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VALIDATION OF DEVELOPED METHOD BY RP-HPLC FOR ESTIMATION OF PRASUGRELIN HUMAN PLASMA AND STUDYING THE STABILITY OF THE DRUGS IN PLASMA

[1]Ashutosh Kumar S.*, [2]Manidipa Debnath, [3]Seshagiri Rao J. V. L. N., [4]Gowri Sankar D.
[1]Department of Pharmacy, Tripura University, (A Central University), Suryamaninagar, Tripura West, Agartala, Tripura, 799022
[2]Inspecting Officer (Drugs), O/o, The Deputy Drugs Controller, Pandit Nehru Complex, Gourkhabasti, Agartala, Tripura, 799006.
[3]Prof. of Pharmaceutical Analysis and Quality Assurance, Srinivasarao College of Pharmacy, Pothinamallayyapalem, Madhurawada, Visakhapatnam, 530041, A.P
[4]Prof. of Pharmaceutical Analysis, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, 530003, A.P

*****Corresponding author's email: ashu.mpharm2007@gmail.com
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ABSTRACT
This paper is concern with a reverse phase high performance liquid chromatography (RP-HPLC) bio-analytical method development and validation for Prasugrel in human plasma using photo diode array detector (PDA detector). The HPLC separation was carried out in an isocratic mode on an X-Terra C_{18} column (4.6 x 150 mm; 5 µm) with a mobile phase consisting of potassium dihydrogen phosphate [pH 3.0] and acetonitrile in the ratio of 30:70 v/v at a flow rate of 1.0 mL/min. The run time was maintained for 5 mins and the detection was monitored at 210 nm. The percentage recovery was found 99.61-100.06 in human plasma. This reveals that the method is quite accurate. The linearity was found 15-40 µg/mL in human plasma. The inter-day and intra-day precision in plasma was found within the limits. The lower limit of quantification (LLOQ) obtained by the proposed method was 0.05 µg/mL. The percentage relative standard deviation (%RSD) obtained for the drug spiked in plasma for stability studies were less than 2 %.

Keywords: High performance liquid chromatography; Potassium dihydrogen phosphate, Acetonitrile, Prasugrel, Accuracy, Lower limit of quantification, Plasma.

INTRODUCTION
Prasugrel is an effective third-generation oral thienopyridine which reduces the tendency for clotting [1]. "It blocks a specific receptor on the platelet surface, which may results in clogged arteries and may lead to heart attack" [2]. "It is effectively preventing ischemic events in patients with acute coronary syndrome undergoing percutaneous coronary intervention, which increases in bleeding and improved net clinical outcome" [3]. It inhibits adenosine diphosphate–induced platelet aggregation more rapidly and consistently in healthy volunteers. "It is most effective in individuals, although there have been several case reports of decreased responsiveness to Prasugrel" [4]. Several analytical methods based on UV [5], RP-HPLC [6-9] and HPTLC [10] were reported for the estimation of Prasugrel. Although literature survey reveals that no methods were reported for estimation of Prasugrel in human plasma. The chemical structure for Prasugrel is represented in figure 1.

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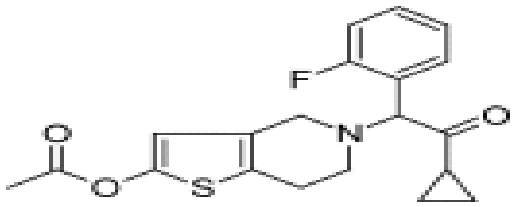

Figure 1. Chemical structure of Prasugrel

MATERIALS AND METHOD [11-12]

Chemicals and reagents used: The reference samples of Prasugrel were supplied by M/s Pharma Train, Hyderabad**.** HPLC grade water was procured from Standard Reagents, Hyderabad, Telagana. HPLC grade acetonitrile was purchased from Merck, Mumbai, Maharashtra. The chemicals used for preparation of buffer includes potassium dihydrogen phosphate (Finar Chemicals, Ahmedabad, Gujarat), and orthophosphoric acid (Standard Reagents, Hyderabad, Telagana).

