Stability Indicating Method Development and Validation for Determination of Alprazolam and Propranolol by RP-HPLC

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ABSTRACT
A simple, economic new stability indicating high performance liquid chromatographic method for Alprazolam and Propranolol in tablet dosage form. The chromatographic separation was achieved using Phenomenex C18 (150mm x 4.6mm, 5µm) column as stationary phase with Phenomenex Security Guard Cartridges C18, 4x3mm and methanol: phosphate buffer pH 7 (70:30) as mobile phase at 1.0ml/min flow rate. Detection was carried out at 221nm on Younglin Acme 9000 HPLC system. ALP and PRO were eluted at 3.8 min and 5.06 min respectively. The method was validated in accordance with ICH guidelines. The described method was linear over concentration range of 0.8-1.2 µg/ml (r2=0.996) for ALP and 64-96 µg/ml (r2=0.991) for PRO. The amounts of ALP and PRO in marketed formulation were 108% and 100% respectively. The developed method has adequate sensitivity, reproducibility and specificity for determination of ALP and PRO in tablet dosage form. The stability of method was demonstrated by forced degradation studies under influence of acid, alkaline, oxidative Stress, photolytic and thermal stress as per ICH Q1A (R2) guidelines.

Results indicated ALP susceptible for acid hydrolysis, oxidative stress and PRO susceptible for alkaline hydrolysis and oxidative stress. Developed method is simple and stability indicating can be used for routine estimation of ALP and PRO in tablet dosage form.

Keywords: Alprazolam, Propranolol hydrochloride, High performance liquid chromatography, Forced degradation study.

INTRODUCTION
Alprazolam (ALP) is an anxiolytic drug belonging to the class of benzodiazepine, chemically it is 8-Chloro-1-methyl-6-phenyl-4H-(1,2,4)triazolo(4,3-α)(1,4)-benzodiazepine (Fig.1a). It is used in the treatment of anxiety disorders, agoraphobia, panic disorders and depression 1-3.

Propranolol hydrochloride (PRO) is 1-[(1-methyl ethyl) amino]-3-(1-naphthylenoxyl) 2 Propranolol hydrochloride (Fig.1b) is a nonselective β blocker, it blocks the action of epinephrine on both β1- and β2-adrenergic receptors. It is used for the treatment of angina pectoris, cardiac arrhythmia, hypertension, anxiety attacks and glaucoma 1-3.

The combination of ALP and PRH is approved in the treatment of anxiety. Literature survey revealed that few analytical methods reported for estimation of ALP alone and combination with other drugs UV-Spectrophotometric 4, RP-HPLC5,6, HPTLC6 similarly methods were reported for the determination of Propranolol by UV-Spectrophotometric7,8, RP-HPLC9,10, individual and combination with other drugs. Spectrophotometric11, HPLC12 method have been reported for simultaneous estimation of ALP and PRO, costly solvent are used, so need to develop economical and accurate method for estimation of ALP and PRO. The objective of present work was to develop stability indicating method for determination of alprazolam and propranolol by RP-HPLC

Figure 1: Structure of Alprazolam and propranolol

MATERIALS AND METHODS

Chemicals and Reagents

Instruments
Chromatographic separation was performed on Younglin 9000 HPLC system. Chromatographic system equipped with Quaternary Gradient HPLC system (SP903D), fitted
with UV-VIS Detector. Phenomenex C18 (150 x 4.6mm, 5µm) column as stationary phase with Phenomenex Security Guard Cartridges C18, 4x3mm. The mobile phase was degassed by sonication using ultrasonic bath (Microclean-103). Shimadzu UV Spectrophotometer 1800 was used for wavelength selection. The standard substances were weighed on, electronic balance Shimadzu, Japan AY220. Vigo Melting point apparatus was used.

**Preparation of Standard Stock Solution**

**Standard Stock Solution of ALP**

10mg of standard ALP was weighed and transferred to a 10ml volumetric flask then dissolved in the methanol LiChrosolv®. The volume was made up to the mark with same solvent to obtain conc. of 1000µg/ml of ALP. From the resulting solution 1ml was diluted to 10ml with same solvent to obtain conc. of 100µg/ml of ALP, 1 ml from above solution was diluted up to 10 ml with same solvent to obtain conc. 10µg/ml and labeled as ‘Std Stock ALP’.

**Standard Stock Solution of PRO**

10mg of standard PRO was weighed and transferred to a 10ml volumetric flask then dissolved in the methanol LiChrosolv®. The volume was made up to the mark with same solvent to obtain conc. of 1000µg/ml of PRO. From the resulting solution 8 ml was diluted to 10ml with same solvent to obtain conc. of 800µg/ml of PRO, 1ml from above solution was diluted up to 10 ml with same solvent to obtain conc. 80µg/ml and labeled as ‘Std Stock PRO’.

