DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF PRASUGRELS AND ASPIRIN

B.A. Patel*, S. Alvi* and S.J. Parmar*

Post Graduation Department of Pharmaceutical Sciences, Sardar Patel University, Vallabh Vidhyanagar – 388120, India.

ABSTRACT

A simple & precise Stability indicating HPTLC method has been developed & validated for the simultaneous estimation of Prasugrel (PRA) & Aspirin (ASP) from Dosage form. The method employed TLC aluminum plates precoated with silica gel 60 GF 254 as the stationary phase. The solvent system comprised Carbon Tetrachloride: Ethyl Acetate: Acetic Acid (7.5:2.4:0.1, v/v/v). This system was found to give good result for both the drugs (Rf value: of ASP 0.34cm & PRA 0.58cm). Spectrodensitometric scanning-integration was performed at a wavelength of 240nm. The calibration curve was found to be linear within the concentration range of 150ng/spot to 900ng/spot for PRA and 1125-6750 ng/spot for ASP. The regression data for calibration curve shows good linear relationship with $r^2 = 0.9981$ & 0.9979 for PRA & ASP respectively. The method was validated as per ICH guidelines. Thus, the proposed method can be used successfully for routine analysis of PRA & ASP from dosage form.

KEY WORD: Validation, Stability indicating HPTLC method, Prasugrel & Aspirin.

INTRODUCTION

Aspirin (ASP), 2-(acetyloxy)benzoic acid, (Figure 1) has anti-inflammatory & antipyretic properties & acts as an inhibitor of cyclo-oxygenase which results in the inhibition of the biosynthesis of prostaglandins. It also inhibits platelet aggregation & is used in the prevention of arterial & venous thrombosis. Aspirin is official in IP, BP & USP.\textsuperscript{[1-4]}

*Correspondence for Author
S. Alvi
Post Graduation Department of Pharmaceutical Sciences, Sardar Patel University, Vallabh Vidhyanagar – 388120, India.
Prasugrel (PRA), 5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4H,5H,6H,7H-thieno[3,2-c]pyridin-2-yl acetate, a thienopyridine derivative (Figure 2), is a platelet activation & aggregation inhibitor structurally & pharmacologically related to clopidogrel & ticlopidine. It is a prodrug that requires enzymatic transformation in the liver to its active metabolite R-138727. R-138727 irreversibly binds to P2Y12 type ADP receptors on platelets thereby inhibiting ADP-mediated platelet activation & aggregation.\textsuperscript{[5]} It is not official in any pharmacopoeia. Aspirin in combination with Prasugrel is used to prevent thrombotic complications of Acute Coronary Syndrome (ACS) & percutaneous coronary intervention (PCI).\textsuperscript{[6]}

Literature survey reveals that some analytical methods have been reported for determination of PRA & ASP individually & aspirin in combination with other drugs, HPLC & UV simultaneous methods are available for estimation of PRA & ASP in their combined dosage form.\textsuperscript{[7-17]} The objective of the current study was to develop simple, accurate & reproducible second order derivative & ratio first order derivative analytical methods for the simultaneous estimation of Prasugrel & Aspirin in presence of excipients. The methods were developed & validated as per ICH guideline Q2 (R1).\textsuperscript{[18]}
MATERIALS & METHODS

Chemicals & Reagents
Methanol, Acetonitrile, Toluene, Ethyl acetate, Carbon tetrachloride & Acetic acid of AR Grade were purchased from Sisco Chem Pvt Ltd.

Chromatographic Conditions
Stationary phase was Precoated Silica gel G60 F_{254} aluminum Sheets 10×10 cm^2, layer thickness 0.2 mm. Activated the TLC plates by prewashing with Toluene & activated in Oven at 50^0C for 5 minute. The Optimized Mobile phase was Carbon Tetrachloride: Ethyl Acetate: Acetic Acid (7.5:2.4:0.1, v/v/v). Chamber saturation time: 30 minute at ambient temperature & migration distance was 75mm. The detection was done at 240 nm.

