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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF ASPIRIN, CLOPIDOGREL BISULPHATE AND ROSUVASTATIN CALCIUM IN BULK AND PHARMACEUTICAL DOSAGE FORMULATION.

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ABSTRACT

A sensitive, accurate, and precise stability-indicating HPTLC method has been developed for the simultaneous estimation of Aspirin (ASP), Clopidogrel Bisulphate (CLOP) and Rosuvastatin Calcium (ROS) in bulk and pharmaceutical dosage formulation. The method employed Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v) as a mobile phase and Silica Gel G 60 F254 TLC plates as stationary phase. Detection was performed at 258 nm. The Rf Value of ASP, CLOP and ROS were found to be 0.22 ±0.02, 0.88 ±0.02 and 0.16 ±0.02 respectively. The method was validated in compliance with ICH Guideline for linearity, limit of detection (LOD), limit of quantification (LOQ), precision, specificity, accuracy and robustness. The linear regression analysis (n=3) showed good linear relationship over the concentration range of 225 - 1350 ng/spot for ASP (r^2=0.9999), 225 – 1350ng /spot for CLOP (r^2=0.9991) and 90 – 180 ng/spot of ROS (r^2=0.9992). The LOD of ASP, CLOP and ROS were found to be 1.671ng/spot, 2.211ng/spot and 0.207ng/spot respectively. The LOQ of ASP, CLOP and ROS were found to be 50.65ng/ spot, 67.01ng/spot and 6.27ng/spot respectively. The % recovery was calculated and found to be 99.27– 100.4% for ASP, 98.2%-99.17% for CLOP and 98.86-101.15% for ROS. ASP, CLOP and ROS were subjected to acidic, alkaline, oxidative, neutral and thermal degradation conditions. The degradation products obtained were well resolved from the pure drugs with significantly different Rf

values. As the method could effectively separate the drugs from its degradation products, it can be used for stability-indicating analysis as well as statistical evaluation proved that the established method was accurate, specific, precise, repeatable and robust for the estimation of ASP, CLOP and ROS in bulk and pharmaceutical dosage form.

KEYWORDS: High-performance thin-layer chromatography (HPTLC), Aspirin (ASP), Clopidogrel Bisulphate (CLOP) and Rosuvastatin Calcium (ROS), Stability indicating method development, Validation.

INTRODUCTION

A heart attack occurs when one or more coronary arteries become blocked. Over time, a coronary artery can narrow from the build-up of various substances, including cholesterol (atherosclerosis). This condition, known as coronary artery disease, causes most heart attacks. During a heart attack, one of these plaques can rupture and spill cholesterol and other substances into the bloodstream. A blood clot forms at the site of the rupture. If large enough, the clot can completely block the flow of blood through the coronary artery.

Another cause of a heart attack is a spasm of a coronary artery that shuts down blood flow to part of the heart muscle. Use of tobacco and of illicit drugs, such as cocaine, can cause a life-threatening spasm. A heart attack can also occur due to a tear in the heart artery (spontaneous coronary artery dissection).

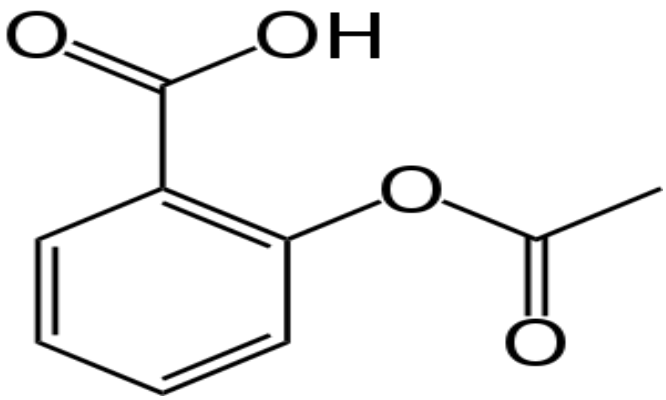

Fig 1: Structure of Aspirin.

Aspirin (ASP) 2-(acetyloxy) benzoic acid interferes with blood's clotting action. It prevents blood clot formation which could block the coronary artery leading to a heart attack. The usual dosage of ASP is 75mg once daily.

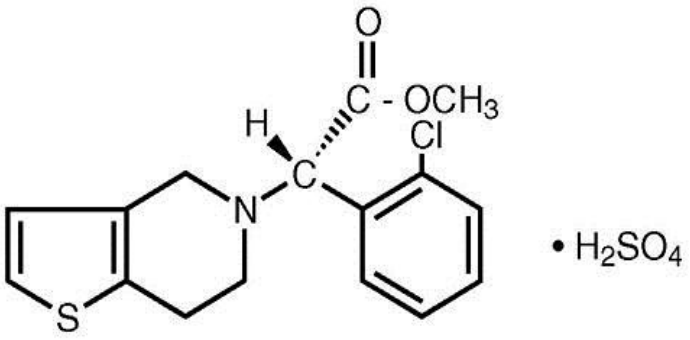

Fig 2: Structure of Clopidogrel Bisulphate.

Clopidogrel Bisulphate (CLOP), methyl (2S)-2-(2-chlorophenyl)-2-(6,7-dihydro-4H-thieno[3,2-c]pyridin-5-yl)acetate sulfuric acid in a anti-thrombotic drug. It helps reduce the risk of heart attack by preventing clot formation. The usual dosage is 75mg once daily.

