids and drug product [15–18.]

USP states that clopidogrel bisulfate contains not less than 97.0 % and not more than 101.5% of clopidogrel bisulfate calculated on dried basis. Estimation of clopidogrel bisulfate by HPLC using phosphate buffer and acetonitrile (75: 25) at 220 nm using L 57 column has been described in USP[19]. Hence it was decided to develop HPLC method for combination of clopidogrel bisulfate and rivaroxaban mixer and tablets for study purpose. Selecting the appropriate detector before starting method development is determined by, for example, whether one component is being measured requiring single detection or whether qualitative analysis is required where universal detection would be preferred.[20]

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After taking the above into consideration, method development should start with the chromatographic separation step which requires selecting an HPLC method and optimization of the experimental conditions. Nowadays, different approaches to HPLC method development are used. Reversed phase – HPLC methods are often selected as an initial choice. It is increasingly considered the best separation technique to achieve high resolution, a short run time and better reproducibility of retention time by manipulating the HPLC conditions[21,22]. The applicability of analytical methods is assessed by a validation process. Validation is the formal and systematic way to demonstrate the suitability of a developed method for testing the analyte to provide useful analytical data within defined limits[23]. ICH guidelines Q2A and Q2B,in the Food and Drug Administration guidance, and by United States of Pharmacopoeia.

MATERIALS AND METHODS

A HPLC SPD- 20AT (Shimadzu) equipped with detector SPD-20AT, pump LC-20AT, injector: Rheodyne injector (20 µl Capacity), syringe: Hamilton (25 µl) and chromatographic software: Spinchrom was used for the study purpose. Other equipments used for the study are pH Meter from Chemiline, India, Ultasonicsonicator from Toshcon, Toshniwal process instrument pvt. Ltd. Ajmer, Analytical Balance: AX 200 etc.

Chromatographic conditions:

Column: BDS hypersil C_{18}, 250mm × 4.6mm, 5µ(particle size), Thermo scientific
Flow rate: 1.0ml/min
Injection volume: 20µl
Column Oven temperature: 25°C /Room Temperature
Wavelength: 220nm
Run Time: 10 minutes

Preparation of mobile phase:

Mobile phase is the mixture of 0.05M potassium dihydrogen ortho phosphate buffer pH 4.0and methanol in ratio of 30:70.

Preparation of Buffer for mobile phase:

0.05M potassium di hydrogen ortho phosphate buffer pH 4.0 was prepared by dissolving 6.8gm potassium dihydrogen orthophosphate in 800ml water. This solution was adjusted to pH 4.0 with 1% orthophosphoric acid (H_3PO_4) and make up volume up to 1000ml with water.

Preparation of diluent:

2.99 gm sodium acetate trihydrtae was dissolved in 1000ml of water and pH was adjusted to 4.5 with glacial acetic acid.In this buffer 2.00 gm SLS was dissolved and mixed well .This pH 4.5 Acetate buffer with 0.2% SLS was used as final diluents for Assay.

Preparation of Blank solution

10 ml of methanol was taken in 100 ml volumetric flask and make up to 100 ml with diluents, this solution is used as blank.

Preparation of standard solution for Assay:

Standard Stock Preparation:

22mg Rivaroxaban working standard and 97.8 mg Clopidogrel Bisulfate working standard was taken into a 100 ml volumetric flask. In this flask 30-70ml methanol was added and sonicated for 10 minutes. Once the standards are dissolved make up the volume up to 100ml with methanol.

Standard preparation:

Further 10 ml from the above stock solution was taken into a 100 ml volumetric flask and volume is made up with diluent. This solution is used as std. (22ppm Rivaroxaban, 97.8 ppm clopidogrel bisulfate)

Preparation of Stock solution of Test:

Twenty tablets of clopidrogel bisulfate tablets 75 mg and rivaroxaban tablets 20 mg were weighed separately and average weight was determined. Tablets of each product were triturated in a mortar pestle separately. Accurately weighed quantity of tablet powder equivalent to 22 mg rivaroxaban and 97.8 mg clopidogrel bisulfate transferred in to 100 ml volumetric flask. 70 ml methanol was added and sonicated till it dissolved completely by maintaining the temperature at 25°C and made volume up to the mark with methanol and mixed. The solution was filtered with Whatman filter paper 1 filter. Initial few ml of filtered solution was discarded and the filtered solution was collected in volumetric flask (220ppm Rivaroxaban, 978.0 ppm clopidogrel bisulfate). This stock solution was used for the preparation of test solution.

