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Development of Reverse Phase Liquid Chromatographic Method for Determination of (+)-(S)-(o-Chlorophenyl)-6,7-Dihydrothieno [3,2-c] pyridine-5(4H)-acetic acid,Hydrochloride and Methyl (+/-) - (o-Chloro phenyl)-4,5-Dihydrothieno[2,3-c]pyridine-6(7H)-acetate, Hydrochloride from Clopidogrel Besylate

Pawaskar PS*[1], Dighe VV[1], Adhyapak SS[1], Shambhu NS[1], Mestry DY[1]
[1]*Department of Chemistry, Ramnarain Ruia College, Matunga (East), Mumbai-400019, India*
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ABSTRACT

Clopidogrel besylate contain single stereogenic center and has impurities like ((+) - (S) - (o-chlorophenyl)-6,7-dihydrothieno[3,2-c] pyridine-5(4H)- acetic acid, hydrochloride) which is known as impurity A and (Methyl (+/-) - (o-chloro phenyl)-4,5-dihydrothieno[2,3-c] pyridine-6(7H)-acetate, hydrochloride) which is known as impurity B. They are introduced during production. A simple, sensitive, precise and high performance liquid chromatographic (HPLC) method has been developed and validated for quantitative determination of impurity A and impurity B from clopidogrel besylate in bulk drug using uv detector at 220 nm. The developed method was able to separate impurity A and impurity B of clopidogrel besylate from its bulk drug within 50 min. The chromatographic separation was carried out by reverse phase chromatography using C_8 column (Zorbax SB C_8 250 mm x 4.6 mm x 5 µm), with mobile phase comprising of buffer solution and acetonitrile in the gradient composition, at a flow rate of 1.0 ml/min, at 25°C temperature. The limit of detection and limit of quantitation of impurity A were found to be 0.07 µg/ml and 0.20 µg/ml and of impurity B were found to be 0.10 µg/ml and 0.30 µg/ml respectively. The linearity of response of impurity A was in the range of 0.20 µg/ml to 3.0 µg/ml with r > 0.9999. The linearity of response of impurity B was in the range of 0.30 µg/ml to 4.5 µg/ml with r > 0.9995. The method was validated and found to be suitable for determination of impurity A and impurity B from clopidogrel besylate bulk drug.

KEYWORDS

Clopidogrel besylate, ((+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetic acid, hydrochloride) i.e. (impurity A), (Methyl (+/-) - (o-chloro phenyl)-4,5-dihydrothieno[2,3-c]pyridine-6(7H)-acetate, hydrochloride) i.e. (impurity B), High performance liquid chromatography, Method validation and quantitation.Nephrotoxicity, Anti-tubercular drugs, Thymoquinone.

INTRODUCTION

Clopidogrel besylate (+)-(S)-α-(2-chlorophenyl)-4, 5, 6, 7- tetrahydrothieno [3, 2-c] pyridine-5-yl-acetic acid methyl ester benzene sulfonate is a new thienopyridine compound, structurally related to ticlopidine[1].

***Address for Correspondence:**
Mr. Pramod S.Pawaskar
Ramnarain Ruia college,
Mumbai University,
Matunga, Mumbai, India.
E-Mail Id: ppawaskar@rediffmail.com

It is used for the reduction of atherosclerotic events in patients with atherosclerosis documented by recent stroke, recent myocardial infarction or cardiovascular disease. It is an analogue of ticlopidine and acts by inhibiting adenosine diphosphate-mediated platelet aggregation. Clopidogrel inhibits platelet aggregation by selectively preventing the binding of adenosine diphosphate (ADP) to its platelet receptor. It is a potent antiplatelet drug used in thromboembolitic disorders with molecular formula $C_{22}H_{22}ClNO_5S_2$[1-3].

Clopidogrel besylate contains one chiral center. Hence is optically active compound. The S-enantiomer has biological activity and is an estabilished drug. Following process related impurities are introduced during production of clopidogrel besylate drug.