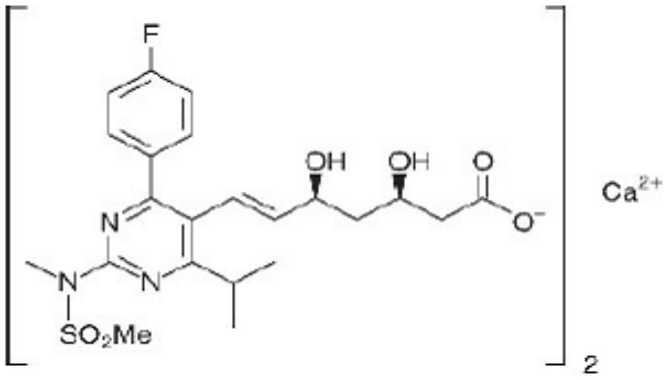

Fig 3: Structure of Rosuvastatin Calcium.

Rosuvastatin Calcium (ROS), 3*R*, 5*S*,6*E*-7-[4-(4-Fluorophenyl)-2-(*N*-methylmethanesulfonamido)-6-(propan-2-yl)pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoicacid is an lipid lowering drug. It is used to reduce the risk of heart attack and stroke by lowering the blood cholesterol levels and reducing the plaque formation. The usual dosage varies from 5 mg – 40mg once daily.

Patients who are at a risk for heart attack are treated with two antiplatelet agents to prevent blood clotting. This is known as Dual Antiplatelet therapy (DAPT). One anti platelet agent is ASP and other is P2Y12 inhibitor (CLOP). Treatment of heart attack using statins to manage cholesterol levels for preventing plaque formation is known as statin therapy. DAPT with statin therapy is associated with an improved clinical outcome. Examples of such

combinations include ASP, CLOP Bisulphate and atorvastatin and ASP, CLOP Bisulphate and ROS. ROS is reported to show greater clinical efficacy than atorvastatin and other statins. After a thorough literature survey it is evident that, there are no articles related to simultaneous estimation of Aspirin, Clopidogrel Bisulphate and Rosuvastatin Calcium. Also, there are no articles related to stability indicating method for estimation of Aspirin, Clopidogrel Bisulphate and Rosuvastatin Calcium.

MATERIALS AND METHODS

Reference standard of Clopidogrel Bisulphate (99.9%); Aspirin (99.99%) and Rosuvastatin Calcium (99.99%) were obtained from Alkem Pharmaceuticals; Bliss GVS Pharma Ltd. and Apoteck India, respectively. All chemicals and reagent were of AR grade and were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. The capsules containing 75mg Clopidogrel Bisulphate, 75mg Aspirin and 10mg Rosuvastatin Calcium, ROSUMAC GOLD were procured from local market.

INSTRUMENTATION

Camag HPTLC System (with TLC Scanner, Win CATS Softwar Version 4.0 and Linomat 5 application device) used for the analysis. Precoated silica gel 60 F254 on aluminum sheets (250µm thick) of E-Merck, Germany were used as stationary phase. Pre-washing of plate was done with methanol and then it was activated by keeping in an oven at 110˚C for 10 minutes. The samples were spotted in the form of bands of width 2 mm with a Camag 100 µl sample (Hamilton, Bonded, Switzerland) syringe. A constant application rate of 0.1 µl/s was used and the space between two bands was 5 mm. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm/s. Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. Each chromatogram was developed over a distance of 9 cm. The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm.

PREPARATION OF SOLUTION

Preparation of standard stock solution I

10mg of Rosuvastatin Calcium; Aspirin and Clopidogrel Bisulphate were weighed separately and transferred to three separate 10ml volumetric flasks and dissolved in methanol and diluted up to the mark with methanol to get standard stock solutions of ROS, R1 (1000µg/ml); ASP, A1 (1000µg/ml) and CLOP, C1 (1000µg/ml), respectively.

Preparation of standard stock solution II

7.5 ml of standard of stock solution of C1 (CLOP 1000 µg/ml) was diluted to 10 ml with methanol to get working standard solution C2 (750 µg/ml Clopidogrel Bisulphate).

7.5 ml of standard solution A1 (ASP 1000 µg/ml) was diluted to 10ml with methanol to get standard stock solution A2 (750 µg/ml Aspirin).

1 ml of standard solution R1 (ROS 1000 µg/ml) was diluted to 10ml with methanol to get standard stock solution R2 (100 µg/ml Rosuvastatin Calcium).

Preparation of Working Standard Solution

1 ml of C2 was diluted to 10ml with methanol to get a working standard solution of Clopidogrel Bisulphate C3 (75 µg/ml).

1 ml of A2 was diluted to 10ml with methanol to get a working standard solution of Aspirin A3 (75 µg/ml).

1 ml of R2 was diluted to 10ml with methanol to get a working standard solution of Rosuvastatin Calcium R3 (10 µg/ml).

Preparation of Mixture Standard Solution

1 ml solution of C2 (750 µg/ml CLOP); 1ml solution of A2 (750 µg/ml ASP) and 1 ml solution of R2 (100 µg/ml ROS) were taken in a 10 ml volumetric flask and the volume was made upto the mark with methanol to get a mixed standard solution, M (75 µg/ml ASP; 75 µg/ml CLOP and 10 µg/ml of ROS).

DETECTION OF SUITABLE WAVELENGTH BY UV SPECTROPHOTOMETER

The working standard solution A3 (Aspirin 75 µg/ml); C3 (Clopidogrel Bisulphate 75 µg/ml) and R3 (ROS 10 µg/ml) were scanned in the range of 200 -400 nm using UV-Visible spectrophotometer model Jasco-V-630 to determine the absorbtion maxixa (λ_{max}). The UV absorption spectra are given in following fig 4,5 and 6 respectively.

IN-SITU DETECTION OF WAVELENGTH BY HPTLC

The mixed standard solution M (75 µg/ml ASP; 75 µg/ml CLOP and 10 µg/ml of ROS) was spotted (10µl) on a TLC plate. The plate was dried and then scanned using camag TLC

scanner III with winCATS software version 1.4.4 in the reflectance-absorbance mode between 200-400nm. Given fig 7 is the In-situ spectrum of ASP, CLOP and ROS.