Preparation of PRA standard stock solution
Accurately weighed quantity of 75 mg PRA was transferred to 25 mL volumetric flask, dissolved & diluted up to the mark with methanol to get 3000 μg/mL of PRA.

Preparation of PRA working standard solution
From the standard stock solution, 0.5 mL aliquot was withdrawn & transferred to 10 mL volumetric flask & diluted up to the mark with methanol to produce working standard solution of 150 μg/mL of PRA.

Preparation of ASP standard stock solution
Accurately weighed quantity of 562.5 mg ASP was transferred to 25 mL volumetric flask, dissolved & diluted up to the mark with methanol to get 22500 μg/mL of ASP.

Preparation of ASP working standard solution
From the stock solution, 0.5 mL was transferred to 10 mL volumetric flask & diluted up to the mark with methanol to produce working standard solution of 1125 μg/mL of ASP.

Preparation of mixture of ASP & PRA for Calibration
Different aliquots i.e. 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL & 3.0 mL were withdrawn from standard stock solution of PRA & transferred to 10 mL flasks each. Then aliquots of 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL & 3.0 mL were withdrawn from standard stock solution of ASP & were transferred to above respective 10 mL flasks & diluted up to the mark with methanol to obtain mixture of PRA & ASP solutions containing 150 - 900 μg/mL of PRA & 1125 - 6750 μg/mL of ASP.
Preparation of sample solution from laboratory prepared synthetic mixture

Synthetic mixture of PRA (10 mg) & ASP (75 mg) was prepared by using common excipients like Corn Starch (50 mg), Lactose (113 mg) & Magnesium Stearate (2 mg) per tablet. Tablet powder was prepared by calculating formula for 20 Tablets having label claim for PRA & ASP, 10 mg & 75 mg, respectively. From this mixture, powder equivalent to 10 mg Prasugrel & 75 mg Aspirin was dissolved in 50 mL methanol & then sonicated for 15 min & filtered through Whatman filter paper. From this solution, 7.5 mL aliquot was taken in 10 mL volumetric flask & diluted up to the mark with methanol to make final concentration of PRA & ASP, 150 μg/mL & 1125 μg/mL, respectively which was used for Assay.

Method Validation

Linearity The calibration curve was linear over the concentration range of 150-900ng/spot for PRA & 1125-6750ng/spot for ASP.

Precision Repeatability of measurement of peak area was checked by repeated scanning of the same spot (n = 6) of PRA (300 ng/spot) & ASP (2250 ng/spot) without changing the position of the plate.

Repeatability of sample application was assessed by spotting PRA (300 ng/spot) & ASP (2250 ng/spot) six times on an HPTLC plate, developing the plate, & recording peak area for the spots. The precision of the method was evaluated by calculating the percent relative standard deviation (% RSD) of mean peak areas obtained from each spot of sample.

Intermediate Precision (Reproducibility)

The intra-day & inter-day precision of the method was determined by estimating the corresponding response three times on the same day & on three different days over a period of one week for three different concentrations of PRA (300, 450, & 600 ng/spot) & ASP (2250, 3375, & 4500 ng/spot). The precision of the method was evaluated by calculating the percent relative standard deviation (% RSD) of mean peak areas obtained from each spot of sample.

Accuracy The accuracy of the method was determined by recovery studies using method of standard additions to laboratory made synthetic mixture. Known amount of PRA & ASP standards were added at three different levels (50, 100 & 150 % of label claim) to constant weight of synthetic mixture respectively.
**Sensitivity** The sensitivity of measurement of ASP & PRA by the use of proposed method was estimated in terms of Limit of Detection (LOD) & Limit of Quantitation (LOQ). The LOD & LOQ were calculated by equation. Based on the standard deviation of the response & the slope, LOD & LOQ were estimated using the formulae.

\[ \text{LOD} = 3.3 \sigma / S \]
\[ \text{LOQ} = 10 \sigma / S \]

Where, \( \sigma \) = the standard deviation of the response 
\( S \) = the slope of the calibration curve

LOD & LOQ were determined from the standard deviations of the responses for six replicate determinations.