Preparation of Test Solution:

10 ml of above stock solution of test was taken separately in to 100 ml volumetric flask and was diluted up to the mark with diluent and mixed. (22 ppm rivaroxaban, 97.8 ppm clopidogrel bisulfate)

Figure 1: Standard Solution

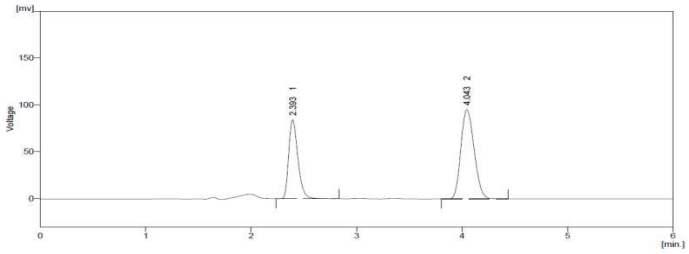


Figure 2: Test Solution

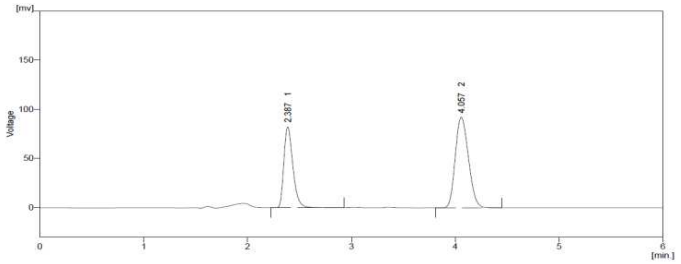
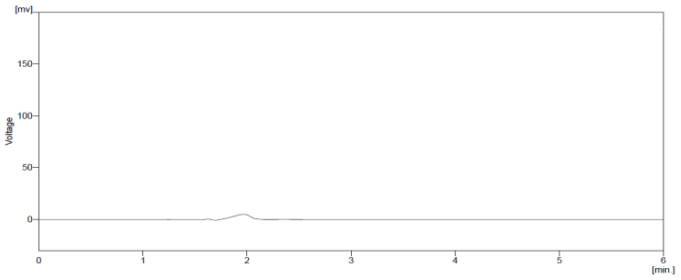


Figure 3: Blank Solution



METHOD VALIDATION

System Precision

Standard solution was prepared and injected into HPLC system for ten replicates.% RSD for the area response was calculated for rivaroxaban and clopidogrel bisulfate and was found to be less than 1%.

Linearity

The linearity of the method was determined by preparing a standard stock solution from which working solutions were prepared by diluting appropriately to yield solutions containing 50%, 60%, 75%, 85%, 100%, 110%, 125%,135%,145% and 150% of the standard solution concentration. Each of these solutions was then analyzed in duplicate and the peak areas obtained for each analyte compound plotted against concentration. Correlation coefficient was calculated for both the analytes. The ICH guidelines recommend that for the establishment of linearity a minimum of five concentrations be utilized over the range of 80 to 120 % [24, 25]. This method was found to be linear over the range tested for all the two compounds.

Precision at Linearity

For precision at linearity; lower and higher concentration prescribed under linearity experiment was injected in replicate (6 times) into the HPLC system and the precision was evaluated at each concentration. Percent relative standard deviation (%RSD) for response factor was calculated.

Method Precision

Six test preparations as per the above method was prepared and injected into the HPLC system by following the conditions prescribed in the Test method. Assay was calculated in mg and in % of label claim of rivaroxaban and clopidogrel bisulfate for each of this test preparation. Now average and % RSD of assay of these six test preparations was determined.

Accuracy

The accuracy of the method was determined by recovery experiments. Test preparation was done in triplicate by spiking active ingredient into the placebo. Percent recovery was calculated. The recovery studies were performed in triplicate. This standard addition method was performed at 50%, 75%, 100%, 125%, 150% level and the percentage recovery was calculated. For both the drugs, recovery was performed in the same way.

Robustness

Robustness of the method was checked by making slight deliberate changes in chromatographic conditions. Test solutions were prepared in triplicate as per the above method and analyzed with changed parameters. Flow rate was changed to ± 10%. As per the test procedure flow rate should be 1.0ml/min. Assay was performed with these test preparations with flow rate 1.1ml/min and 0.9ml/min. Assay of these triplicate preparations were calculated. Average, standard

deviations and % RSD was determined for these three assay results. Similarly robustness was performed for temperature ± 5°C. For temperature change these three test preparations were analyzed maintaining column oven temperature at 20°C for -5°C and 30°C for +5°C. Assay of these triplicate preparations were determined. Average, standard deviations and % RSD was determined for these three assay results. For change in wavelength these three test solutions were injected in the HPLC system at ± 5nm. For -5nm select 215nm and for + 5 select 225nm. Assay of these triplicate preparations were determined. Average, standard deviations and % RSD was determined for these three assay results.

Robustness was also performed for change in organic phase for ± 5 %. As per the test method ratio of buffer and methanol should be 30:70. To determine robustness for organic phase at +5% buffer & methanol ratio was 26.5:73.5 and for -5%, buffer & methanol ratio was 33.5:66.5. Three preparations stated above were injected in HPLC system with ratio 26.5:73.5 (+5%) and 33.5:66.5 (-5%).