**Combined Standard Stock Solution of ALP and PRO**

5ml of ‘Std Stock ALP’ (10µg/ml) and 5ml of ‘Std Stock PRO’ (800µg/ml) mixed to get conc. of 5µg/ml of ALP and 400µg/ml of PRO. The solution was labeled as ‘Std Stock MIX’.

**Selection of Analytical Wavelength**

To investigate the appropriate wavelength for simultaneous determination of ALP and PRO, individual solutions in the mobile phase were scanned in the range of 200-300nm.

**Chromatographic Condition**

**Analytical Column:** Phenomenex C18 column (150 mm × 4.6 mm, 5 µm)

**Mobile Phase:** Methanol Phosphate Buffer pH7 (70:30)

**Flow Rate:** 1ml/min

**Column temperature:** Ambient

**Injection Volume:** 20 µl

**Detection Wavelength:** 221nm

**Preparation of phosphate buffer pH7**

Accurately weighed 0.136gm of potassium dihydrogen phosphate and 0.174gm of dipotassium hydrogen phosphate transferred in the 100ml volumetric flask and dissolved in HPLC water, then volume was made up to the mark with HPLC water.

**Preparation of Mobile phase**

Various combination of mobile phase including water, methanol, acetonitrile, phosphate buffer were tried finally Methanol and phosphate buffer of pH7 in the ratio (70:30) was selected for analysis of ALP and PRO.

**Identification of Separated Peak of the Drugs**

For identification of peak of the drugs; the standard solutions of ALP (10µg/ml) and PRO (10µg/ml) were injected separately into HPLC system and retention time were matched with retention time of mixture.

**Method Validation**

The method was validated according to ICH guidelines with respect to specificity, accuracy, precision, linearity, robustness, ruggedness, system suitability.

**Specificity**

The chromatogram of standard solution of mixture of ALP and PRO was compared with formulation to observe the interference of excipient.

**Linearity**

From the ‘Std Stock MIX’ (ALP 5µg/ml & PRO 400µg/ml) solution, 1.6, 1.8, 2, 2.2 and 2.4ml were transferred in a series of 10ml volumetric flasks. The volume was made up to the mark with mobile phase to obtain the conc. of 0.8, 0.9, 1, 1.1 and 1.2µg/ml of ALP and 64, 72, 80, 88 and 96µg/ml of PRO.

The solutions were filtered through syringe filter and 20µl injected into the HPLC system and their chromatogram were recorded for 10mins. Peak areas were recorded for all the peaks. Calibration curves of ALP and PRO were constructed by plotting the peak area v/s conc. The correlation coefficient (r²) of least square linear regression for ALP and PRO was calculated.

**Range**

The range of analytical method was decided from the interval between upper and lower level of calibration curves by plotting the curve.

**Accuracy**

From the ‘Sample Stock 1’ solution 1 ml was transferred to four different 10ml volumetric flasks separately along with 0, 0.8, 1, 1.2 ml from the ‘Std Stock MIX’ (ALP-5µg/ml and PRO -400µg/ml) solution. The volume was made up to the mark with mobile phase. All the solutions were filtered through syringe filter and injected into the HPLC system and their chromatograms were recorded under the same chromatographic conditions after getting a stable baseline. Peak areas were recorded for all the peaks and percent recoveries were calculated.
**Precision**
The precision of an analytical method was studied by performing Repeatability and intermediate precision.

**Repeatability**
Repeatability was determined by analyzing the standard solution of ALP (1µg/ml) and PRO (80µg/ml) six times and %RSD calculated

**Intermediate Precision Intra-day Precision**
Intra-day precision was determined by analyzing the standard solution of ALP (1µg/ml) and PRO (80µg/ml) at 9.00am and 6.00pm on same day following the procedure of repeatability.

**Robustness**
Combined standard solution of ALP (1µg/ml) and PRO (80µg/ml) was prepared and analyzed at different flow rates (0.9, 1.0, 1.1 ml/min) separately.

**System Suitability**
Sample solutions of ALP (1µg/ml) and PRO (80µg/ml) were prepared and analyzed six times. Chromatograms were studied for different parameters such as tailing factor, resolution and theoretical plates to see that whether they comply with the recommended limit or not.

**Assay of Tablet Formulation**
Twenty Tablets (Alprazolam 0.25mg, Propranolol hydrochloride 20mg) were weighed and finely powdered. Tablet powder equivalent to 0.25mg of ALP & 20mg of PRO was transferred to 50ml volumetric flask. 5ml methanol was added, sonicated for 10min, diluted with methanol to volume, and filtered through whatman filter paper No. 41 (‘Sample Stock1’). 2ml of resulting solution diluted up to 10ml with mobile phase to obtain 1µg/ml ALP and 80µg/ml PRO (‘Sample Stock2’).