- ((+)-(S)-(o-chlorophenyl)-6, 7-dihydrothieno [3, 2-c] pyridine-5(4H)-acetic acid, hydrochloride) is known as impurity A.
- (Methyl(+/-)-(o-chlorophenyl)-4,5-dihydrothieno[2,3-c]pyridine-6(7H)-acetate, hydrochloride) is known as impurity B.
- (Methyl (-)-(R)-(o-chlorophenyl)-6, 7-dihydrothieno [3, 2-c] pyridine-5(4H)-acetate, hydrogen sulfate) is known as impurity C.

Above process related impurities are also present as impurities in clopidogrel besylate and impurity A and impurity B are quantitated in the present research work.

Owing to the pharmacological and toxicological differences between these isomers, it is quite important to develop stereo specific assay for separation of these drugs.

Literature survey revealed that few methods has been reported for the estimation of clopidogrel by spectrophotometric [4], liquid chromatography [5-7], capillary electrophoresis[8] and TLC[9] methods. However reverse phase LC method for quantitation of impurity A and impurity B from clopidogrel besylate using Zorbax SB C_8 250 mm x 4.6 mm x 5 µm column within 50 min. has not been reported in the literature.

In the present research work, a simple, sensitive and accurate normal phase HPLC method to separate impurity A and impurity B of clopidogrel besylate in bulk drug using Zorbax SB C_8 column has been reported for first time. The method was also validated to ensure the compliance in accordance with the ICH guidelines.[10]

MATERIAL AND METHODS

Materials and Reagents

Potassium dihydrogen phosphate (purity 99.0 %) used in the present research work was of AR Grade and was purchased from Merck (Mumbai, India). Orthophosporic acid (purity 88.0%) used in the present research work was of GR Grade and was purchased from Merck (Mumbai, India). Acetonitrile (purity 99.0 %) and Water used in the present research work were of HPLC Grade and was purchased from Merck (Mumbai, India).

Clopidogrel besylate working standard (B. No. : CLA1DKG92D Purity: 99.31%) was obtained from Cadila Health Care Limited, Tal-vadodara, Gujarat, India.

Clopidogrel impurity A ((+)-(S)-(o-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetic acid, hydrochloride) reference standard (no.: LOT GOH250 Purity: 0.99 mg/mg) and impurity B (Methyl(+/-)-(o-chlorophenyl)-4,5-dihydrothieno[2,3-c] pyridine-6(7H)-acetate, hydrochloride) reference standard (no.: LOT G1H047 Purity: 0.99 mg/mg) was obtained from LGC Promochem India Private Limited.

Sample of clopidogrel besylate bulk drug (clopidogrel besylate API B. No. : RD/CBE-00/1112/038) was obtained from the Process Development Laboratory of active pharmaceutical ingredient plant unit of RPG Life Sciences Ltd, Navi Mumbai, India.

Instrumentation

The HPLC was performed on Water alliance with variable wavelength UV-Visible detector, auto injector and Empower software.

Column Zorbax SB C_8 (250 x 4.6 mm) x 5µ, (Agilent, USA) was used for separation.

Chromatographic Conditions

Chromatographic separation was achieved on Zorbax SB C_8 (250 x 4.6 mm) x 5µ column with mobile phase consisting of buffer solution and acetonitrile in the gradient composition. The flow rate was 1.0 ml/min and detector

wavelength was kept at 220 nm for monitoring separation. 10 µl volume was injected into the system with total run time of 50 min.

Preparation of Buffer Solution

About 1.36 gm of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 100 ml of HPLC grade water first and 1.0 ml of orthophosphoric acid was added to it and the solution was diluted to the mark in 1000 ml standard volumetric flask with HPLC grade water to get the concentration of 10 mM.

Preparation of Mobile Phase

The mobile phase was prepared by buffer solution (A) and acetonitrile (B) in the gradient composition. The HPLC gradient was T/B (where T is time in minutes and B is % concentration of acetonitrile in terms of volume by volume i.e. v/v): 0 min / 20%, 5 min /20% , 15 min / 40% , 35 min /60 % , 40 min / 20%,and 50 min / 20% v/v.