OPTIMISATION OF MOBILE PHASE

The mobile phase containing Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v), with the saturation time of 45 min shows well defined and resolved peak. The wavelength used for detection was 258nm. The retention factors were found to be 0.88±0.2 for CLOP Bisulphate; 0.22±0.2 for ASP and 0.16±0.2 for ROS Calcium. Representative densitogram of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) is shown in fig. 8.

VALIDATION OF METHOD

The developed HPTLC method was validated as per the ICH guidelines Q2 (R1) for linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and specificity.

Linearity

3, 6, 9,12, 15 µl of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) were spotted on TLC plate to get concentration in the range of 225 - 1350 ng/spot of Clopidogerl Bisilphate ; 225 – 1350 ng /spot of Aspirin and 30 – 180 ng/spot of Rosuvastatin Calcium. The plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v). The plates were dried and scanned at 258 nm using Camagwin software version 1.4.4.6337 and densitograms were recorded. The procedure was repeated three times (n = 3) and the calibration curves were developed by plotting peak area v/s concentration.

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

To determine the Limit of detection (LOD) and limit of quantification (LOQ) aliquots of mixed standard solution, M (Lamivudine 30µg/ml and Dolutegravir sodium 5µg/ml) were spotted on TLC plate and plate was run with mobile phase Toluene: Ethyl Acetate: Methanol Ammonia (6:3.5:1.5:0.2 v/v/v/v). The plate was dried and scanned at 258nm using Camagwin software version 1.4.4.6337 and densitograms were recorded and areas were reported. LOD and LOQ were calculated.

Precision

The precision of the method was verified by intraday precision and inter day precision studies. To evaluate intraday precision, three spots of 6 µl, 12 µl, and 15 µl of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) were spotted on a TLC plate. The plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v), dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded. This procedure was repeated three times on a same day (n = 3). To evaluate interday precision, three spots of 6 µl, 12 µl, and 15 µl of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) were spotted on a TLC plate. The plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v), dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded. This procedure was repeated three times on three different days (n = 3). The AUC were reported. The precision of the method was evaluated by %RSD. The precision of method was evaluated by %RSD.

Repeatability of measurement and sample application

Repeatability was assessed by spotting 9µl of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) on a TLC Plate. The plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v), dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded. The above procedure was repeatecd six times (n = 6). The AUC were reported. The repeatability of method was evaluated by %RSD. (Table.16, 17 and 18).

Robustness

Robustness studies were done by making small, deliberate changes in optimized condition like mobile phase composition; saturation time and development distance. By applying concentration of 9µl of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) on a TLC Plate. The plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v), dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded and AUC and Rf were reported. The %RSD of peak area was calculated for each parameter. (Table. 19,20 and 21).

Recovery study

Accuracy of the method was determined by applying the method to marketed formulation to which known amount of standard API corresponding to 80, 100 and 120% of label claim had

been added (standard addition method). The contents of 1 capsule were dissolved in methanol and diluted suitably to get a concentration equivalent to mixed standard solution. Percentage recovery was calculated.

Assay

To determine the content of Clopidogrel Bisulphate; Aspirin and Rosuvastatin Calcium in tablets (containing 75mg Clopidogrel Bisulphate; 75mg Aspirin and 10mg Rosuvastatin Calcium), contents of 20 Capsules were weighed accurately and average weight was calculated. The amount equivalent to 1 capsule was dissolved and diluted in methanol to obtain a concentration equivalent 10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP. 9µl spots of the prepared solution and of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) were spotted on the TLC plate. The plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v), dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded and AUC and Rf were reported. The drug content was calculated and the possibility of excipient interference with analysis was examined. (Fig 17 and 18).

FORCED DEGRADATION STUDIES

In order to develop a SIM for simultaneous determination of ASP, CLOP Bisulphate and ROS Calcium Forced Degradation studies were carried out according to ICH guidelines.

Preparation of acid induced degradation product

A mixture of 5 ml mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and 5 ml of 0.1N HCl was heated for 2hr at 60˚C. The resultant solution was cooled at room temperature. Spots of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and resultant degraded solution was applied on TLC plates with spot concentration equivalent to 675ng/spot of Clopidogrel Bisulphate; 675ng/spot of Aspirin 90ng/spot of Rosuvastatin Calcium and the plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v). The plates were dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded.

Preparation of base induced degradation product

A mixture of 5 ml mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and 5 ml of 0.1N NaOH was heated for 2hr at 60˚C. The resultant solution was cooled at room temperature. Spots of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml

ASP; 75 µg/ml CLOP) and resultant degraded solution was applied on TLC plates with spot concentration equivalent to 675ng/spot of Clopidogrel Bisulphate; 675ng/spot of Aspirin 90ng/spot of Rosuvastatin Calcium and the plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v). The plates were dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded.

Preparation of hydrogen-peroxide induced degradation product

A mixture of 5 ml mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and 5 ml of 3% Hydrogen Peroxide solution was heated for 30 min at 60˚C. The resultant solution was cooled at room temperature. Spots of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and resultant degraded solution was applied on TLC plates with spot concentration equivalent to 675ng/spot of Clopidogrel Bisulphate; 675ng/spot of Aspirin 90ng/spot of Rosuvastatin Calcium and the plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v). The plates were dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded.

Preparation of Thermal induced degradation product

750mg of Clopidogerl Bisulphate; 750mg of Aspirin and 100mg Rosuvastain Calcium were mixed in a petri plate and kept in a Hot Air Oven at 60^{O}C for 2hrs. Powdered samples of 10mg were withdrawn after 30min, 1hr, 2hr, 3hr and 4hr intervals. The degradation sample was cooled at room temperature and was subjected to analysis after suitable dilutions to make spot concentration equivalent to Mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and the plate was run with mobile phase Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v). The plates were dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded.

