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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING REVERSE-PHASE HPLC METHOD FOR THE DETERMINATION OF PRASUGREL HYDROCHLORIDE AND ITS RELATED SUBSTANCES

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Keywords:

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ABSTRACT: A gradient reverse-phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the determination of Prasugrel hydrochloride and its related substances. The well chromatographic separation of prasugrel from its seven related substances and degradation products was achieved on Sunfire C18, 5µm (250mm x 4.6mm) column temperature maintained at 45°C with a mobile phase A: 0.1% v/v orthophosphoric acid in water and mobile phase B: 0.1% v/v orthophosphoric acid in acetonitrile. The flow rate was 1.0mL/min, and the detection wavelength was 220nm. The developed method was validated for specificity, forced degradation studies, sensitivity (LOD and LOQ), linearity, precision (system precision, method precision and intermediate precision), accuracy, stability of standard and sample solutions and robustness. The method is linear with a concentration range of 0.085-3.218µg/ml with correlation coefficients more than 0.9997 for prasugrel and its related substances. The method recoveries obtained are ranged between 96.4% -101.1% for LOQ levels and 94.7%-103.3% for remaining levels. The method was found to be specific, linear, sensitive, precise, rugged, accurate, robust and stability indicating in nature. The more results are detailed in research paper.

INTRODUCTION: Prasugrel hydrochloride is chemically known as 5-[(1*RS*)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydro thieno[3,2-*c*]pyridin-2-yl acetate hydrochloride **(Fig. 1)** and molecular weight is 409.9.

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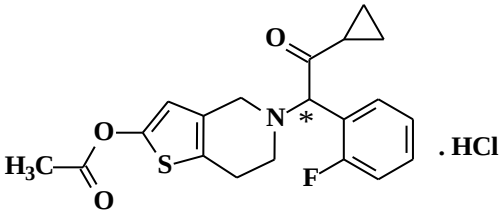
Prasugrel is a member of the thienopyridine class of antiplatelet agents along with clopidogrel and ticlopidine. Prasugrel is an orally bioavailable prodrug metabolized to an active adenosine diphosphate (ADP) receptor antagonist, which is a potent inhibitor of platelet activation and aggregation mediated by the P2Y12 ADP receptor. Prasugrel is more effective by preventing ischemic events in patients with acute coronary syndrome undergoing percutaneous coronary intervention, increase in bleeding and improved net clinical outcome [1-4].

Prasugrel hydrochloride is a prodrug. In aqueous media, cleavage of the ester moiety forms the hydrolysis product, which exists as a mixture of diastereomers, and which are the precursors of the active metabolite. The hydrochloride is used because of its better hydrolytic stability and because it provides a better solubility at relevant physiological pH's [5].

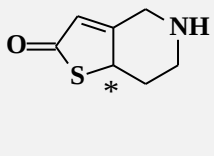
Many analytical methods have been reported in the literature for the determination of prasugrel hydrochloride and its related substances in both active ingredient and formulated products. Mohammed Ishaq and *et al* published the article for the determination of prasugrel in both bulk and tablets by using Intertsil ODS-3V column in 2011 [6]. Determination of both the active and inactive metabolites of prasugrel in human plasma by using LC –Tandem mass spectroscopy technique and determination of Prasugrel pharmaceutical formulation by RP-HPLC using X-terra RP C18, 250mm x 4.6mm column with 5µm particle size have been reported in literature [7, 8].

T.C Borole and *et al* reported stability indicating HPTLC method for the determination of prasugrel [9]. So far there is no method reported in literature for the determination of seven related substances of Prasugrel hydrochloride mentioned in this paper by RP-HPLC. This paper describes the development of RP-HPLC method for the determination of related substances of Prasugrel hydrochloride and also included stress degradation studies under acid, base, oxidative, humidity, heat and photolytic conditions to prove that the method is stability indicating. The developed method was validated to ensure the compliance in accordance with International Conference on Harmonization guidelines [10].