0.45 µ membrane filters (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) were used for filtration of various solvents and solutions intended for injection into the column.

Instrumentation: A Waters Alliance 2695 separation module equipped with a 2487 UV detector was employed throughout this study. Column that was employed in the method was X-Terra C_{18} (4.6 x 150 mm, 3.5 µm, Make: ACE). The samples were injected with an automatic injector. The 20 µL volume of sample was injected. The input and output operations of the chromatographic system were monitored by Waters Empower software. The flow rate selected was 1.0 mL per min. The detection was done at 210 nm. The temperature and run time was monitored at 25°C and 8.0 min respectively.

The ultra violet spectra of the drug used for the investigation was taken on a Lab India UV 3000 spectrophotometer for finding out their λ_{max} values.

Solubility of the compounds was enhanced by sonication on an ultra sonicator (Model: Power Sonic 510, Hwashin Technology).

All the weighings in the experiments were done with an Afcoset electronic balance. The Herm Le microlitre centrifuge Z100 (model no 292 P01) was used for the centrifugation process and Remi equipments (Model no- CM101DX) Cyclomixer was used.

Glassware: All the volumetric glassware used in the study was of Grade A quality Borosil.

Preparation of potassium dihydrogen phosphate (pH 3.0): The buffer solution was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in 900 mL of HPLC grade water in a 1000 mL clean and dry flask. The mixture was stirred well until complete dissolution of the salt. Further 100 mL of water was added the pH was adjusted to 3.0 with 1 % ortho phosphoric acid.

Preparation of mobile phase: The mobile phase was prepared by mixing 300 mL phosphate buffer (pH 3.0), and 700 mL of acetonitrile in a 1000 mL clean and dry flask. The mixture was degassed in an ultra

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sonicator for 5 min and the resultant mobile phase was filtered through a 0.45 µ membrane filter (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) under vacuum.

Preparation of diluent: The diluent was prepared by mixing sodium phosphate buffer (pH 3.0) and acetonitrile (HPLC grade) in the ratio of 30:70 v/v. This solution was used for diluting the drug solutions in the study.

Preparation of standard solution of Prasugrel: 10 mg Prasugrel was weighed accurately and transferred to a 100 mL clean and dry volumetric flask. Initially, the drug was mixed with 70 mL of diluent. The solution was sonicated for 15 min for complete dissolution of the drug. The final volume was made up to the mark. From the above prepared stock solution 0.3 mL solution was pipetted out and transferred to a 10 mL volumetric flask and it was diluted up to the mark. The resultant solution was mixed well and then it was filtered through a 0.45 µm filter. This stock solution contains 30 µg/mL of Prasugrel.

Spiking of Prasugrel to plasma and its extraction from plasma (By precipitation method): From the above prepared stock solution (30 µg/mL of Prasugrel), 0.5 mL was pipetted out and spiked into 0.5 mL of plasma in a polypropylene tubes (Tarson's). Then all the tubes were cyclo mixed for 5 min. Then 1.0 mL of acetonitrile was added to the tube and centrifuged for 20 min at 3000 rpm. Further the supernatant liquids were collected in another Eppendorf tube and 20 µL supernatant was injected into the analytical column.

VALIDATION DEVELOPMENT [13-18]

1. **Selectivity:** No interference was observed at the retention time of Prasugrel extracted from plasma. A typical chromatogram of Prasugrel in plasma is shown in Figure2.

