

Development and validation of RP-HPLC-UV method for simultaneous quantitation of clopidogrel bisulphate and aspirin in bulk drug

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ABSTRACT

A simple, selective, linear, precise, and accurate RP-HPLC method was developed and validated for the simultaneous estimation of Clopidogrel Bi Sulphate and Aspirin from bulk drug. Chromatographic separation was achieved isocratically on a Shimadzu Phenomenex Luna, C18 column (250×4.6 mm, 5 μ particle size) using a mobile phase, (0.3% ortho phosphoric acid (v/v)-acetonitrile (40:60 v/v). The flow rate was 1 ml/min and effluent was detected at 226 nm and 20 μl of sample was injected. The retention time of Clopidogrel bisulphate and Aspirin were 6.6 and 8.4 min respectively. Linearity was observed in the concentration range of 0.030-0.120 mg/ml for aspirin and 0.015-0.060 mg/ml for clopidogrel. Percent recoveries obtained for aspirin was 99.12-99.83% and 98.20-100.35 % for clopidogrel. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision, LOD and LOQ. The method developed was successfully applied for the analysis of simultaneous estimation of Clopidogrel Bi Sulphate and Aspirin bulk drug.

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KEYWORDS

Clopidogrel bi sulphate;
Aspirin;
Bulk drug;
OPA buffer.

INTRODUCTION

Clopidogrel bisulfate, chemically it is [S- (a) (2-chlorophenyl)-6,7- dihydrothieno (3,2-C) pyridine-5 (4H) acetic acid methyl ester sulphate] (Figure 2). The empirical formula of clopidogrel bisulfate is $C_{16}H_{16}ClNO_2S \cdot H_2SO_4$ and its molecular weight is 419.9 g/mole^[1]. It is a white to off-white powder. It is practically insoluble in water at neutral pH but freely soluble at pH 1. It also dissolves freely in methanol, dissolves sparingly in methylene chloride and is practi-

cally insoluble in ethyl ether. It has a specific optical rotation of about +56°. The structural formula is as follows:

Clopidogrel is an inhibitor of platelet aggregation. A variety of drugs that inhibit platelet function have been shown to decrease morbid events in people with established cardiovascular atherosclerotic disease as evidenced by stroke or transient ischemic attacks, myocardial infarction, unstable angina or the need for vascular by-pass or angioplasty. This indicates that platelets participate in the initiation and/or evolution of these

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events and that inhibiting them can reduce the event rate.

Clopidogrel selectively inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex, thereby inhibiting platelet aggregation. Biotransformation of clopidogrel is necessary to produce inhibition of platelet aggregation, but an active metabolite responsible for the activity of the drug has not been isolated. Clopidogrel also inhibits platelet aggregation induced by agonists other than ADP by blocking the amplification of platelet activation by released ADP. Clopidogrel does not inhibit phosphor diesterase activity.

Clopidogrel acts by irreversibly modifying the platelet ADP receptor. Consequently, platelets exposed to clopidogrel are affected for the remainder of their lifespan. Dose dependent inhibition of platelet aggregation can be seen 2 hours after single oral doses of clopidogrel bisulfate. Repeated doses of 75 mg clopidogrel bisulfate per day inhibit ADP-induced platelet aggregation on the first day and inhibition reaches steady state between day 3 and day 7. At steady state, the average inhibition level observed with a dose of 75 mg clopidogrel bisulfate per day was between 40% and 60%. Platelet aggregation and bleeding time gradually return to baseline values after treatment is discontinued, generally in about 5 days.

Aspirin is chemically acetylsalicylic acid (Figure 1) Its molecular formula is $C_9H_8O_4$ having molecular weight 180 g/mole^[1]. It is slightly soluble in water, freely soluble in alcohol, soluble in chloroform and ether, sparingly soluble in absolute ether.

Aspirin, one of the first drugs to come into common usage, is still the most widely used drug in the world,