Preparation of Stock Solution of Reference Standard Impurity A (100 µg/ml)

About 5.0 mg of reference standard impurity A was accurately weighed and dissolved in 10 ml diluent containing buffer and acetonitrile in the volume ratio (80:20) first and diluted to the mark in 50 ml standard volumetric flask with diluent to get the concentration of 100 µg/ml. This stock solution was stored in a refrigerator at 5°C.

Preparation of Stock Solution of Reference Standard Impurity B (100 µg/ml)

About 5.0 mg of reference standard impurity B was accurately weighed and dissolved in 10 ml diluent containing buffer and acetonitrile in the volume ratio (80:20) first and diluted to the mark in 50 ml standard volumetric flask with diluent to get the concentration of 100 µg/ml. This stock solution was stored in a refrigerator at 5°C.

Preparation of Stock Solution of Standard Clopidogrel Besylate (1000 µg/ml)

About 10.0 mg of clopidogrel besylate standard was accurately weighed and dissolved in 10 ml diluent containing buffer and acetonitrile in the volume ratio (80:20) first and diluted to the mark in 10 ml standard volumetric flask with diluent to get the concentration of 1000 µg/ml.

Preparation of Stock Solution of Standard Clopidogrel Besylate (100 µg/ml)

About 10.0 mg of clopidogrel besylate working standard was accurately weighed and dissolved in 100 ml diluent containing buffer and acetonitrile in the volume ratio (80:20) first and diluted to the mark in 100 ml standard volumetric flask with diluent to get the concentration of 100 µg/ml and stored in a refrigerator at 5°C

Preparation of Working Standard Solution of Clopidogrel Besylate (1.0 µg/ml)

1.0 ml of standard stock solution (100 µg/ml) of clopidogrel besylate was diluted to the mark in 100 ml standard volumetric flask with buffer and acetonitrile in the volume ratio (80:20) as diluents to give solution of concentration of 1.0 µg/ml of clopidogrel besylate.

Preparation of Sample solution of clopidogrel besylate from Bulk Drug (1000µg/ml)

25.0 mg of clopidogrel besylate bulk drug was accurately weighed and dissolved in 5.0 ml with buffer and acetonitrile in the volume ratio (80:20) as diluents and diluted to the mark in 25 ml standard volumetric flask with diluents to get the concentration of 1000 µg/ml.

Preparation of Sample solution of clopidogrel besylate from Bulk Drug (100 µg/ml)

10.0 mg of clopidogrel besylate bulk drug was accurately weighed and dissolved in 5.0 ml with buffer and acetonitrile in the volume ratio (80:20) as diluents and diluted to the mark in 100 ml standard volumetric flask with diluents to get the concentration of 100 µg/ml.

METHOD VALIDATION

Method Validation Parameters

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of

its impurity. Baseline separation of impurity A, impurity B and clopidogrel besylate was achieved using Zorbax SB C_8 column. There were no interfering peaks which co-eluted with the compound of interest. This has indicated appropriate specificity of elaborated procedure. The order of elution was determined using UV detector. The retention times of impurity A, clopidogrel besylate and impurity B were approximately 11.0 min, 22.0 min and 25.0 min. respectively. A typical chromatogram of impurity A, impurity B and clopidogrel besylate has been represented in Figure 1 to Figure 3.