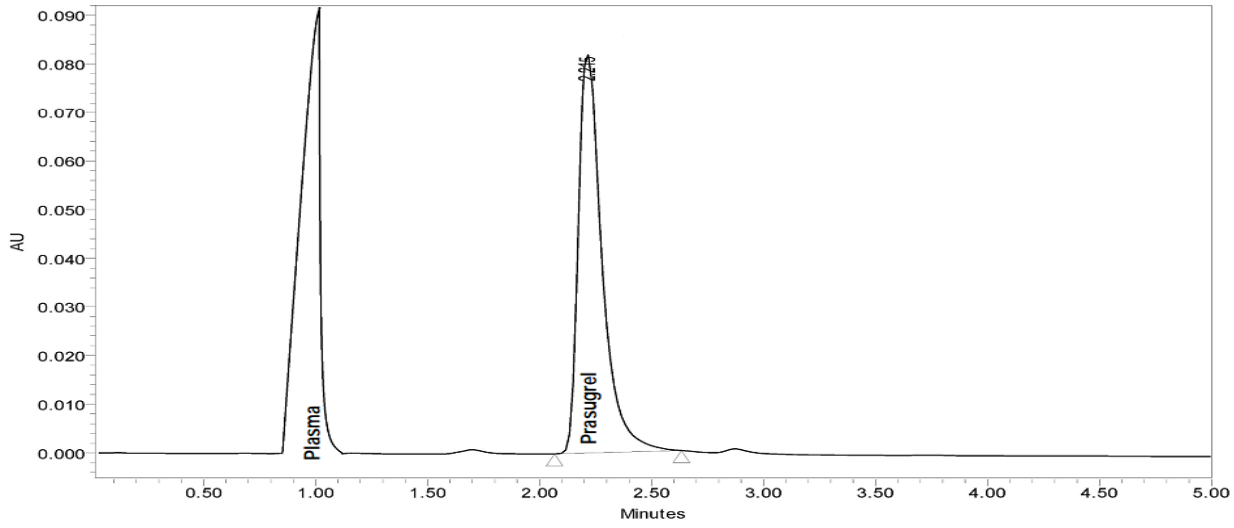

Figure 2. A typical chromatogram of Prasugrel standard drug in plasma

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2. **Sensitivity:** The LLOQ obtained by the proposed method was 0.05 µg/mL for Prasugrel.

3. **Precision:** The precision of the proposed method i.e. the intra and inter-day variations in the peak areas of the drug solutions in plasma were calculated in terms of percent RSD and the results are represented in table 1 and 2. A statistical evaluation revealed that the relative standard deviation of the drug at linearity level for 6 injections was less than 2.0. Typical chromatogram of Prasugrel in plasma for intra and inter-day precision are shown in figure 3 and 4.

Table 1. Intra-day precision of the proposed method for Prasugrel in plasma

Injection	Retention Time	Peak area
Injection-1	2.216	616156
Injection-2	2.215	616196
Injection-3	2.214	616187
Injection-4	2.215	616198
Injection-5	2.216	616124
Injection-6	2.215	616184
Average	2.215	616174.17
Standard Deviation	0.001	28.82
%RSD	0.03	0.005

Table 2. Inter-day precision of the proposed method for Prasugrel (on three consecutive days n = 6) in plasma

Days	Retention Time	Peak area
Day-1*	2.215	616141
Day -2*	2.216	616099
Day -3*	2.215	616289
Average	2.215	616176.3
Standard Deviation	0.0006	99.08
%RSD	0.03	0.02