Preparation of Neutral induced degradation product

A mixture of 5 ml mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and 5 ml of distilled water was heated for 30 min at 60˚C. The resultant solution was cooled at room temperature. Spots of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) and resultant degraded solution was applied on TLC plates with spot concentration equivalent to 675ng/spot of Clopidogrel Bisulphate; 675ng/spot of Aspirin 90ng/spot of Rosuvastatin Calcium and the plate was run with mobile phase Toluene: Ethyl

Acetate: Methanol: Ammonia (6:3.5:1.5:0.2 v/v/v/v). The plates were dried and scanned at 258 nm using Camagwin softare versiom 1.4.4.6337 and densitograms were recorded.

RESULT AND DISCUSSION

VALIDATION OF THE METHOD

Linearity

The linear regression data for the calibration curves (n=3) showed good linear relationship over the concentration range 30 – 180 ng/spot for ROS Calcium (r^2=0.9992). The residual plot shows random and equal distribution of residues. The data was subjected to regression analysis. Values of significance and correlation coefficient were confirmed by linearity in the respective concentration ranges.

The linear regression data for the calibration curves (n=3) showed good linear relationship over the concentration range of 225 - 1350 ng/spot for Aspirin (r^2=0.9999). The residual plot shows random and equal distribution of residues. The data was subjected to regression analysis. Values of significance and correlation coefficient were confirmed by linearity in the respective concentration ranges.

The linear regression data for the calibration curves (n=3) showed good linear relationship over the concentration range of 225 - 1350 ng/spot for CLOP Bisilphate (r^2=0.9991). The residual plot shows random and equal distribution of residues. The data was subjected to regression analysis. Values of significance and correlation coefficient were confirmed by linearity in the respective concentration ranges. The results were summarised in table no1-3 and fig no 5-7.

Precision

The Precision of the method was verified by inter day and intraday precision with nine replicate analysis of mixture standard solution. The developed method was found to be precise as the RSD values for intraday and inter-day precision studies were <2% as recommended by ICH guidelines.

Intra-day precision

For Intraday precision (n=3) %RSD were found to be 0.03%-0.2% for ASP; 0.01%-0.02% for CLOP and 0.04%-0.06% for ROS. %RSD was <2% which is in the acceptable range and results were given in the table 5-7.

Inter-day precision details

For Inter day precision (n=3) %RSD were found to be 0.03%-0.2% for ASP; 0.01%-0.06% for CLOP and 0.03%-0.09% for ROS. %RSD was <2% which is in the acceptable range and results were given in the table 8-10.

LOD and LOQ

LOD and LOQ of Rosuvastatin Calcium were found to be **0.207ng** and **6.27 ng**.

LOD and LOQ of Aspirin were found to be **1.671ng** and **50.65ng**.

LOD and LOQ of Clopidogrel bisulphate were found to be **2.211ng** and **67.01ng** given in table 4.

Repeatability

Repeatability of the method was checked by analysing of mixed standard solution, M (10 µg/ml ROS; 75 µg/ml ASP; 75 µg/ml CLOP) after application of 9µl spot on TLC Plate (n=6) and RSD and %RSD were found. %RSD was <2% which is in the acceptable range and results were given in the table 11-13.

Robustness

Robustness studies were done by making small, deliberate changes in optimized condition like mobile phase composition; saturation time and wavelength. %RSD was found to be <2% which is within the accepted range. The results of robustness studies are given in the following table 14-16.

Recovery

The % recovery was calculated and found to be **98.86-101.15%** for ROS and **99.27– 100.4%** for ASP and **98.2%-99.17%** for CLOP. The %recovery values obtained are given in the table 17-19.

Assay

Three peaks were observed at Rf of 0.16; 0.22; and 0.88 in the chromatogram of the drug Rosuvastatin calcium; Aspirin and Clopidogrel Bisulphate respectively from conventional tablets. There is no interference from the excipients commonly present in the conventional tablets. The drug content was found to be 99.48% for ASP; 100.96%for CLOP and 98.57% ROS given in table no 20, Fig No. 8-9.

Forced Degradation Studies

In order to develop SIM for simultaneous estimation of Lamivudine and Dolutegravir sodium forced degradation studies were carried out according to ICH guidelines.results shown in fig no 10-15, Table no 21-25.

Acid Degradation

The densitogram of acid degradation study (Fig.3.15) shows peaks for Rosuvastatin (90ng/spot); Aspirin (675 ng/spot) and Clopidogrel (675ng/spot) at R*f* 0.16; 0.22 and 0.89 respectively**.** On comparison it showed additional peaks in chromatogram at Rf 0.39 and 0.59, which are well separated from principle drug peaks Fig 3.15 and Table 3.13. There was **7.13%** degradation of Rosuvastatin Calcium **9.65%** degradation of Aspirin and **10.1%** of Clopidogrel Bisulphate sodium with well resolved peak.

Base Degradation

The densitogram of base degradation study (Fig.3.16) shows peaks for Rosuvastatin (90ng/spot); Aspirin (675 ng/spot) and Clopidogrel (675ng/spot) at R*f* 0.16; 0.22 and 0.88 respectively**.** On comparison it showed additional peak in chromatogram which at Rf of 0.75 which is well separated from principle drug peaks. Fig 3.16 and Table 3.14 There was **6.36%** degradation of Rosuvastatin Calcium; **7.72%** degradation of Aspirin and **6.12%** of Clopidogrel Bisulphate sodium with well resolved peak.