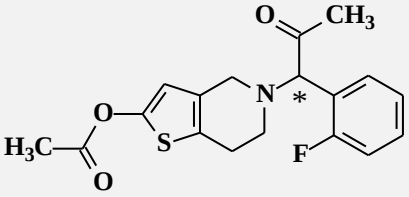
PRASUGREL HYDROCHLORIDE

FIG 1: CHEMICAL STRUCTURES OF PRASUGREL HYDROCHLORIDE AND ITS RELATED SUBSTANCES



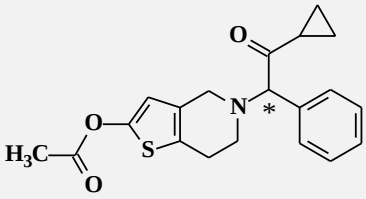
[[3,2-c]OHTP]

[RS-1]



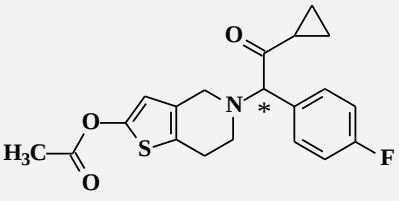
Methyl Ketone Prasugrel

[RS-2]



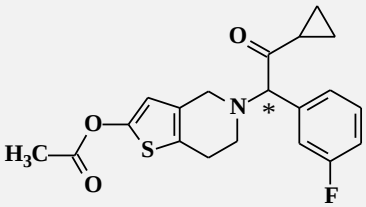
Desfluoro Prasugrel

[RS-3]



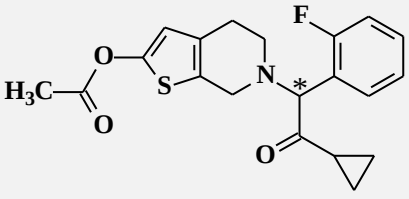
4-Fluoro Prasugrel

[RS-4]



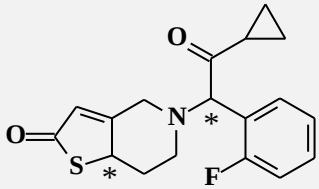
3-Fluoro Prasugrel

[RS-5]



[[2,3-c]Prasugrel

[RS-6]



Desacetyl Prasugrel

[RS-7]

EXPERIMENTAL:

Chemicals, reagents and samples: The investigated samples, Prasugrel hydrochloride reference sample, Prasugrel hydrochloride drug substance and its related substances: [3,2-c]OHTP(RS-1), Methyl ketone prasugrel(RS-2), Desfluoro prasugrel(RS-3), 4-Fluoro prasugrel(RS-4), 3-Fluoro prasugrel(RS-5), [2,3-c] prasugrel(RS-6), Desacetyl prasugrel (RS-7) were gifted from APL Research Centre laboratories (A division of Aurobindo Pharma Ltd., Hyderabad.). Chemical structures of Prasugrel hydrochloride and its related substances are given in **Fig 1.** Analytical reagent (AR grade) Orthophosphoric acid and acetonitrile (HPLC grade) were procured from Merck (India) limited and pure milli-Q water was used with the help of millipore purification system (Millipore®, Milford, MA, USA). Photo stability studies were carried out in a photo stability chamber (Make: Sanyo, Model: PSC062.AHA.C, Sanyo Gallenkamp PLC, Leics, UK).Thermal studies were carried out in thermal oven (Make: Newtronic, Model: NW-CON-51, Mumbai, India) and humidity studies were carried out in humidity chamber (containing saturated aqueous solution of potassium nitrate by creating 90% relative humidity at 25°C).

High performance liquid chromatography: A Waters Alliance 2695 separation module equipped with 2996 photodiode array detector with Empower pro data handling system [Waters Corporation, MILFORD, MA 01757, USA] was used.

The analysis was carried out on a stainless steel column 250 mm long, 4.6 mm internal diameter filled with Octadecyl silane chemically bonded to porous silica particles of 5 µm diameter [Sunfire C18, 250 mm × 4.6 mm, 5 µ (Make: Waters)] maintained at temperature 45°C. Mobile phase A was prepared by mixing 1ml of orthophosphoric acid in 1000 ml of water and mobile phase B was prepared by mixing 1 ml of orthophosphoric acid in 1000ml of acetonitrile. Diluent was prepared by mixing water and acetonitrile in the ratio of 60:40%v/v. Flow rate was kept as 1.0 ml/min, injection volume was 20µl, chromatographic data acquisition time was 55 min and UV detection was carried out at 220 nm. Retention time of prasugrel is about 22 minutes. The pump was in gradient mode and the program was as follows: Time (min)/ A (v/v): B(v/v); $T_{0.01}$/85:15, T_{17}/80:20, T_{29}/65:35, T_{45}/25:75, T_{55}/10:90, T_{57}/85:15, T_{65}/85:15.