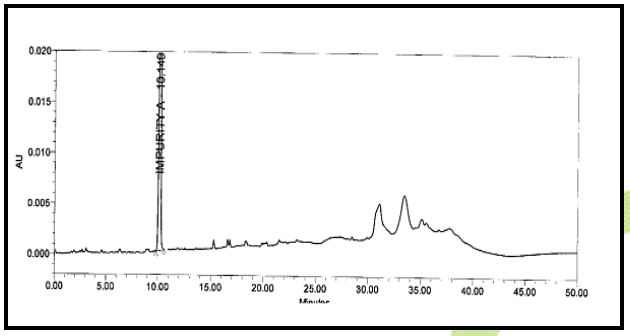

Figure 1: A typical chromatogram of impurity A standard

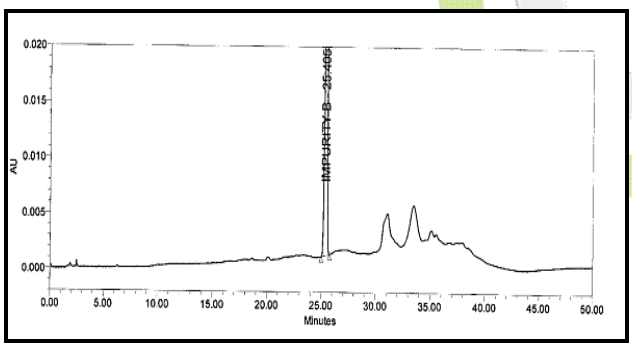

Figure 2: A typical chromatogram of impurity B standard

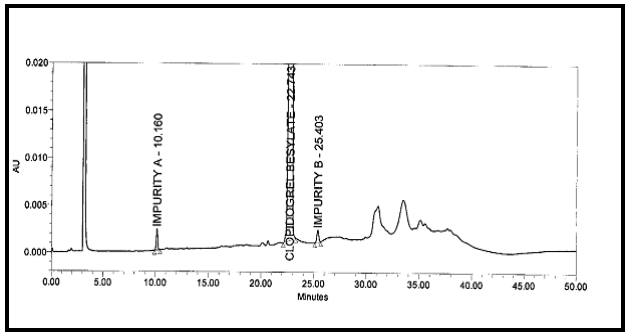

Figure 3: A typical chromatogram of clopidogrel besylate bulk drug

Precision

The method was validated in terms of system precision, method precision and intermediate precision. The system precision was studied by separate, repetitive injections (n = 6) of standard solution of clopidogrel besylate working standard (1.0 µg/ml), in the chromatographic system under the specified conditions. The percent relative standard deviation was found to be 0.96. The method precision was evaluated by carrying out six replicates of test sample (1000 µg/ml). The values of percent relative standard deviation of retention times and peak areas for impurity A and impurity B were found to be less than 1. The intermediate precision of the method was evaluated on different days in the same laboratory. The values of percent relative standard deviation of peak area of impurity A and impurity B for intermediate precision was found to be less than 1.The results indicate that method is precise and reproducible.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection and limit of Quantitation of the impurity A and impurity B was estimated at a signal to noise ratio of 3:1and 10:1 respectively by injecting a series of diluted solution of impurity A and impurity B with known concentration. The value of LOD and LOQ of impurity A were found to be 0.07 µg/ml and 0.20 µg/ml respectively. The value of LOD and LOQ of impurity B were found to be 0.10 µg/ml and 0.30 µg/ml respectively. The value of LOD and LOQ of clopidogrel besylate were found to be 0.03 µg/ml and 0.10 µg/ml respectively.

Linearity of Clopidogrel Besylate, Impurity A and Impurity B

Linearity was evaluated by analyzing working standard solutions of impurity A, impurity B and clopidogrel besylate in the concentration range 0.10 µg/ml to 3.0 µg/ml, 0.30 µg/ml to 4.5 µg/ml and 0.10 µg/ml to 200 µg/ml. The solutions were injected in duplicate in the chromatographic system under optimized conditions described earlier. The calibration plot

for impurity A was found to be linear in the concentration range 0.10 µg/ml to 3.0 µg/ml with correlation coefficient r as 0.9999. The calibration plot for impurity B was found to be linear in the concentration range 0.30 µg/ml to 4.5 µg/ml with correlation coefficient r as 0.9995. The calibration plot for clopidogrel besylate was found to be linear in the concentration range 0.30 µg/ml to 4.5 µg/ml with correlation coefficient r as 0.9999.