Oxidative Degradation

The densitogram of acid degradation study (Fig.3.17) shows peaks for Rosuvastatin (90ng/spot); Aspirin (675 ng/spot) and Clopidogrel (675ng/spot) at R*f* 0.16; 0.22 and 0.88 respectively**.** On comparison it showed additional peak in chromatogram at Rf of 0.72 and 0.78 which are well separated from principle drug peaks Fig 3.17 and Table 3.15. There was **9.47%** degradation of Rosuvastatin Calcium and **6.62%** of Clopidogrel Bisulphate sodium and **12.08%** degradation of Aspirin with well resolved peak.

Thermal Degradation

The densitogram of base degradation study (Fig.3.18) shows peaks for Rosuvastatin (90ng/spot); Aspirin (675 ng/spot) and Clopidogrel (675ng/spot) at R*f* 0.16; 0.22 and 0.88 respectively On comparison it showed additional peak at Rf of 0.42, 0.54, 0.55 and 0.58 which are well separated from principle drug peaks Fig 3.18 and table 3.16. There was

8.83% degradation of Rosuvastatin Calcium and **7.34%** of Clopidogrel Bisulphate sodium and **9.12%** degradation of Aspirin with well resolved peak.

Neutral Degradation

The densitogram of neutral degradation study (Fig.3.19) shows peaks for Rosuvastatin (90ng/spot); Aspirin (675 ng/spot) and Clopidogrel (675ng/spot) at R*f* 0.16; 0.22 and 0.89 respectively**.** On comparison it showed additional peaks in chromatogram at Rf 0.39 and 0.59, which are well separated from principle drug peaks Fig 3.15 and Table 3.13. There was **6.11%** degradation of Rosuvastatin Calcium **10.54%** degradation of Aspirin and **14.78%** of Clopidogrel Bisulphate sodium with well resolved peak.

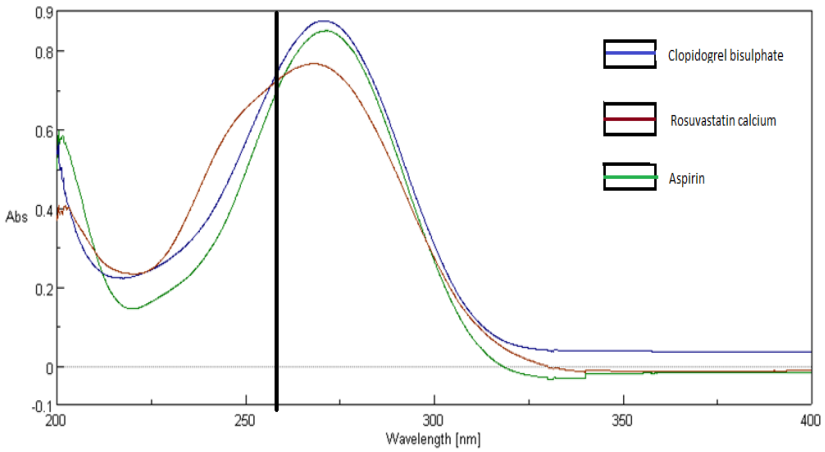

Fig 4: Overlay Spectra of Clopidogrel Bisulphate; Aspirin and Rosuvastatin Calcium.

VALIDATION OF THE METHOD

Linearity

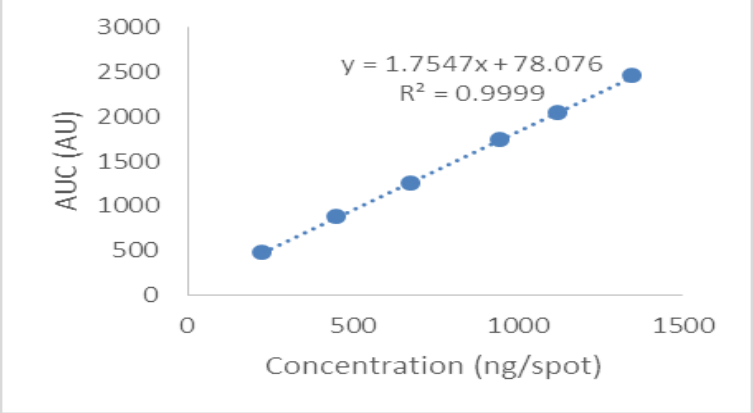

Fig 5: Linearity Graph of Aspirin.

Table 1: Regression details of Aspirin.

Parameter	Range
Linearity Range (ng/spot)	225 – 1350
r^2	0.9999
Slope	1.7547
Intercept	78.076

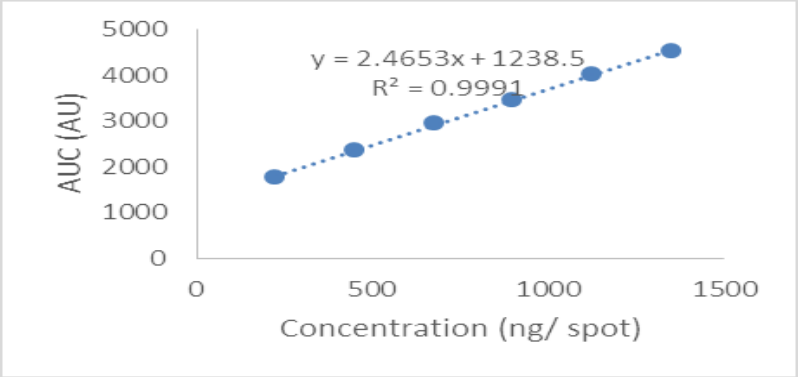

Fig 6: Linearity Graph of Clopidogrel Bisulphate.

Table 2 Regression details of Clopidogrel Bisulphate.