Preparation of solutions:

(a) **System suitability solution:** Prepare 1mg/ml solution using Prasugrel hydrochloride enriched with 3-Fluoro prasugrel reference sample with diluent. Filter through 0.45 µ or finer porosity membrane filter.

(b) **Standard solution:** Prepare 1.5µg/ml using Prasugrel hydrochloride reference sample with diluent. Filter through 0.45 µ or finer porosity membrane filter.

(c) **Sample solution:** Prepare 1mg/ml using Prasugrel hydrochloride sample with diluent. Filter through 0.45 µ or finer porosity membrane filter.

Suitability requirements: The column efficiency as determined from the Prasugrel peak is not less than 20000 USP plate count, USP tailing for the same peak is not more than 2.0 and USP resolution between Prasugrel and 3-Fluoro Prasugrel peak is not less than 2.5 obtained from system suitability solution. Relative standard deviation (RSD) for peak areas of prasugrel obtained from six injections of the standard solution is not more than 5.0%.

Validation of the method:

1. **Specificity:** As per ICH, specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the developed LC method for prasugrel hydrochloride was carried out in the presence of its related substances; namely, RS-1, RS-2, RS-3, RS-4, RS-5, RS-6 and RS-7. Injection of all individual related substances solutions to confirm the retention times, injection of sample solution, injection of sample solution spiked with all related substances into HPLC and monitored the peak purities of prasugrel and its related substances and the results are found to be passed. Forced degradation studies were conducted for prasugrel hydrochloride to prove the stability-indicating property and specificity of the projected method. In this study, prasugrel hydrochloride was subjected to following stress conditions.

Thermal stress: Two samples were subjected to dry heat at 105°C one for 48hours and another for 120 hours.

Hydrolytic conditions:

(a) Acidic hydrolysis: Two sample solutions were mixed with 1M hydrochloric acid solution. One solution kept aside for 15min at room temperature and another solution was exposed at 85°C for 30 min.

(b) Alkaline hydrolysis: Sample solution was mixed with 0.02M sodium hydroxide/Initial.

Oxidative condition: Two sample solutions were mixed with 10% H_2O_2 solution exposed at 85°C, one solution was kept for 15 min and another solution was kept for 60min.

Photolytic condition: Two samples were exposed to photolytic degradation i.e., one for 10K Lux / 120 hours and another for 10K Lux / 120 hours + UV light/200watts/m^2.

Humidity condition: The sample was exposed under 90% RH at 25°C for 120 hours.

2. **Limit of detection and Limit of quantitation:** The limit of detection (LOD) and limit of quantitation (LOQ) values of prasugrel and its related substances were determined using the values of slope, standard deviation and responses of individual analytes. The predicted concentrations of LOD and LOQ for the related substances and prasugrel were verified for precision by preparing the solutions containing at about predicted concentrations and injected each six times into HPLC and calculated the %RSD of peak areas.

3. **Linearity:** A series of solutions were prepared using prasugrel hydrochloride and its related substances at concentration levels from LOQ to 150% of specification levels (for RS-1, 2, 3, 4, 6 - 0.15% and for RS-5 & 7 - 0.2% has chosen as specification) and each solution was injected in to HPLC and calculated the statistical values like slope, intercept, STEYX and correlation coefficient from linearity plot drawn for concentration versus area.

4. **Precision:** The precision (system precision) was evaluated by injecting out six injections of standard solution in to HPLC and calculated the % relative standard deviation (RSD). The method precision was checked by injecting six individual preparations of prasugrel hydrochloride (1000µg/mL) spiked with 0.15% of RS-1, 2, 3, 4 & 6 and with 0.2% of RS-5 & 7 with respect to sample concentration in to HPLC. The ruggedness of the method was also evaluated by injecting six individual preparations of prasugrel hydrochloride (1000µg/mL) (same batch used in method precision) spiked with 0.15% of RS-1, 2, 3, 4 & 6 and with 0.2% of RS-5 & 7 with respect to sample concentration using different analyst, different instrument, different lot of column on different day. The % RSD of content of each related substance was calculated.