***Average of Six injections**

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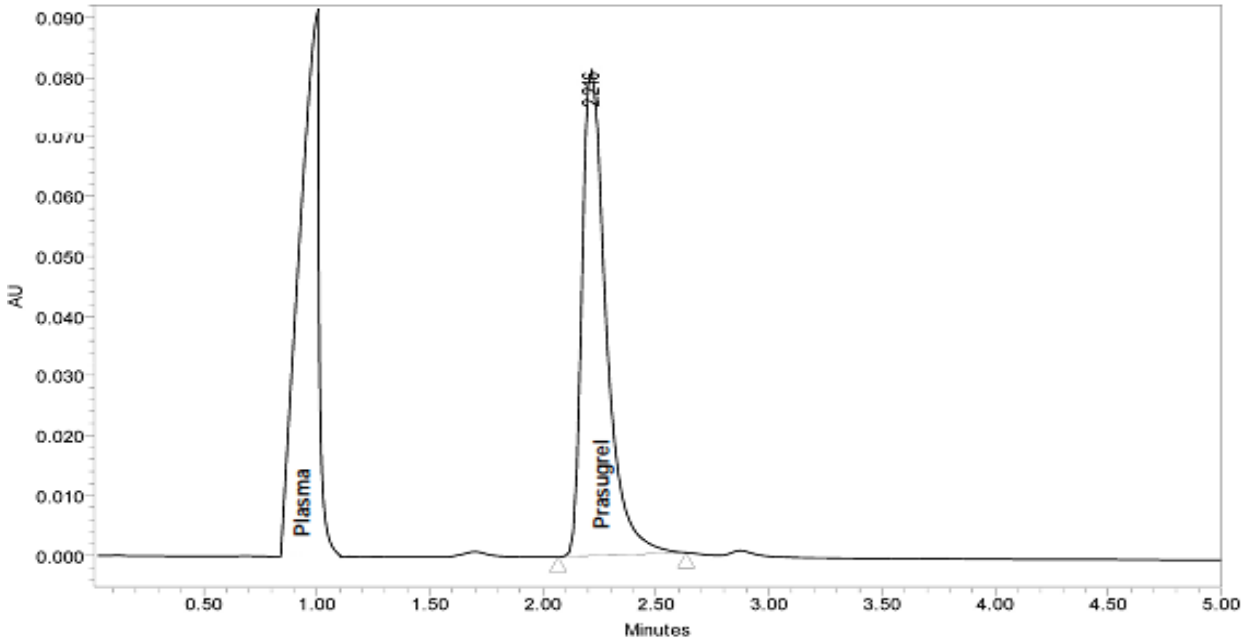