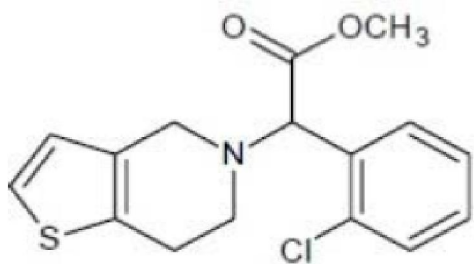


Figure 1 : Chemical Structure of clopidogrel

Figure 1 : Chemical structure of Clopidogrel bisulphate

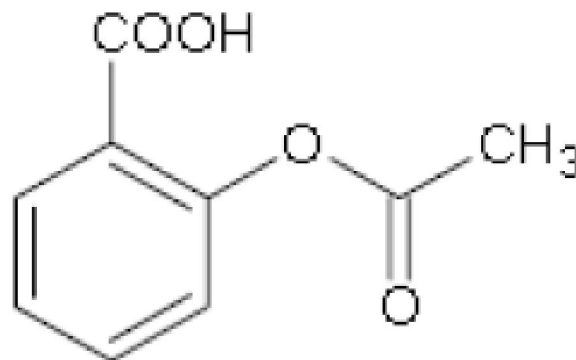


Figure 2 : Chemical structure of Aspirin

is a non-steroidal anti-inflammatory drug that exhibits anti-inflammatory, analgesic and antipyretic activities. Aspirin is now accepted as an important weapon in the prevention of heart disease. A single dose of 300 mg is now recommended for patients in the acute stages of a heart attack followed by a daily dose of 75-100 mg.

A similar low dose treatment regime is recommended for patients with angina, a history of heart problem or who have undergone coronary by-pass surgery. Major use of aspirin is as an anti-platelet aggregating agent.

Aspirin is an inhibitor of the enzyme cyclooxygenase, the reaction being considered to be due to an irreversible acetylation process. In blood platelets such enzyme inhibition prevents the synthesis of thromboxane A₂, a compound that is a vasoconstrictor, causes platelet aggregation and is thus potentially thrombotic. Thus aspirin inhibits platelet inhibition. Aspirin is an effective antithrombotic at doses as low as 80 mg, but the rapid, acute effect probably requires 162.5 mg.

Different publications are available regarding determination method of aspirin and clopidogrel but most of the methods are applicable to alone aspirin or clopidogrel in pharmaceutical dosage form or in biological fluids. Only three methods are reported for the simultaneous determination of aspirin and clopidogrel. One is semi-micro column HPLC-UV method for simultaneous determination of clopidogrel metabolite, aspirin and salicylic acid in rat plasma. Second is a spectrophotometric method, which is able to determine aspirin and clopidogrel in combine dosage form and third is simple high performance liquid chromatography, which applicable to routine quality control sample analysis. The separation is performed by high performance liquid chromatography for reasons of robustness and fa-

miliarity of analysts with this technique. To our knowledge, no stability-indicating analytical method for the determination of aspirin and Clopidogrel in combine dosage forms has been published. The previous published methods are not directly applicable for this issue and need more investigation for method development and validation.

Some analytical methods for the quantitative determination of clopidogrel in pharmaceutical bulk drug analysis are described in the literature like. Metabolite of Clopidogrel in human plasma & its liquid extraction was detected by high performance liquid chromatography (HPLC)^[2] and its determination of carboxylic acid metabolite in rat plasma by HPLC method^[3] Bio analytical method to determine unchanged clopidogrel in human plasma^[4] Method available for the determination of aspirin include HPLC are simultaneous determination of acetylsalicylic acid, paracetamol, caffeine and phenobarbital in tablets^[5], quantitative high performance liquid chromatographic method for the determination of aspirin and related substances in tablets^[6], simple reversed phase high-performance liquid chromatography method for the simultaneous estimation of aspirin and isosorbide 5-mononitrate in combined formulation^[7], determination of acetylsalicylic acid and salicylic acid in skin and plasma by high-performance liquid chromatography^[8].

No HPLC-UV method has been reported in the literature for the simultaneous determination of clopidogrel and aspirin in their pharmaceutical bulk drug. It would therefore be beneficial to provide accurate, precise, and reliable methods for simultaneous determination of clopidogrel and aspirin. The present work describes an analytical procedure for the quantitation of clopidogrel with aspirin using reversed phase HPLC.

MATERIALS AND METHODS

Chemicals

Clopidogrel and aspirin API's were obtained as a gift sample from Mepro Pharmaceuticals Pvt.Limited (Surendranagar, India) and their percentage purity of 99.90% and 100.00%, respectively. HPLC grade acetonitrile was obtained from Merck Limited. Ortho phosphoric acid was obtained from SD Fine (Mumbai,

India). HPLC grade water was obtained by Rankem Limited.. All other chemicals used were of pharmaceutical or analytical grade.