5. **Accuracy:** The accuracy study of impurities was carried out by preparing Prasugrel hydrochloride sample solutions (1000µg/ml) in triplicate by spiking related substances at levels LOQ, 50, 100% and 150% of specification level, injected in to HPLC and calculated the percentage recovery.

6. **Robustness:** To determine the robustness of the developed method, system suitability solution and sample solution spiked with all related substances at specification level were injected in to HPLC at different deliberately altered experimental conditions; flow rate (±10% i.e 0.9ml/min & 1.1ml/min), detection wavelength (±5nm i.e 215nm & 225nm), organic in mobile phase (±2% absolute) and column oven temperature (±5°C i.e 40°C & 50°C) and evaluated the system suitability and method's ability to remain unaffected.

7. **Stability of standard and sample solutions:** Standard solution and sample solution spiked with related substances were prepared and analyzed initially and at different time intervals by keeping the solutions at room temperature (≈25°C) and at refrigerator temperature (≈6°C). Percentage difference between the areas obtained at initial and different time intervals of standard solution and sample solution was calculated.

Results obtained proved that standard solution was stable up to 48hrs at room temperature. But sample solution was not stable at room temperature and as well as at refrigerator temperature.

RESULTS AND DISCUSSION:

Development and optimization of method: The main objective of the chromatographic method development was focused on the separation of prasugrel peak from its related substances. The related substances are [3,2-c]OHTP, Methyl ketone prasugrel, Desfluoro prasugrel, 4-Fluoro prasugrel, 3-Fluoro prasugrel, [2,3-c] prasugrel and Desacetyl prasugrel [Desacetyl prasugrel exhibits as two peaks named as Diastereoisomer-1 & 2]. Prasugrel hydrochloride samples spiked with these impurities were injected in gradient mode using different combinations of the following chromatographic parameters:

1) Different stationary phases like C8, C18, Cyano, phenyl …etc
2) Different mobile phases like phosphate, sulfates and acetate buffers with different pH (2.0-8.0)
3) Different organic modifiers like acetonitrile, methanol and ethanol.

Satisfactory separations were achieved on Sunfire C18 250mm x 4.6mm, 5µ(Make: Waters) column with 0.1% OPA in water and 0.1% OPA in acetonitrile as mobile phases A & B, column oven temperature as 45°C, flow rate 1.0ml/min with injection volume 20µl. The choice of monitoring UV wave length 220nm was prasugrel and most of its related substances shows maximum UV absorbance at 220nm. The gradient elution given as follows.

Time (min)/ A (v/v): B(v/v); $T_{0.01}$/85:15, T_{17}/80:20, T_{29}/65:35,T_{45}/25:75,T_{55}/10:90, T_{57}/85:15, T_{65}/85: 15.

The typical relative retention times of [3, 2-c] OHTP, Methyl ketone prasugrel, desfluoro prasugrel, 4-Fluoro prasugrel, 3-Fluoro prasugrel, [2, 3-c] prasugrel, Desacetyl prasugrel diastereoisomer-1 and Desacetyl prasugrel diastereoisomer-2 are 0.09, 0.61, 0.71, 0.93, 1.07, 1.10, 1.28 & 1.35 with respect to prasugrel. (Prasugrel retention time is about 22 min). A typical representative HPLC chromatogram of prasugrel hydrochloride spiked with all related substances is given in **Fig. 2.**