Ruggedness

Ruggedness of the proposed analytical method was evaluated for variability studies like system variability, analyst variability, and column variability. Six test solutions were prepared as per the proposed test method and analyzed for each of the variability. Precision studies on replicate six samples at each of the variables was performed and effect of ruggedness parameter was evaluated. Results were compared with method precision data obtained under precision studies.

System variability

Six test preparations were done as per the above test method and injected into the two different HPLC systems by the same analyst using the same HPLC column. % assay was calculated for each of the test preparation. Average assay of six preparations and % RSD for six preparations was calculated and recorded. This set is considered as set II. This data was compared with the data obtained in the method precision data (Set I) and overall average, overall standard deviation, overall % RSD for twelve determinations was calculated and recorded which should be less than 2%.

Analyst variability

Six test preparations were prepared as per the proposed test method by two different analysts by using same HPLC and same column and injected into same HPLC systems. % assay was calculated for each of the test preparation. Average assay of six preparations and %RSD for six preparations was calculated and recorded. This set is considered as set III. This data was compared with the data obtained in the method precision data (Set I) and overall average, overall standard deviation, overall % RSD for twelve determinations was calculated and recorded which should be less than 2%.

Column variability

Six test preparations were done as per the above test method and injected into the same HPLC, by the same analyst but using different HPLC column. % assay was calculated for each of the test preparation. Average assay of six preparations and %RSD for six preparations was calculated and recorded. This set is considered as set IV. This data was compared with the data obtained in the method precision data (Set I) and overall average, overall standard deviation, overall % RSD for twelve determinations was calculated and recorded which should be less than 2%.

Filter paper Validation

To perform filter paper validation standard preparation and test preparation was done in duplicate. Some portion of stock solution of each of the standard and test preparation was filtered through Whatman filter paper 1 and some of the test preparation was centrifuged. Each of these preparations was further diluted as per the test method and injected in HPLC system. Assay of centrifuged and filtered solution was calculated. For comparison the % difference between average response of filtered and unfiltered standard and % assay of filtered and centrifuged test solution was calculated.% difference between average response of filtered and unfiltered standard and % difference between assay of filtered and centrifuged samples shall be NMT 2.0%.

Solution stability

In order to demonstrate the stability of both standard and sample solutions, the solutions were kept at room temperature

and refrigerator at 2 – 8°C and the standard & samples were analyzed at 0hrs,6hrs,12hrs,18hrs, 24 hrs.

Specificity

To perform the specificity for placebo interference triplicate preparations were done as per the proposed method. To prepare the solution of placebo, placebo equivalent to 97.8mg clopidogrel bisulfate and 22 mg rivaroxaban was taken and preparation was done as per the test procedure.

For specificity of as such sample, test preparation was done as per the above proposed method and injected in HPLC system and % assay was calculated. The peak purity result was also determined.

RESULTS AND DISCUSSION

The retention times of Clopidogrel bisulfate and Rivaroxaban were found about 2.39 min and 4.04 min respectively.

Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. [25]

System Precision: For System Precision % RSD values should be less than 1%.Current method was obtained precise as % RSD for Clopidogrel Bisulfate was obtained 0.734 and for Rivaroxaban was 0.948.Which is less than 1%.Hence complies the acceptance criteria of System Precision.

Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample as per ICH Q2R1[25]. Linearity of method was performed between the concentration range of 50% to 150%.

The data obtained from the linearity determination experiments was subjected to linear regression analysis for both Rivaroxaban and Clopidogrel Bisulfate. The correlation coefficient for Rivaroxaban was obtained 0.999 and for Clopidogrel Bisulfate was also obtained as 0.999 indicating a strong correlation between the concentrations of the analytes and the peak areas and therefore the method could be applied in the assay of these two analyte compounds. It complies with the acceptance criteria of linearity. A method to be linear correlation coefficient should not be less than 0.999. Hence it is concluded that the proposed method was found to be linear in this concentration range for both Clopidogrel Bisulfate and Rivaroxaban.

Precision at Linearity: The %RSD of Clopidogrel Bisulfate at Lower Level was 0.487 and at higher level 0.760. For Rivaroxaban at Lower Level was 0.533 and at higher level 0.596. Which is less than 2%.From the data obtained for the developed RP-HPLC method was found to be precise.

Method Precision: Average, SD & % RSD of assay of six test preparations for method precision for Clopidogrel Bisulfate was calculated. Method is precise because % RSD of these six preparations was obtained less than 2.0%.

Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. [25] Accuracy of the method was indicated by percent recovery which was within the range of 99.73 to 101.07 for Rivaroxaban and for Clopidogrel bisulfate 100.05 to 101.38 which indicates that the method is accurate. It complies the acceptance criteria that the recovery at each level shall be NLT 98.0% and NMT 102.0% of the added amount.