**Specificity**
The specificity of an analytical method is ability to measure accurately an analyte in presence of interferences like synthetic precursor, excipients, degradants or matrix component. Purity of spectra was determined at three different levels, at starting, middle & end. Correlation between the spectra of standard & spectra of drug in sample track was considered for determination of peak purity. The specificity was also determined by checking whether the sample matrix, solvent or mobile phase interfered in the analysis.

**Robustness**
Robustness was studied in six replicates at the concentration 300 ng/spot of PRA & 2250 ng/spot of ASP. By introducing various changes in the previous chromatographic conditions the effects on the results were examined. In this experiment, seven parameters (mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography & chromatography to spotting) were studied. The standard deviation of peak areas was calculated for each parameter & % R.S.D. was determined.

**Forced Degradation Study of standard mixture**
Forced degradation studies for pure drugs & formulation were conducted in order to establish whether the developed analytical method for the simultaneous assay of PRA & ASP was stability indicating.
Stress studies were carried out under the acidic, basic, neutral & oxidation conditions as mentioned in ICH Q1A (R2). Photo-degradation of drug substances & drug product was performed in the solution under day light.

**Preparation of standard stock solution for forced degradation studies**

Accurately weighed 75 mg of PRA & 562.5 mg of ASP were transferred to 25 mL volumetric flask, dissolved & diluted up to the mark with methanol to produce mixture of 3000 μg/mL of PRA & 22500 μg/mL of ASP.

**Degradation under acid catalyzed hydrolytic condition**

2 mL of standard stock solution of PRA & ASP of concentration 3000 μg/mL & 22500 μg/mL was mixed with 1 mL of 0.1 N HCl. The solution was diluted to 10 mL with methanol & kept for 4 hours at room temperature. Appropriate volume of resultant solution (1 μL) (600 ng/spot of PRA & 4500 ng/spot of ASP) was applied on TLC plate & developed & scanned as per optimized chromatographic conditions.

**Degradation under alkali catalysed hydrolytic condition**

2 mL of standard stock solution of PRA & ASP of concentration 3000 μg/mL & 22500 μg/mL was mixed with 1 mL of 0.01 N NaOH. Appropriate volume of resultant solution (1 μL) (600 ng/spot of PRA & 4500 ng/spot of ASP) was applied on TLC.

**Degradation under neutral hydrolytic condition**

5 mL of standard stock solution of PRA & ASP of concentration 3000 μg/mL & 22500 μg/mL was mixed with 5 mL distilled water. The solution was diluted to 50 mL with methanol & refluxed for 1 hour. Appropriate volume of resultant solution (2 μL) (600 ng/spot of PRA & 4500 ng/spot of ASP) was applied on TLC plate & developed & scanned as per optimized chromatographic conditions.

**Oxidative degradation**

5 mL of standard stock solution of PRA & ASP of concentration 3000 μg/mL & 22500 μg/mL was mixed with 5 mL of 3% H₂O₂. The solution was diluted to 50 mL with methanol & refluxed for 1 hour. Appropriate volume of resultant solution (2 μL) (600 ng/spot of PRA & 4500 ng/spot of ASP) was applied on TLC plate & developed & scanned as per optimized chromatographic conditions.
Photo degradation
2 mL aliquot of standard stock solution was transferred to 10 mL volumetric flask & diluted up to the mark with methanol to produce mixture of 600 μg/mL of PRA & 4500 μg/mL of ASP which was used as control. The resulting solution was subjected to photo degradation in day light for hours.

Forced Degradation Study of synthetic mixture
Sample was exposed to stress condition as mentioned under study for bulk drugs. Then the sample was filtered & appropriate volume was spotted on to TLC plate.