Chromatographic conditions

The HPLC system (LC 20AD, Shimadzu, Japan) consisted of binary gradient system, in-line degasser, UV detector (Shimadzu, FPD-20A model) and rheodyne injector (Shimadzu, 7725i). Data was processed using LC Solution ver. 1.2 software (Shimadzu, Japan). Isocratic elution of the mobile phase Acetonitrile: buffer pH was obtained to 4.15 by using 0.3% ortho phosphoric acid as a buffer in the ratio of 60:40 v/v with the flow rate of 1 ml/min. Separation was performed on a Phenomenex Luna C18 (250 x 4.6 mm i.d, 5 μ particle size) analytical column and a precolumn to protect the analytical column from strongly bonded material. Integration of the detector output was performed using the Shimadzu LC Solution software to determine the peak area. The contents of the mobile phase were filtered through a 0.45 μ m membrane filter and degassed by sonication before use. Mobile phase was used as diluents. The flow rate of the mobile phase was optimized to 1 ml/min which yields a column back pressure of 120 bar. The run time was set at 13 min and a column temperature was maintained at 30°C. The volume of injection was 20 μ l, prior to injection of the analyte, the column was equilibrated for 30–40 min with the mobile phase. The eluent was detected at 226 nm. The developed method was validated in terms of specificity, linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), intra-day and inter-day precision and robustness for the assay of Clopidogrel and Aspirin as per ICH guidelines^[9].

Preparation of standard stock solutions of clopidogrel and aspirin

Standard solution containing Aspirin Standard (0.075 mg/ml) and Clopidogrel Standard (0.0375 mg/ml) were prepared by dissolving 37.5 mg aspirin and 24.46 mg clopidogrel bisulphate (equivalent to 18.5 mg clopidogrel) in 100 ml volumetric flask by mobile phase (stock standard solution). Pipette out 10 ml stock solution into 50 ml volumetric flask and dilute up to mark with mobile phase.

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Preparation of test Solution (Analysis of clopidogrel and aspirin in bulk drug)

Standard solution containing Aspirin Bulk Drug Sample (0.075 mg/ml) and Clopidogrel Bulk Drug Sample (0.0375 mg/ml) were prepared by dissolving 37.5 mg aspirin and 24.46 mg clopidogrel bisulphate (equivalent to 18.5 mg clopidogrel) in 100 ml volumetric flask by mobile phase (stock standard solution). Pipette out 10 ml stock solution into 50 ml volumetric flask and dilute up to mark with mobile phase.

RESULTS AND DISCUSSION

The present research work was designed at developing a rapid, sensitive, precise and accurate HPLC method for the simultaneous estimation of clopidogrel and aspirin in pharmaceutical dosage forms. In order to affect analysis of the component peaks under isocratic conditions, mixtures of buffer and acetonitrile in different combinations with different pH were tested as mobile phase on a Phenomenex Luna C18 stationary phase. A binary mixture of Acetonitrile: buffer pH adjusted to 4.15 with ortho phosphoric acid in the ratio of 60:40 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were better defined and resolved and free from tailing. A flow rate of 1.0 ml/min of the mobile phase was found to be suitable.

Method development

After various trials, the following chromatographic conditions were finally optimized for the simultaneous estimation of clopidogrel and aspirin in a bulk drug form. Mobile phase constitutes of Acetonitrile: buffer pH adjusted to 4.15 with ortho phosphoric acid in the ratio of

60:40 v/v. Detection wave length 226 nm flow rate 1.0 ml/min, after a steady baseline the standard solution were injected and chromatograms were recorded until the reproducibility of the peak areas were found and finally 100 µg/ml of the standard solution of the individual samples of clopidogrel and aspirin and mixed standard solutions were injected and the chromatograms were recorded. The separation of clopidogrel and aspirin with retention times of 6.6 and 8.4 min respectively. The typical chromatograms of the standard solutions were recorded for the repeatability and the respective chromatogram was given in Figure 3.

Method validation

After development of method, validation of the method for simultaneous estimation of clopidogrel and aspirin was performed in accordance with ICH guidelines (International Conference on Harmonization (ICH) 2000) which include System suitability, Linearity, Accuracy, Precision, LOD and LOQ, Specificity and Robustness.

Linearity

Calibration graphs were constructed by plotting peak area vs. concentration of clopidogrel and aspirin and the regression equations were calculated. The calibration graphs were plotted over 5 different linear concentrations in the range of 0.030-0.120 mg/ml for aspirin and 0.015-0.060 mg/ml for clopidogrel. Aliquots (20 µl) of each solution were injected under the operating chromatographic condition described above [Number of replicates (n = 6)]. The method was found linear over the concentration range of 0.030-0.120 mg/ml for aspirin and 0.015-0.060 mg/ml for clopidogrel. Linearity curves of clopidogrel and aspirin were shown in figure 4 & 5 respectively.