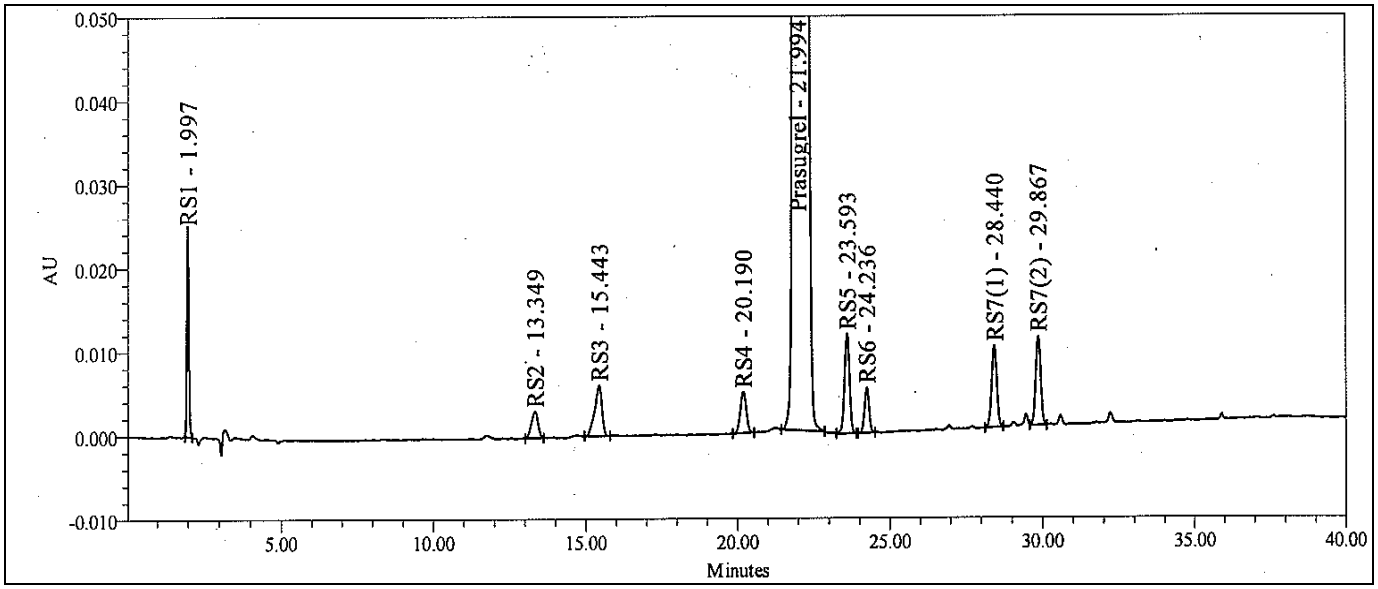


FIG. 2: A TYPICAL REPRESENTATIVE HPLC CHROMATOGRAM OF PRASUGREL HYDROCHLORIDE SPIKED WITH ITS RELATED SUBSTANCES

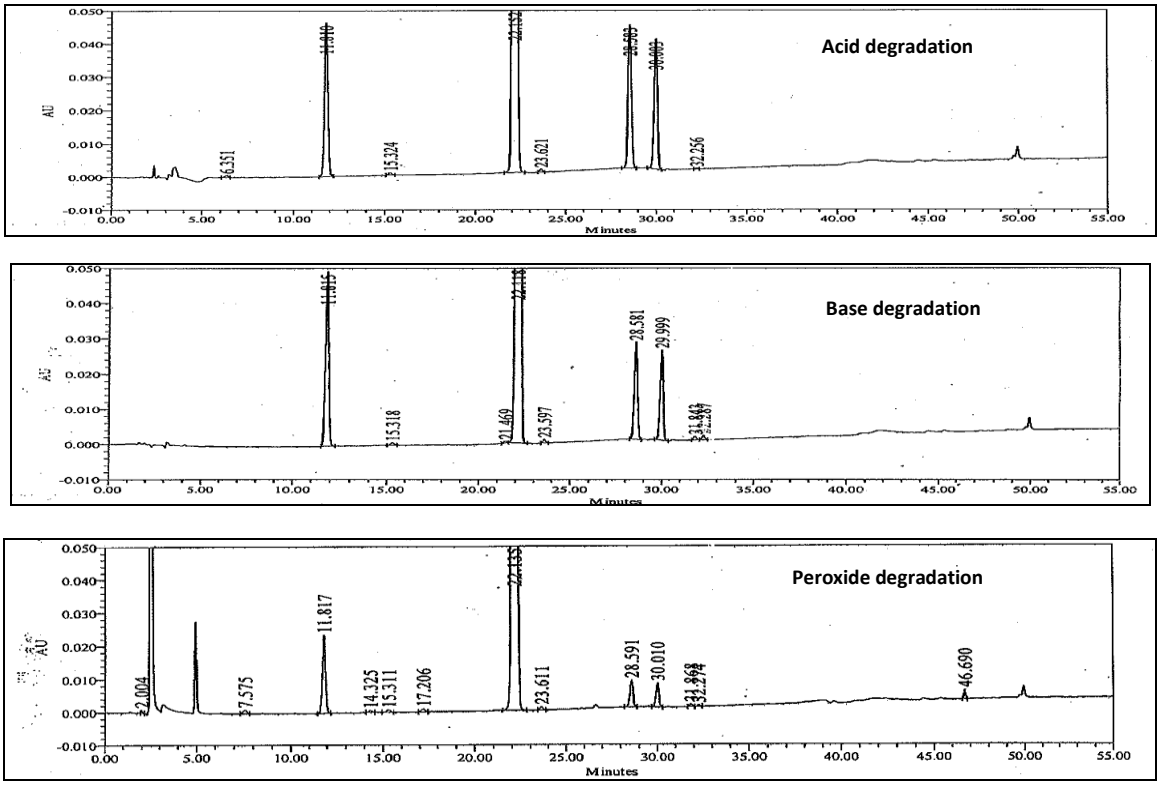
Forced degradation studies: The unstressed sample and each stressed sample were prepared to the required concentration and injected into HPLC using the analytical conditions. The obtained results are reported in **Table 1** and the typical representative stressed test samples chromatograms are given in **Fig 3.** The results of various stress conditions employed to degrade prasugrel hydrochloride drug substance indicated that, prasugrel hydrochloride is susceptible to degrade