Accuracy

The accuracy of the method was established by performing recovery experiment using standard addition method. For zero level, only sample solution was analyzed by HPLC in triplicates. To 1000 µg/ml of clopidogrel besylate bulk drug sample, pure standard of impurity A with varying concentrations (0.20 µg/ml, 1.0 µg/ml, 2.0 µg/ml, 3.0 µg/ml) and pure standard of impurity B with varying concentrations (0.30 µg/ml, 1.5 µg/ml, 3.0 µg/ml, 4.5 µg/ml) respectively were added. The solutions were prepared and analyzed by HPLC for each level and mean amounts of impurity A and impurity B present in each level of sample solution were determined. The percentage recovery of impurity A and impurity B was calculated against 1.0 µg/ml of clopidogrel besylate working standard .The value of mean percent recovery of impurity A was found to be 98.70. The value of mean percent recovery of impurity B was found to be 99.55.The results are given in Table 1 and Table 2.

Accuracy of the method was determined at three different concentration levels, i.e. at 80 %, 100 % and 120 % (w/w) of the assay concentration of clopidogrel besylate (100 µg/ml) as described in the method for three different determinations each triplicate. For each determination fresh samples were prepared and calculated against the clopidogrel besylate working standard concentration of 100 µg/ml. The value of mean percent recovery of clopidogrel besylate was found to be 99.03. The results are given in Table 3.

Table 3: Recovery results of studies of clopidogrel besylate bulk drug using the proposed HPLC method

Recovery level	Theoretical recovery (%)	*Mean amount of clopidogrel besyalte found (%)		
		Actual Recovery	% Avg. Recovery	% RSD
1	80	79.17	98.96	0.07
2	100	99.27	99.27	0.05
3	120	118.63	98.86	0.14

*n=3

Table 1: Recovery results of studies of impurity A from clopidogrel besylate bulk drug using the proposed HPLC method

Recovery level	Amount of impurity A added (µg/ml)	*Mean amount of impurity A found (µg/ml)		
		Mean amount found	% Average Recovery	% RSD
1	0.2	1.51	98.62	0.90
2	1.0	2.30	98.49	0.44
3	2.0	3.30	99.12	0.16
4	3.0	4.27	98.60	0.58

*n=3

Table 2: Recovery results of studies of impurity B from clopidogrel besylate bulk drug using the proposed HPLC method

Recovery level	Amount of impurity B added (µg/ml)	*Mean amount of impurity B found (µg/ml)		
		Mean amount found	% Average Recovery	% RSD
1	0.3	1.08	99.34	0.51
2	1.5	2.28	99.95	0.42
3	3.0	3.76	99.04	0.46
4	4.5	5.28	99.86	0.69

*n=3

Robustness

Robustness of the method was determined by making small deliberate changes in the chromatographic conditions utilized in present method. The robustness of the method is evaluated by replicate analysis of standard solutions of clopidogrel besylate (1.0 µg/ml).The chromatographic conditions which changed were ortho phosphoric acid of buffer preparation composition (+/- 10.0 %), flow rate (+/- 10.0 %) and wavelength (+/- 2 nm). The amount of impurity A and impurity B from clopidogrel besylate bulk drug obtained by normal method did not affect the system suitability criteria. The resolution between impurity B and clopidogrel besylate was greater than 6; under all separation conditions tested, demonstrating sufficient robustness. As deliberate changes made to the chromatographic method did not affect the results, it can be concluded that method was found to be robust.

RESULTS AND DISCUSSION

In the present research work, HPLC method has been developed for the quantitation of impurity A and impurity B from clopidogrel besylate bulk drug. The chromatographic analysis was carried out on Zorbax SB C_8 column, (250 mm x 4.6 mm x 5 µm)**,** using a mobile phase comprising buffer and acetonitrile, in the gradient composition. The detection was carried out at λ = 220 nm. The flow rate of mobile phase was 1.0 ml/min. and column temperature used was 25°C.