Parameter	Range
Linearity Range (ng/spot)	225 – 1350
r^2	0.9991
Slope	2.4653
Intercept	1238.5

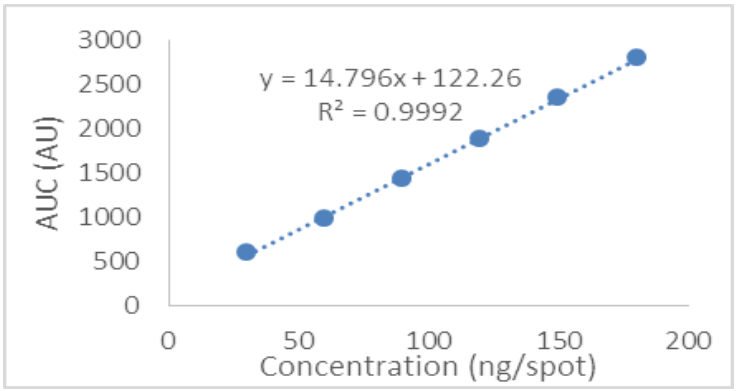

Fig 7: Linearity Graph of Rosuvastatin Calcium.

Table 3: Regression details of Rosuvastatin Calcium.

Parameter	Range
Linearity Range (ng/spot)	10 – 180
r^2	0.992
Slope	14.796
Intercept	122.26

LOD AND LOQ

Table 4: LOD and LOQ details of ASP, CLOP and ROS.

Drug	LOD (ng/spot)	LOQ (ng/spot)
Rosuvastatin Calcium	0.206	6.27
Aspirin	1.167	50.65
Clopidogrel Bisulphate	2.211	67.01

PRECISION

INTER-DAY PRECISION DETAILS

Table 5 Intra-day precision details of ASP.

Concentration (ng/spot)	Std. Deviation	%RSD
675	5.1643	0.41
900	0.611	0.03
1125	1.4106	0.06

Table 6: Intra-day precision details of CLOP.

Concentration (ng/spot)	Std. Deviation	%RSD
675	0.98657	0.03
900	2.2722	0.06
1125	0.75045	0.01

Table 7: Intra-day precision details of ROS.

Concentration (ng/spot)	Std. Deviation	%RSD
90	0.9073	0.06
120	0.7023	0.03
150	2.2715	0.09

INTER DAY PRECISION

Table 8: Inter-day precision details of Aspirin.

Concentration (ng/spot)	Std. Deviation	%RSD
675	2.29194	0.2
900	0.5686	0.03
1125	0.9073	0.04

Table 9: Inter-day precision details of Clopidogrel Bisulphate.

Concentration (ng/spot)	Std. Deviation	%RSD
675	0.611	0.02
900	0.7023	0.02
1125	0.5	0.01

Table 10: Inter-day precision details of Rosuvastatin Calcium.

Concentration (ng/spot)	Std. Deviation	%RSD
90	0.8888	0.06
120	0.8185	0.04
150	1.1060	0.04

REPEATABILITY

Table 11: Repeatability study of Aspirin.

Sr. No.	Concentration (ng/spot)	AUC	Avg. AUC	Std. Deviation	%RSD
1	675	1254.9	1255.25	0.582237	0.04
2	675	1255.6			
3	675	1254.7			
4	675	1254.6			
5	675	1255.8			
6	675	1255.9			

Table 12: Repeatability study of Clopidogrel Bisulphate.

Sr. No.	Concentration (ng/spot)	AUC	Avg. AUC	Std. Deviation	%RSD
1	675	2956.2	2956.15	0.350714	0.01
2	675	2955.9			
3	675	2955.8			
4	675	2956.1			
5	675	2956.1			
6	675	2956.8			

Table 13: Repeatability study of Rosuvastatin Calcium.

Sr. No.	Concentration (ng/spot)	AUC	Avg. AUC	Std. Deviation	%RSD
1	90	1427.6	1428.26	0.4219	0.029
2	90	1428.1			
3	90	1427.9			
4	90	1428.4			
5	90	1428.6			
6	90	1428.6			

ROBUSTNESS

Table 14: Robustness details of Aspirin.

Condition	Rf	Peak Area	%RSD
Mobile Phase Composition (±0.2 ethyl acetate)			
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.3:1.5:0.2)	0.21	1252.6	0.125
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2)	0.21	1255.6	
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.7:1.5:0.2)	0.22	1254.9	
Detection Wavelength (±5 nm)			

251 nm	0.22	1255.5	0.71
256 nm	0.21	1253.7	
261 nm	0.2	1254.6	
Duration of Saturation (±10min)			
60	0.22	1255.2	0.06
50	0.23	1254.8	
40	0.22	1256.4	

Table 15: Robustness details of Clopidogrel Bisulphate.

Condition	Rf	Peak Area	%RSD
Mobile Phase Composition (±0.2 ethyl acetate)			
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.3:1.5:0.2)	0.9	2936.2	0.02
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2)	0.88	2937.8	
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.7:1.5:0.3)	0.88	2936.6	
Detection Wavelength (±5nm)			
251 nm	0.88	2938.4	0.01
256 nm	0.91	2937.5	
261 nm	0.92	2937.3	
Duration of Saturation (±10min)			
60	0.88	2937.1	0.16
50	0.88	2939	
40	0.89	2930	

Table 16: Robustness details of ROS Calcium.

Condition	Rf	Peak Area	%RSD
Mobile Phase Composition (±0.2 ethyl acetate)			
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.3:1.5:0.2)	0.14	1428.8	0.03
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.2)	0.16	1429.5	
Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.7:1.5:0.3)	0.16	1428.6	
Detection Wavelength (±5nm)			
251 nm	0.16	1428.9	0.41
256 nm	0.16	1428.8	
261 nm	0.15	1429.8	
Duration of Saturation (±10min)			
60	0.16	1428.6	0.03
50	0.16	1428.5	
40	0.16	1429.3	

RECOVERY

Table 17: Recovery study of Aspirin.