Figure 3. Typical chromatogram of Prasugrel in plasma for intra-day precision

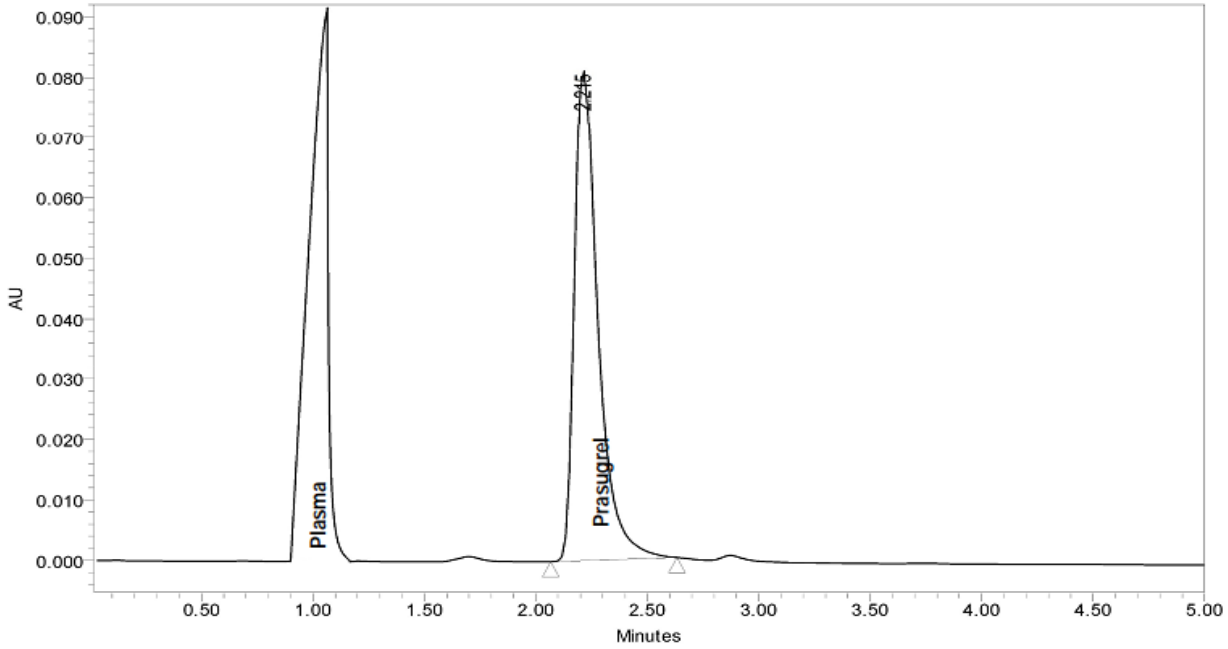

Figure 4. Typical chromatogram of Prasugrel in plasma for inter-day precision

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4. **Accuracy:** The drug solutions were diluted at standard concentration (30.0 µg/mL of Prasugrel) with (8.0, 10.0, 12.0 mg of Prasugrel) of pure drug. Then each dilution was injected thrice (n=3). The percent recoveries of the drug were determined. The results are shown in table 3.

Table 3. Accuracy data of the proposed method for Prasugrel in plasma

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered	SD	% RSD
80 %	99.71	99.61	7.98	0.010	0.13
	99.61		7.97		
	99.52		7.96		
100 %	99.62	100.06	9.96	0.064	0.64
	99.79		9.98		
	100.76		10.08		
120 %	99.72	99.97	11.97	0.055	0.46
	100.52		12.06		
	99.67		11.96		

5. **Linearity:** The relevant regression equation was found y = 20539 x (r^2=1) (where y is the peak area and x is the concentration of Prasugrel (µg/mL)). The slope, intercept and the correlation coefficient of the plot are shown in table 4. The linearity ranges for Prasugrel and its corresponding graph is shown in figure5.

Table 4. Linearity range of Prasugrel in plasma

Concentration (µg/mL)	Area of the peak	Statistical analysis
15	308082	Slope=20539x Intercept= 0 Correlation coefficient=1
20	410776	
25	513470	
30	616164	
35	718858	
40	821552	

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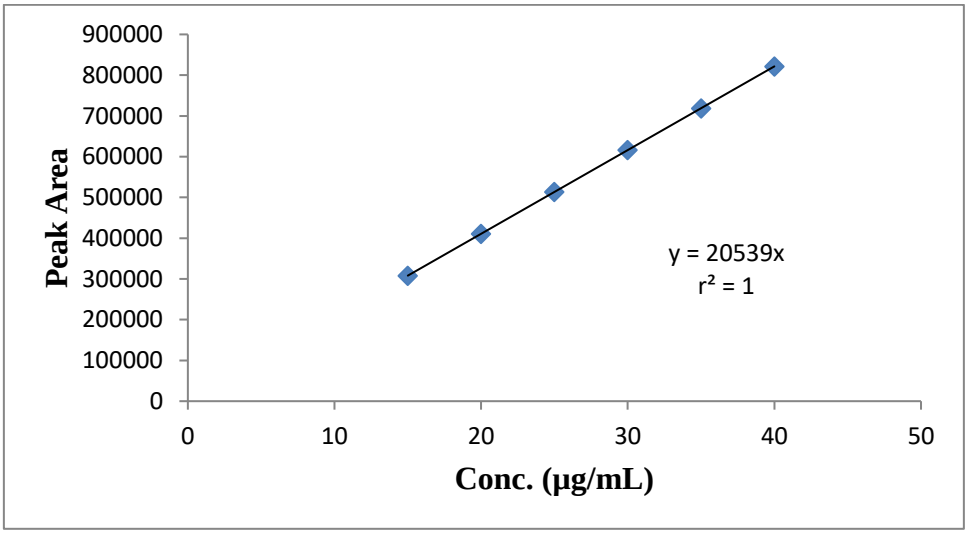

Figure 5. Calibration curve for Prasugrel

6. **Stability [19]:** The stock solutions of the analyte for stability evaluation were prepared in an appropriate solvent at known concentrations. To test the stability of the drug extract, it was subjected to

   a) Freeze and thaw stability at -20ºC ± 2ºC,
   b) Short term stability for period of 24 hours stored at room temperature,
   c) Long term stability for period of 15 days stored at 4ºC.