under acid, alkaline, oxidative, thermal and photolytic conditions, where as it was found be stable in humidity condition. Further, the obtained information from this study is almost comparable with literature [11-13]. Further the peak purity data of prasugrel peak at different degradation conditions show that it is homogeneous and there are no co-eluting peaks proving that the method is specific and stability indicating for the determination of prasugrel hydrochloride and its related substances.

TABLE 1: EVALUATION OF FORCED DEGRADATION STUDIES OF PRASUGREL HYDROCHLORIDE

Type of Degradation	Degradation Condition	Area	% Degradation (Area)	Purity Angle	Purity Threshold	Remarks
Sample as such	-	10338293	-	0.069	0.299	-
Acid degradation	1M HCl / RT/ 15min	10437765	Nil	-	-	-
	1M HCl / 85°C / 30min*	8646609	16.5	0.102	0.320	Degraded to RS-7(1) and 7(2)
Alkaline degradation	0.02M NaOH / Initial*	9163833	11.3	0.064	0.283	Degraded to RS-7(1) and 7(2)
Peroxide degradation	10% H_2O_2 / 85°C / 15 min*	9296938	10.1	0.072	0.293	Degraded to RS-7(1) and 7(2)
	10% H_2O_2 / 85°C / 60 min	6894706	33.6	-	-	Degraded to RS-7(1) and 7(2)
Thermal degradation	105° C / 48 Hrs*	8601794	16.8	0.060	0.265	Degraded to RS-7(1) and 7(2)
	105° C / 120 Hrs	5434929	47.4	-	-	Degraded to RS-7(1) and 7(2)
Photolytic degradation	10 K Lux / 120 Hrs	10372275	Nil	-	-	-
	10K Lux / 120 Hours + UV light/200watts*	9982773	3.4	0.085	0.288	Degraded to RS-3 and 5
Humidity degradation	90% RH / 25°C /120 Hrs*	10369872	Nil	0.005	0.265	-

*Considered for stressed test samples chromatograms for fig 3.