RESULTS & DISCUSSION
Method development
The solvent system was developed & optimized using trial & error method. Various proportions of different solvents as mobile phase were tried to get resolution of both the compounds. The optimized mobile phase was Carbon Tetrachloride: Ethyl Acetate: Acetic Acid (7.5:2.4:0.1, v/v/v) . The optimized mobile phase could resolve both the compounds apart from each other & the bands obtained were compact too. The maximum absorption of PRA & ASP together as detected at 235 nm & this wavelength was chosen for the analysis.

The optimized solvent system yielded a symmetrical peak for the both drugs with Rf 0.35 & 0.58 for ASP & PRA respectively. The HPTLC Chromatogram of ASP & PRA is shown in Figure 3.

Figure 3: A Typical Chromatogram of ASP & PRA
The developed method was then validated & successfully applied for quantification of ASP & PRA from the formulation. Regression analysis data is shown in Table 1.

**Table 1: Statistical Data of ASP & PRA drug**

<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>Linearity Range</th>
<th>Regression equation</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA</td>
<td>150 – 900 (ng/spot)</td>
<td>y = 3.4997x + 293.5092</td>
<td>0.9981</td>
</tr>
<tr>
<td>ASP</td>
<td>1125 – 6750 (ng/spot)</td>
<td>y = 1.3432x + 2,714.4037</td>
<td>0.9979</td>
</tr>
</tbody>
</table>

Precision, expressed in terms of %RSD was determined in terms of intra-day & inter-day precisions, analyzing the drugs at three different concentrations, determining each concentration thrice summarized in Table 2 & 3.

**Table 2: Intra- day Precision Data for ASP & PRA**

<table>
<thead>
<tr>
<th>Concentration (ng/spot)</th>
<th>Mean Area (n=3) ± SD ASP</th>
<th>%RSD ASP</th>
<th>Concentration (ng/spot)</th>
<th>Mean Area (n=3) ± SD PRA</th>
<th>%RSD PRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1125</td>
<td>4074.57 ± 33.35</td>
<td>0.82</td>
<td>150</td>
<td>762.35 ± 13.72</td>
<td>1.80</td>
</tr>
<tr>
<td>2250</td>
<td>5812.46 ± 47.58</td>
<td>0.82</td>
<td>300</td>
<td>1362.76 ± 10.73</td>
<td>0.79</td>
</tr>
<tr>
<td>3375</td>
<td>7361.24 ± 22.83</td>
<td>0.31</td>
<td>450</td>
<td>1919.24 ± 15.74</td>
<td>0.82</td>
</tr>
</tbody>
</table>

**Table 3: Inter- day Precision Data for ASP & PRA**

<table>
<thead>
<tr>
<th>Concentration (ng/spot)</th>
<th>Mean Area (n=3) ± SD ASP</th>
<th>%RSD ASP</th>
<th>Concentration (ng/spot)</th>
<th>Mean Area (n=3) ± SD PRA</th>
<th>%RSD PRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1125</td>
<td>4000.53 ± 70.81</td>
<td>1.78</td>
<td>150</td>
<td>759.93 ± 14.36</td>
<td>1.89</td>
</tr>
<tr>
<td>2250</td>
<td>5922.89 ± 70.65</td>
<td>1.30</td>
<td>300</td>
<td>1350.57 ± 20.03</td>
<td>1.48</td>
</tr>
<tr>
<td>3375</td>
<td>7373.60 ± 55.64</td>
<td>1.85</td>
<td>450</td>
<td>1930.01 ± 12.08</td>
<td>0.63</td>
</tr>
</tbody>
</table>

To ensure accuracy of the method, recovery studies were performed by standard addition method at three different levels I, II & III (50%, 100%, & 150%), to the pre-analyzed samples & the subsequent solutions were re-analyzed. At each level, three determinations were performed & the results obtained are shown in Table 4.