Similar to the preparation of the standard preparation, the above samples were spiked into the plasma and extracted and collected in vial and injected into HPLC system. All the stability samples compared against the standard stock solution assessed for stability. The results are presented in table 5 (the figures in the table are in peak area units). Typical chromatograms for standard samples, freeze and thaw stability samples, short term stability samples and long term stability samples were represented in Figure 6, 7, 8 and 9.

Table 5. The stability data for Prasugrel in plasma

Sr. No.	Standard Sample	Freeze and Thaw Stability Sample	Short Term Stability Sample	Long Term Stability Sample
1.	616162	608154	590526	594128
2.	616120	600198	599972	584157
3.	616189	605108	599054	584587
Mean	616157	604487	596517	587624
SD	35	4014	5209	5637
% RSD	0.01	0.66	0.87	0.96
Assay		98.11	96.81	95.37

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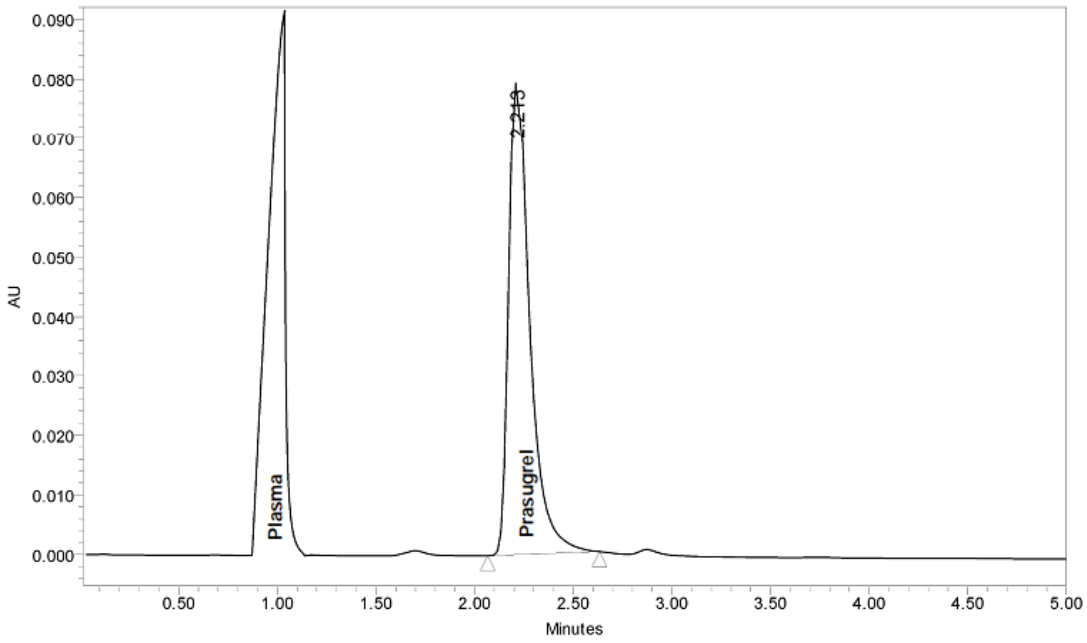

Figure 6. Typical chromatogram for standard samples of Prasugrel in plasma for stability studies