Accuracy

The accuracy of the method was established by recovery studies i.e., external standard addition method. The known amount of standard was added at three different levels to pre analyzed sample. Each determination was performed in triplicate. The mean recoveries obtained were 98.00% and 100.00% for clopidogrel and aspirin. The results of accuracy were tabulated in TABLE 1.

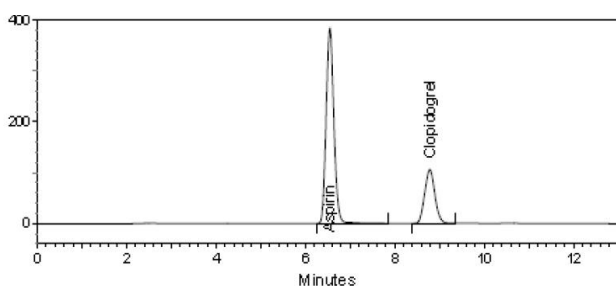


Figure 3 : Typical chromatogram of clopidogrel and aspirin with detection at 226 nm.

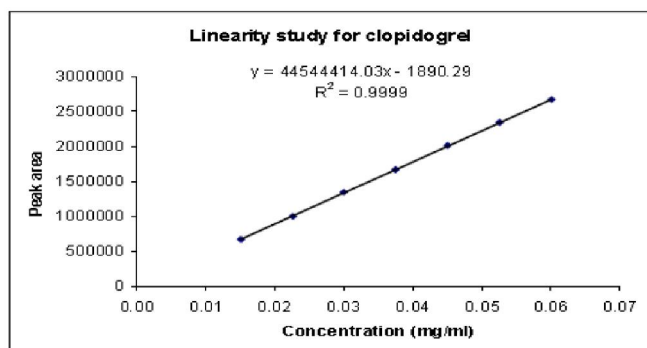


Figure 4 : Linearity of Aspirin

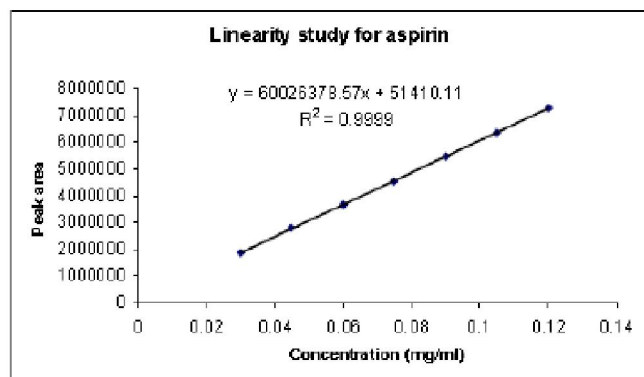


Figure 5 : Linearity of Aspirin

Precision

The intraday and interday precision of the proposed method was determined by analyzing mixed standard

TABLE 1 : Summary of validation parameters

S. No	Validation Parameters	Results	
		Clopidogrel	Aspirin
1	Accuracy (% Recovery)	98-100	98-100
2	Precession (% RSD)	0.53	0.48
3	Interday precession (% RSD)	0.47	0.40
4	LOD	0.15 ng ?ml ⁻¹	0.05 ng ?ml ⁻¹
5	LOQ	0.30 ng ?ml ⁻¹	0.20 ng ?ml ⁻¹

solution of clopidogrel and aspirin at concentration 100 μ g/ml 3 times on the same day and on 3 different days. The results are reported in terms of relative standard deviation. The % RSD values for clopidogrel and aspirin were found to be 0.53% and 0.48% respectively.

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

The limit of detection (LOD) and limit of quantitation (LOQ) of clopidogrel and aspirin were determined by calculating the signal-to noise (S/N) ratio of 3:1 and

10:1, respectively according to International Conference on Harmonization guidelines. LOD values for clopidogrel and aspirin were found to be 0.15 ng ml⁻¹ and 0.05 ng ml⁻¹ respectively. LOQ values for clopidogrel and aspirin were found to be 0.30 ng ml⁻¹ and 0.20 ng ml⁻¹ respectively.

Robustness

The robustness of the method was evaluated by assaying the test solutions after slight but deliberate changes in the analytical conditions like flow rate (0.1 ml min⁻¹), and pH of the mobile phase (\pm 0.2). Stability of standard and test solution (prepared from the dosage form) was established by storage at 25 °C and 15 °C for 48 h. During the storage period, the test solutions were reanalyzed at intervals of 6, 12, 24, 36 and 48 h and assay was determined against appropriate fresh standard preparations.

CONCLUSION

The results evident that the presented method is successfully used for simultaneous determination of clopidogrel and aspirin content in bulk drug.

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