Label Claim	% level	Initial Amount	Total amount added	%Recovery	%RSD
Aspirin (10mg)	80	75 mg	60 mg	99.27	0.01
	100	75 mg	75 mg	99.55	0.03
	120	75 mg	90 mg	100.4	0.08

Table 18: Recovery study of Clopidogrel Bisulphate.

Label Claim	% level	Initial Amount	Total amount added	%Recovery	%RSD
Clopidogrel Bisulphate (75mg)	80	75 mg	60 mg	98.2	0.4
	100	75 mg	75 mg	99.9	0.1
	120	75 mg	90 mg	99.17	0.03

Table 19: Recovery study of Rosuvastatin Calcium.

Label Claim	% level	Initial Amount	Total amount added	%Recovery	%RSD
Rosuvastatin calcium (10mg)	80	10 mg	8 mg	98.86	0.1
	100	10 mg	10 mg	99.23	0.06
	120	10 mg	12 mg	100.15	0.07

ASSAY

Table 20: Assay studies of Rosuvastatin calcium; Aspirin and Clopidogrel Bisulphate.

Drug	Rf	Drug Content(%)	Mean Drug Content
Rosuvastatin Calcium	0.16	98.12	98.57%
		99.04	
		98.56	
Aspirin	0.22	99.41	99.48
		99.35	
		99.68	
Clopidogrel Bisulphate	0.88	100.6	100.96%
		101.2	
		101.1	

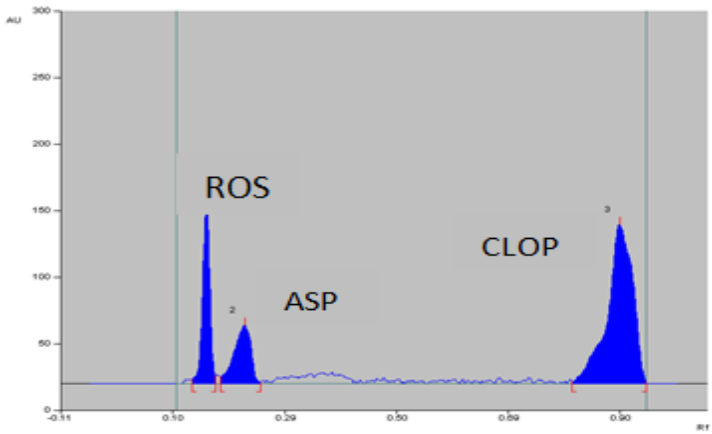

Fig 8: Representative densitogram of ROSUMAC Gold (Marketed Formulation).

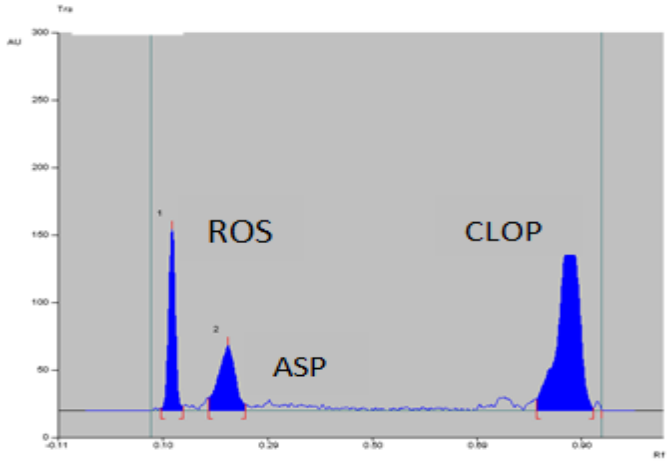

Fig 9: Representative densitogram of standard Bisulphate; Aspirin and Rosuvastatin Calcium.

FORCE DEGRADATION STUDY

Table 21 Details of Acid Degradation Study

	Rf
Standard Rosuvastatin Calcium	0.16
Standard Clopidogrel Bisulphate	0.88
Standard Aspirin	0.24
Degradation Product	0.39
Degradation Product	0.59

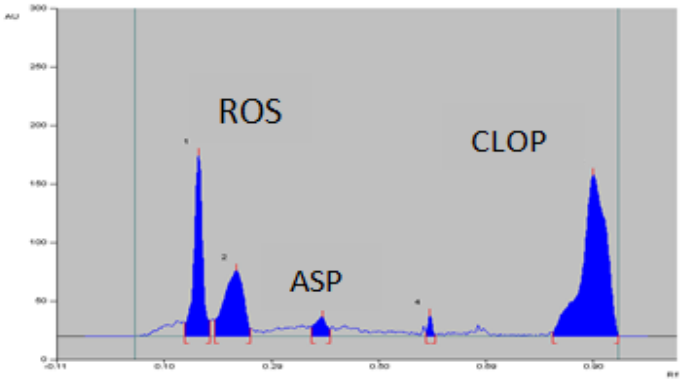

Fig 10: Acid Degradation of Clopidogrel Bisulphate; Aspirin and Rosuvastatin Calcium.

Table 22 Details of Base Degradation Study.