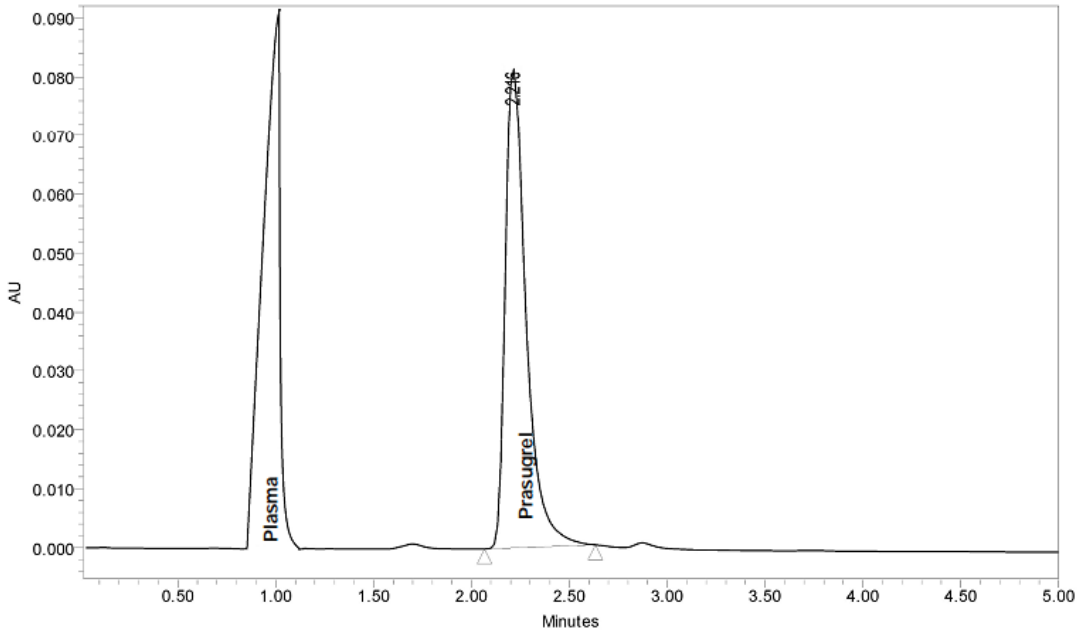

Figure 7. Typical chromatogram for freeze thaw samples of Prasugrel in plasma for stability studies

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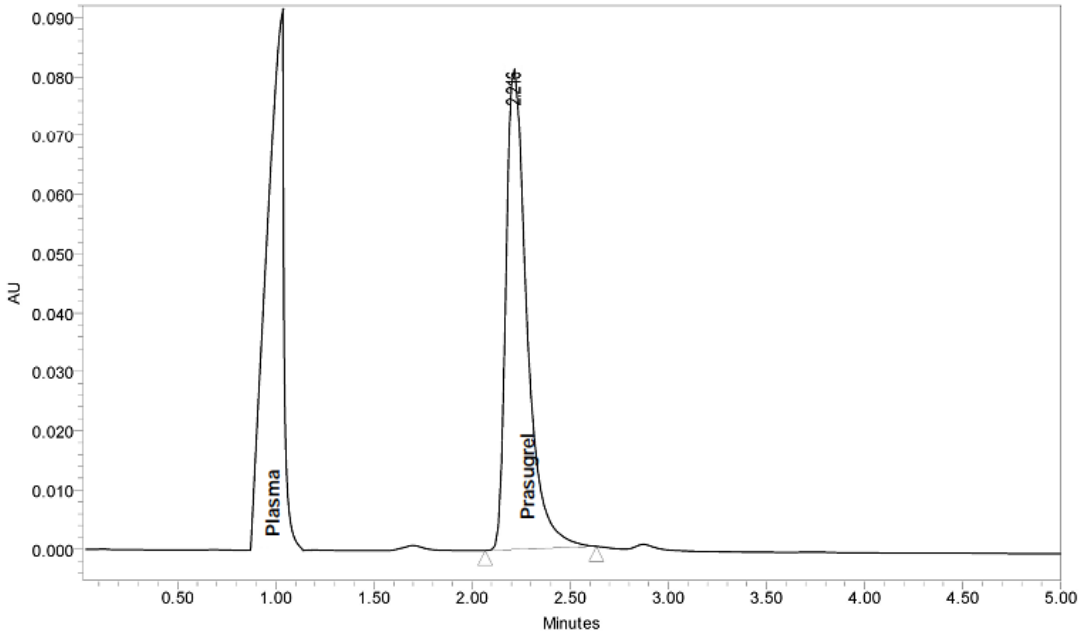