**Table 4: Data of Recovery Studies**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration of drug sample (ng)</th>
<th>Amount of Standard Spiked (ng)</th>
<th>Total amount of drug (mg)</th>
<th>Recovery (%Mean ± SD)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA</td>
<td>300</td>
<td>150</td>
<td>450</td>
<td>99.46 ± 1.15</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>300</td>
<td>600</td>
<td>101.26 ± 0.96</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>450</td>
<td>750</td>
<td>100.27 ± 1.70</td>
<td>1.70</td>
</tr>
<tr>
<td>ASP</td>
<td>2250</td>
<td>1125</td>
<td>3375</td>
<td>100.57 ± 1.32</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>2250</td>
<td>2250</td>
<td>4500</td>
<td>101.10 ± 0.76</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>2250</td>
<td>3375</td>
<td>5625</td>
<td>98.93 ± 0.88</td>
<td>0.89</td>
</tr>
</tbody>
</table>
The sensitivity of measurement of ASO & PRA by the use of proposed method was estimated in terms of Limit of Detection (LOD) & Limit of Quantitation (LOQ). The LOD & LOQ were calculated by equation. Based on the standard deviation of the response & the slope values obtained are shown in Table 5.

Table 5: Results of sensitivity data for ASP & PRA

<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>Limit of Detection</th>
<th>Limit of Quantitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA (ng/spot)</td>
<td>12.61</td>
<td>38.21</td>
</tr>
<tr>
<td>ASP (ng/spot)</td>
<td>108.85</td>
<td>329.83</td>
</tr>
</tbody>
</table>

The peak purity of ASP & PRA were assessed by comparing their respective spectra at peak start, apex & peak end positions of the spot i.e., r (S, M) & r (M, E) Figure 4.

Figure 4: Purity Comparison Spectra of (a). PRA & (b). ASP in standard & formulation

forced degradation study was carried out using standard and marketed formulation in various stressed conditions like: Acidic degradation, Alkali degradation, Oxidative degradation, Neutral hydrolysis, Photo degradation, Dry Heat degradation.

Result from various stress condition for standard mixture of PRA and ASP are shown in Table 6.

Table 6: Results of Percentage Degradation

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>% degradation PRA</th>
<th>% degradation ASP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>Formulation</td>
</tr>
<tr>
<td>Alkali</td>
<td>30.30</td>
<td>25.78</td>
</tr>
<tr>
<td>Acidic</td>
<td>36.32</td>
<td>32.02</td>
</tr>
<tr>
<td>Neutral</td>
<td>13.55</td>
<td>12.79</td>
</tr>
<tr>
<td>(H_2O_2)</td>
<td>44.10</td>
<td>48.45</td>
</tr>
<tr>
<td>Photo degradation</td>
<td>15.70</td>
<td>11.30</td>
</tr>
<tr>
<td>Dry Heat</td>
<td>37.53</td>
<td>40.66</td>
</tr>
</tbody>
</table>
Assay of Marketed Formulation

The amount of PRA & ASP was determined by estimating the concentration of both the drugs in sample solution. The amount of each drug in term of %w/w of label claim was determined. The results are shown in Table 7. Nearly 100% assay of PRA & ASP from market formulation with low RSD value confirmed the applicability of the developed method.

Table 7: Assay data of Marketed Formulation (n=3)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount of drug (mg)</th>
<th>% Amount found (Mean% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prasugrel</td>
<td>10</td>
<td>100.58% ± 1.15%</td>
</tr>
<tr>
<td>Aspirin</td>
<td>75</td>
<td>101.44% ± 0.42%</td>
</tr>
</tbody>
</table>

CONCLUSION

From the above study we can conclude that the PRA & ASP undergo degradation to different extent under different, above mentioned, stress conditions. Also the spectrum of product of degradation was completely different from the spectrum of original drug & non interference by the degraded product can be confirmed by peak purity values. The study on synthetic mixture revealed that the formulation is more stable than the bulk drug. Thus stability indicating HPTLC method was developed & validated which is capable of estimating both the drugs simultaneously in presence of the degradants of both the drugs. The method can thus be applied for the stability study in the marketed formulation.

REFERENCES