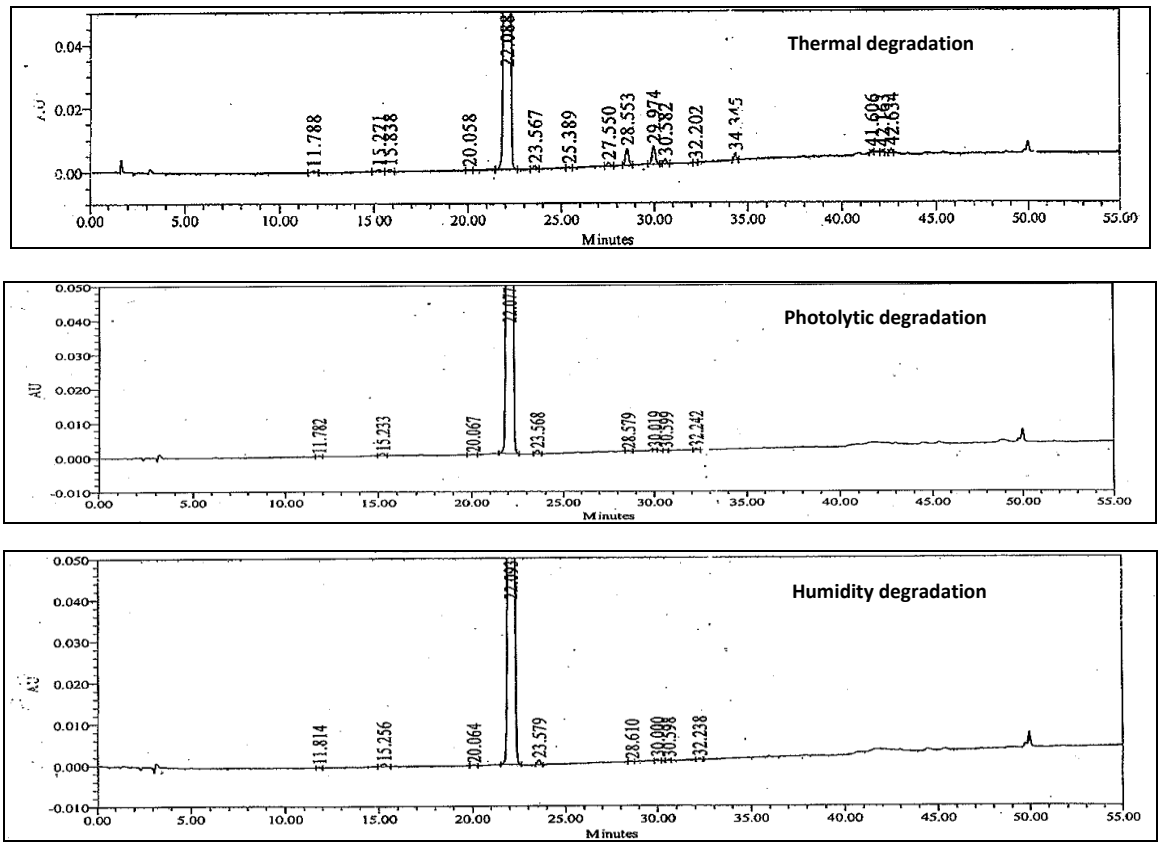


FIG. 3: TYPICAL REPRESENTATIVE HPLC CHROMATOGRAMS OF STRESSED TEST SAMPLES OF PRASUGREL HYDROCHLORIDE

LOD/LOQ precision and linearity: The RSD of peak areas of all related substances at LOQ precision was found to be less than 3.0% and for LOD precision less than 7.0%. In linearity experiment, the correlation coefficient values for all related substances were found to be greater than 0.9997. The LOD, LOQ values and statistical data of linearity experiment are reported in **Table 2.**

TABLE 2: STATISTICAL DATA OF LINEARITY, LOD/LOQ FOR PRASUGREL HYDROCHLORIDE AND ITS RELATED SUBSTANCES

Statistical parameters	RS-1	RS-2	RS-3	RS-4	Prasugrel	RS-5	RS-6	RS-7(1)	RS-7(2)
Correlation coefficient	1.0000	0.9998	0.9998	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
Intercept	135	261	516	35	328	212	161	256	193
STEYX	182	417	625	484	251	454	198	394	345
Slope	74657	31903	42090	40111	37970	42256	34531	54496	53771
Concentration range (µg/ml)	0.085-2.269	0.195-2.255	0.177-2.281	0.152-2.265	0.204-3.024	0.150-3.088	0.209-2.273	0.143-3.014	0.153-3.218
Response factor	0.51	1.19	0.90	0.95	1.00	0.90	1.10	0.70	0.71
LOD (µg/ml)	0.028	0.064	0.058	0.050	0.067	0.049	0.069	0.047	0.050
LOQ (µg/ml)	0.085	0.195	0.177	0.152	0.204	0.150	0.209	0.143	0.153
Precision for LOD (%R.S.D)	5.7	6.0	6.6	2.6	4.8	6.8	2.8	2.9	4.1
Precision for LOQ (%R.S.D)	1.2	2.3	1.0	0.5	0.6	0.7	0.6	1.1	1.0

Precision: The RSD of peak areas of prasugrel in system precision experiment was found to be less than 1.0% and RSD for the values of all related substances from the analysis of six individual preparations in method precision and ruggedness experiments was found to be less than 2.0%.