The limit of Quantitation (LOQ) of impurity A was 0.20 µg/ml and limit of detection (LOD) was found to be 0.07 µg/ml. The limit of Quantitation (LOQ) of impurity B was 0.30 µg/ml and limit of detection (LOD) was found to be 0.10 µg/ml.The limit of Quantitation (LOQ) of clopidogrel besylate was 0.10 µg/mL and limit of detection (LOD) was found to be 0.03 µg/ml. Good linearity was observed for impurity A over the concentration range of 0.2 µg/ml – 3.0 µg/ml, with linear regression equation y = 18683 x – 160.44 and correlation coefficient r > 0.9999. Good linearity was observed for impurity B over the concentration range of 0.3 µg/ml – 4.5 µg/ml, with linear regression equation y = 15559 x – 51.60 and correlation coefficient with r > 0.9995. Good linearity was observed for clopidogrel besylate over the concentration range of 0.1 µg/ml – 200 µg/ml, with linear regression equation = 14065 x + 1370.25 and correlation coefficient r > 0.9999. The accuracy of method of clopidogrel besylate was evaluated by using freshly prepared solution at three concentration levels

of 80 % w/w, 100 % w/w and 120 % of analyte concentration (100µg/ml). The percentage recovery of clopidogrel besylate values were in the range of 98.73 to 99.29. The accuracy of method was evaluated by using freshly prepared solution of clopidogrel besylate drug at four concentration levels 0.20 µg/ml, 1.0 µg/ml, 2.0 µg/ml, 3.0 µg/ml of impurity A and 0.30 µg/ml, 1.5 µg/ml, 3.0 µg/ml, 4.5 µg/ml of impurity B. The percentage recovery of impurity A values were in the range of 97.76 to 99.78. The percentage recoveries of impurity B values were in the range of 98.70 to 100.61. The resolution between clopidogrel and impurity B was greater than 6.0; under all separation conditions tested, demonstrating sufficient robustness.

Reverse phase HPLC method has been reported in literature for determination of impurity A, and impurity B from clopidogrel bisulphate using chiral-recognition protein column (Ultron ES-OVM) (150 mm x 4.6 mm x 5µm) reversed-phase column in the USP. [11]But after 20 to 30 injections of sample in HPLC system, the resolution of impurities from clopidogrel gets minimized using Ultron ES-OVM column. Column life is very short and column was also costly. In present research work, impurity A and impurity B were quantitated at single wavelength 220 nm with good sensitivity and using Zorbax SB C_8 column (250 mm x 4.6 mm x 5 µm).This column has longer life and is less costly. It increased the separation efficiency of impurity A, impurity B and clopidogrel besylate from it bulk drug and gave symmetrical, sharp and well-resolved peaks of impurity A, impurity B and clopidogrel besylate.

Literature survey also reveals HPLC method for determination of impurity A and impurity B from another salt of clopidogrel hydrogen sulphate by reverse phase HPLC.[12] The method uses gradient mode of elution of impurity A and impurity B using pentane sulphonic acid sodium salt as an ion pairing reagent. Column used was water symmetry (150 mm x 3.9 mm x 5µ). Total run time was 60 min. Retention time of impurity A is about 13.0, retention time of clopidogrel is about 25.0 min and retention time of impurity B is about 27.0 min. The method used in present research work uses gradient mode of elution to obtain baseline with good peak resolution from clopidogrel besylate and is used for quantitation of impurity A & impurity B from clopidogrel besylate bulk drug. Column used was Zorbax SB C_8 (250 mm x 4.6 mm) x 5 µm. Total run time was 50 min. Retention time of impurity A is about 10 min., retention time of clopidogrel is about 22.0 min and retention time of impurity B is about 25.0 min. the present method is adventagous as it does not need ion pairing reagent.

In present research work, simple HPLC method has been developed. The column used was Zorbax SB C_8 (250 mm x 4.6 mm) x 5µm, which provides a better resolution for both the standards impurity A & impurity B.

CONCLUSION

The simple gradient, reverse phase liquid chromatographic method has been developed for quantitation of clopidogrel besylate, impurity A and impurity B from its bulk drug The method was found to be precise, sensitive, accurate, linear, robust and specific in bulk active substance. The method was completely validated showing satisfactory data for all validation parameters tested. The developed method could be used for quantification of clopidogrel besylate, impurity A and impurity B in clopidogrel besylate bulk drug samples. Based on the validation exercise performed and the results tabulated above, the HPLC method for estimation of clopidogrel besylate and quantitation of impurity A and impurity B in clopidogrel besylate bulk drug is specific, sensitive, precise, and robust and hence can be used as a routine quality control testing method.

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