	Rf
Standard Rosuvastatin Calcium	0.16
Standard Aspirin	0.22
Standard Clopidogrel Bisulphate	0.88
Degradation Product	0.75

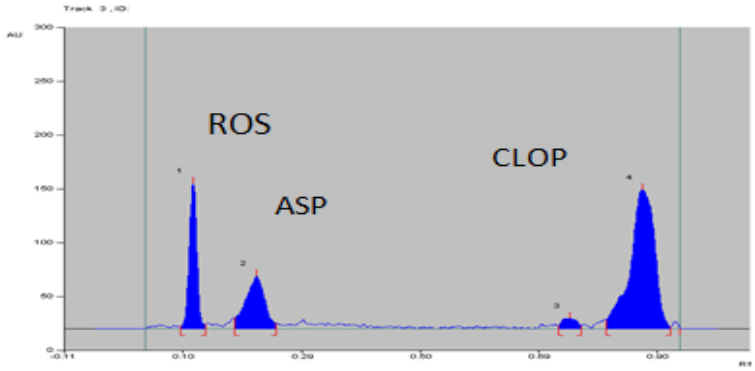

Fig 11: Base Degradation of Clopidogrel Bisulphate; Aspirin and Rosuvastatin Calcium.

Table 23: Details of oxidative degradation study.

	Rf
Standard Rosuvastatin Calcium	0.16
Standard Aspirin	0.22
Standard Clopidogrel Bisulphate	0.88
Degradation Product	0.72
Degradation Product	0.78
Degradation Product	0.78

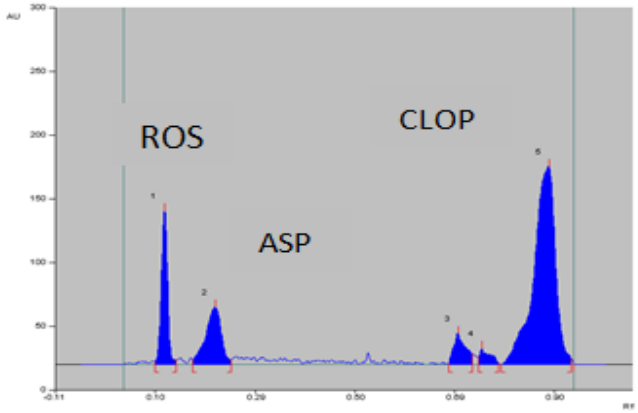

Fig 24 Oxidative Degradation of Clopidogrel Bisulphate; Aspirin and Rosuvastatin Calcium.

Table 24: Details of thermal degradation study.

	Rf
Standard Rosuvastatin Calcium	0.16
Standard Aspirin	0.22
Standard Clopidogrel Bisulphate	0.88
Degradation Product	0.42
Degradation Product	0.54
Degradation Product	0.55
Degradation Product	0.68

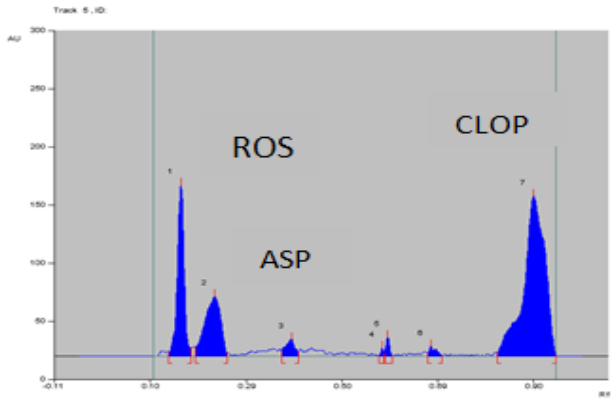

Fig 13: Thermal Degradation of Clopidogrel Bisulphate; Aspirin and Rosuvastatin Calcium.

Table 25: Details of neutral degradation study.

	Rf
Standard Rosuvastatin Calcium	0.16
Standard Clopidogrel Bisulphate	0.88
Standard Aspirin	0.24
Degradation Product	0.39
Degradation Product	0.59

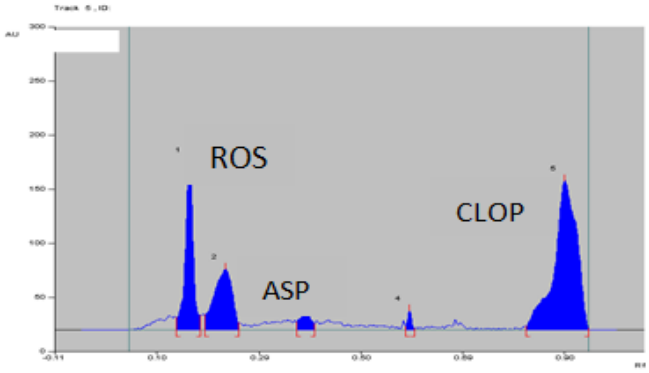

Fig 14: Neutral Degradation of Clopidogrel Bisulphate; Aspirin and Rosuvastatin Calcium.

CONCLUSION

A simple, specific, precise and accurate HPTLC method has been developed for quantitative determination of rosuvastatin calcium, aspirin and clopidogrel bisulphate in bulk drug and in capsule formulation. The separation was achieved using Silica gel pre-coated Aluminium plate 60F254 (10X10cm) with 250µm thickness as a stationery phase and using Toluene: Ethyl Acetate: Methanol: Ammonia (6:3.5:1.5:0.1 v/v/v/v) as a mobile phase. The developed HPTLC method was validated based on ICH guidelines. Statistical analysis proves that the

method is reproducible for the analysis of rosuvastatin calcium, aspirin and clopidogrel bisulphate as bulk drug and in pharmaceutical formulations without any interference from the excipients. In this study, intrinsic stability of rosuvastatin calcium, aspirin and clopidogrel bisulphate was established using various ICH recommended stress conditions. The drug was found to be degraded in acid, base, oxidative, neutral and thermal conditions. As the method could effectively separate principle drug peak from degradation product peaks, it can be employed as a stability indicating method. The advantages of the proposed methods involve a simple procedure for sample preparation and relatively short time of analysis. The proposed HPTLC method is suitable for the analysis of rosuvastatin calcium, aspirin and clopidogrel bisulphate in commercial capsules.

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