Accuracy: The recovery of all related substances of prasugrel hydrochloride obtained by taking average of three replicates of amount found to amount added. The obtained % recovery results were varied from 96.4-101.1% at LOQ level and from 94.7-103.3% at 50, 100 & 150% of specification levels. The recovery values are reported in **Table 3.**

TABLE 3: RECOVERY RESULTS OF PRASUGREL HYDROCHLORIDE RELATED SUBSTANCES

Accuracy (Average of 3 replicates)	RS1, RS2, RS3, RS4, RS5, RS6, RS7-1 & RS7-2		
	Added (µg/g)	Recovered (µg/g)	Recovery (%)
LOQ level	0.0090	0.0091	101.1
50% level	0.075	0.072	96.0
100% level	0.150	0.150	100.0
150% level	0.225	0.213	94.7
LOQ level	0.0210	0.0212	101.0
50% level	0.076	0.076	100.0
100% level	0.152	0.154	101.3
150% level	0.227	0.226	99.6
LOQ level	0.0198	0.0197	99.5
50% level	0.075	0.075	100.0
100% level	0.151	0.154	102.0
150% level	0.226	0.222	98.2
LOQ level	0.0170	0.0171	100.6
50% level	0.077	0.079	102.6
100% level	0.154	0.158	102.6
150% level	0.231	0.231	100.0
LOQ level	0.0167	0.0166	99.4
50% level	0.099	0.099	100.0
100% level	0.199	0.199	100.0
150% level	0.298	0.289	97.0
LOQ level	0.0222	0.0214	96.4
50% level	0.075	0.075	100.0
100% level	0.150	0.152	101.3
150% level	0.224	0.223	99.6
LOQ level	0.0158	0.0156	98.7
50% level	0.100	0.095	95.0
100% level	0.200	0.195	97.5
150% level	0.300	0.294	98.0
LOQ level	0.0168	0.0168	100.0
50% level	0.107	0.110	102.8
100% level	0.214	0.221	103.3
150% level	0.320	0.329	102.8

Robustness: In all the deliberate varied chromatographic conditions (flow rate, detection wavelength, organic in mobile phase and column oven temperature), the resolution, tailing, plate count values were found to be within the system suitability acceptance criteria from system suitability and spiked sample chromatograms illustrating that the method is robust. The system suitability results are reported in **Table 4.**

TABLE 4: SYSTEM SUITABILITY RESULTS AT DIFFERENT CHROMATOGRAPHIC CONDITIONS

Condition	Variation	USP Resolution	USP Tailing	USP Plate count
STP	-	4.3	1.4	43016
Flow	-10%	4.3	1.3	60572
	+10%	4.2	1.4	29093
Column oven temperature	-5°C	4.1	1.6	35348
	+5°C	4.4	1.1	50865
% of Organic in MP	-2%	4.0	1.2	101981
	+2%	4.4	1.6	27388
Wavelength	-5 nm	4.2	1.4	41617
	+5 nm	4.3	1.4	43686

Stability of standard and sample solutions: The % difference between the areas obtained at initial and different time intervals was found to be less than 1.0 for standard solution and was found to be stable up to 48 hours. But the sample solution was not stable at room temperature and as well as at refrigerator temperature. Hence, it is recommended to prepare the sample solution freshly and to be injected immediately.

CONCLUSIONS: The RP-HPLC method developed for the determination of prasugrel hydrochloride and its related substances is specific, sensitive, linear, precise, accurate and rugged. The method was fully validated, showing satisfactory data for all the method validation parameters tested. The developed method is stability-indicating and can be very useful for quality monitoring of regular production samples and can also be employed to check the quality during stability studies.

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