Robustness: Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. [25] As stated in Robustness Average, SD and % RSD of three test preparations was determined for change in flow rate(at +10%, -10%), change in temperature (at+5°C & -5°C),change in wavelength(+5nm & -5nm) and change in organic phase (+5% & -5%). The data obtained was compared with method precision data. Average of triplicate tests were determined

which was compared with the average of method precision average assay result. For all these test preparation %RSD was obtained NMT2%. The % difference between this average assay and with method precision average assay was also obtained within±2.0%.

For these changes in flow rate, temperature, wavelength and organic phase system suitability parameters were found as per the acceptance criteria. The % RSD and % difference with method precision's average assay as % label content in triplicate samples were found within acceptance criteria and hence it is concluded that the analytical results remain unaffected even there is change flow rate, temperature, wavelength and organic phase of mobile phase.
It was also observed that there were no marked changes in chromatograms, which demonstrated that the developed RP-HPLC method is robust**.**

Ruggedness: Ruggedness was performed for system variability, column variability and day variability.

System variability: For system variability overall average, overall SD and the overall % RSD of twelve determinations (six assay results obtained with method precision assay of set I and six assay results obtained with system variability preparations of set II) was obtained NMT 2%.The result found within acceptance criteria concluding that ,the method is rugged for system variability.

Analyst variability: For analyst variability overall average, overall SD and the overall % RSD of twelve determinations (six assay results obtained with method precision of set I and six assay results obtained with system variability preparations of set III) was obtained NMT 2%. The result found within acceptance criteria concluding that, the method is rugged for analyst variability.

Column variability: For column variability overall average, overall SD and the overall % RSD of twelve determinations (six assay results obtained with method precision of set I and six assay results obtained with system variability preparations of set IV) was obtained NMT 2%.The result found within acceptance criteria concluding that, the method is rugged for column variability.

Filter paper validation: Filter paper validation was performed and it complies with the acceptance criteria. % difference between average response of filtered and unfiltered standard was obtained NMT 2.0%.% difference between average % assay of filtered and centrifuged test solution was also found NMT 2.0%. The results found within acceptance criteria showing that filtration does not affect the assay value response of standard. Hence it is concluded that the sample filtration by whatman filter paper 1 does not affect the result.

Solution stability: The results of solution stability solutions at 0hrs, 6hrs, 12hrs, 18hrs, 24 hrs. show that, the retention time and peak area of Clopidogrel bisulfate and Rivaroxaban remained unchanged and no significant degradation within the indicated period was observed. This indicates that both solutions were stable for at least 24 hours, which is sufficient to complete the analytical procedure.

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. as per ICH Q2R1. [25] The method was specific since excipients/placebo in the formulation did not interfere in the estimation of Clopidogrel bisulfate and Rivaroxaban.

There were no peaks at the same RT of Clopidogrel bisulfate and rivaroxaban in chromatograms of placebo solutions. For as such test preparation purity angle was less than purity threshold. Hence it could be concluded that as peak purity of the principle peak was passed and method is specific. The retention times of Clopidogrel bisulfate and rivaroxaban were found about 2.39 min and 4.03 min respectively. The proposed method was found to be linear in the concentration range of 50% to 150% for both Clopidogrel bisulfate and Rivaroxaban.

The method was specific since excipients in the formulation did not interfere in the estimation of Clopidogrel bisulfate and rivaroxaban. Accuracy of the method was indicated by percent Recovery which was within the range of 99.75 to 101.07 for

Rivaroxaban and for Clopidogrel bisulfate 100.05 to 101.38 which indicates that the method was accurate. Precision is reflected by %RSD values less than 2.Method was obtained as Robust as Flow rate was changed to ± 10%, Temperature ± 5°C,Wavelength ± 5nm, Organic phase ± 5 % and % RSD was obtained less than 2% .Ruggedness was also studied for system variability, column variability and analyst variability and system was obtained as Rugged.

CONCLUSION

A fast simple, reliable, precise and robust isocratic reverse phase HPLC method with UV detection was developed for the simultaneous estimation of Clopidogrel bisulfate and Rivaroxaban. The optimized conditions for the separation of the two analytes BDS hypersil C_{18}, 250mm × 4.6mm, 5µ, Thermo scientific. Mobile phase consisted of buffer (0.05M KH_2PO_4; pH 4.0): methanol in the ratio of 30:70 v/v delivered at a flow rate of 1.0 ml / min and wavelength of detection at 220 nm. The retention times of Clopidogrel bisulphate and Rivaroxaban were 2.39 min and 4.04 min respectively. The method was validated for precision, specificity, accuracy, linearity, solution stability, robustness, ruggedness and filter paper validation.

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