Figure 8. Typical chromatogram for short term stability samples of Prasugrel in plasma for stability studies

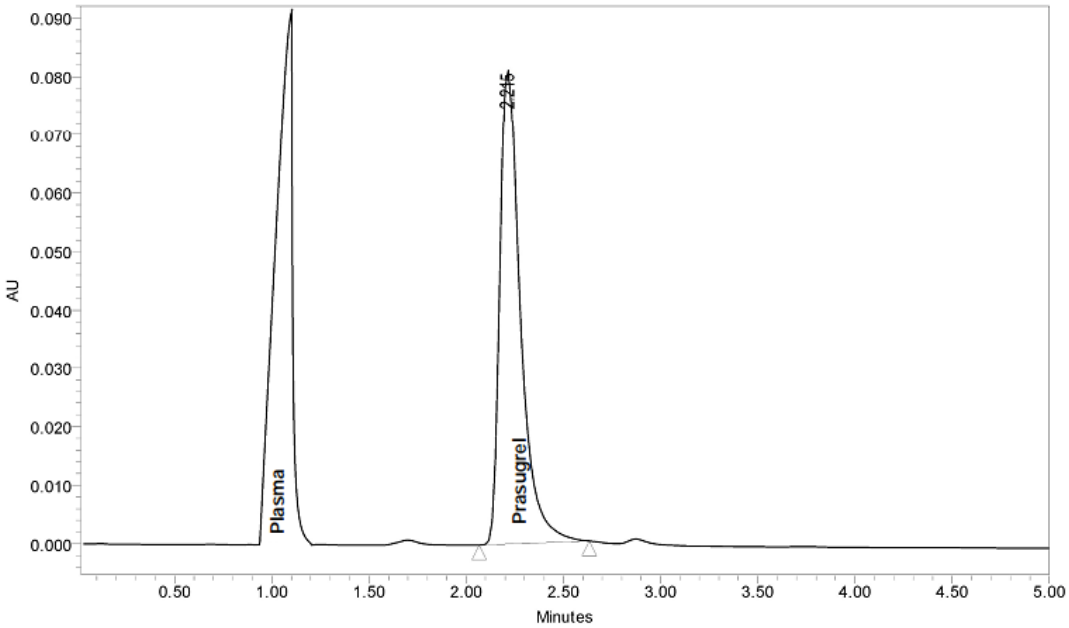

Figure 9. Typical chromatogram for long term stability samples of Prasugrel in plasma for stability studies

RESULTS AND DISCUSSION

To optimize the mobile phase, various proportions of phosphate buffer (pH 3.0) with acetonitrile (HPLC Grade) were tested. Phosphate buffer (pH 3.0) and acetonitrile (HPLC Grade) in the ratio of 30:70 (v/v) resulted in peak with good shape and resolution. A flow rate of 1.0mL /min was found to be optimum.

The LLOQ obtained for Prasugrel in plasma was 0.05 µg/mL.The retention time obtained in plasma was 2.216 min. Quantitative linearity of drug in plasma was obeyed in the concentration ranges of 15-40 µg/mL for Prasugrel. The relevant regression equation was y = 20539x(r^2=1) (where y is the peak area and x is the concentration of Prasugrel(µg/mL))**.**The intra-day and inter-day drugs variations in plasma by the proposed method in plasma showed an RSD less than 2 %, indicating that the method is precise. The corresponding average recoveries of the drug in plasma were 99.61-100.06 %. This reveals that the method is quite accurate. The RSD obtained for the drug spiked in plasma for stability studies were less than 2 %.

CONCLUSION

The optimized HPLC method was found accurate and sensitive for the determination of Prasugrel in plasma. The method was well validated as per ICH guidelines and all the parameters met within the acceptance criteria. The applicability of this method for estimation of Prasugrel in plasma was confirmed.

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