Sample Stock2 was filtered through syringe filter and injected into HPLC system. The amount of ALP and PRO present in the tablets were calculated using calibration curve equations, y= 142x+6.2 and y= 130.91x+1058.4 respectively.

**Forced degradation study**
Forced degradation study performed on ALP and PRO both standard sample and tablet formulation

**Preparation of standard stock solution of ALP (API)**
20mg of standard ALP was weighed and transferred to a 20ml volumetric flask then dissolved in the methanol LiChrosolv®. The volume was made up to the mark with methanol to obtain conc. of 1000µg/ml of ALP (Sample stock A).1 ml of above solution dilute up to 10 ml with methanol resulting concentration 100µg/ml. 4ml of this solution diluted with mobile phase up to 10ml concentration of resulting solution 40µg/ml solution filtered through syringe filter and injected in HPLC. This is chromatograph of fresh sample.

**Preparation of standard stock solution of PRO (API)**
20mg of standard PRO was weighed and transferred to a 20ml volumetric flask then dissolved in the methanol LiChrosolv®. The volume was made up to the mark with methanol to obtain conc. of 1000µg/ml of PRO (Sample stock A).1 ml of above solution dilute up to 10 ml with methanol resulting concentration 100µg/ml. 4ml of this solution diluted with mobile phase up to 10ml concentration of resulting solution 40µg/ml. solution diluted with mobile phase up to 10ml concentration of resulting solution 40µg/ml solution.

**RESULT and DISCUSSION**

**Wavelength selection**
ALP (10µg/ml) and PRO (10µg/ml) in mobile phase were scanned separately in range of 200-400 nm. λ max of ALP 221nm and PRO 290nm. Based on spectral characteristic and overlain spectra 221 selected as wavelength for analysis.

![Figure 2: Overlaid spectra of ALP and PRO between 200-400nm in mobile phase](Image)

**Linearity and Range**
Graphs were plotted concentration in µg/ml on X axis verses area on Y axis and the correlation coefficients were determined. The method was found to be linear in concentration range of 0.8-1.2µg/ml for ALP and 64-96 µg/ml for PRO.
**Figure 3:** Calibration curve of ALP and PRO by RP-HPLC method

**Accuracy**

This technique involves the addition of standard drug solution to preanalyzed sample solution at 80%, 100%, and 120%. The % recoveries were 100-106% (ALP), 105-107% (PRO).

**Table 1:** Result of Accuracy for RP-HPLC Method (n=3)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Level of % Recovery</th>
<th>Amount of Sample (µg/ml)</th>
<th>Amount of Standard Drug Added (µg/ml)</th>
<th>Total Amount Found (µg/ml)</th>
<th>Amount Recovered (µg/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>PRO</td>
<td>ALP</td>
<td>PRO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.5</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>0.5</td>
<td>40</td>
<td>0.4</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>0.5</td>
<td>40</td>
<td>0.5</td>
<td>40</td>
<td>105</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>0.5</td>
<td>40</td>
<td>0.6</td>
<td>48</td>
<td>106</td>
</tr>
</tbody>
</table>

**Precision**

**Table 2:** Result of Repeatability and intermediate precision Study for ALP and PRO

<table>
<thead>
<tr>
<th>Inj.</th>
<th>Peak Area(mV) ALP</th>
<th>Peak Area at 9 am ALP</th>
<th>Peak Area at 6 pm ALP</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>155.96</td>
<td>11951.27</td>
<td>11542</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>153.82</td>
<td>11845</td>
<td>11681.9</td>
<td>0.69</td>
</tr>
<tr>
<td>3</td>
<td>154.84</td>
<td>11964.5</td>
<td>11677.8</td>
<td>0.51</td>
</tr>
<tr>
<td>4</td>
<td>151.5</td>
<td>10998</td>
<td>11700.2</td>
<td>0.76</td>
</tr>
<tr>
<td>5</td>
<td>152.75</td>
<td>11580</td>
<td>11650.3</td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>154.05</td>
<td>11700</td>
<td>11600.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**Robustness**

**Table 3:** Result of Robustness Study: Variation in Flow Rate (ml/min)

<table>
<thead>
<tr>
<th>Inj.</th>
<th>Flow Rate (ml/min)</th>
<th>Analyte</th>
<th>Retention Time (min)</th>
<th>Tailing Factor (T)</th>
<th>Theoretical Plates (N)</th>
<th>Resolution (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>ALP</td>
<td>3.87</td>
<td>1.31</td>
<td>4250</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PRO</td>
<td>5.06</td>
<td>1.3</td>
<td>5517</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>ALP</td>
<td>3.88</td>
<td>1.31</td>
<td>4111</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PRO</td>
<td>5.06</td>
<td>1.3</td>
<td>6327</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.1</td>
<td>ALP</td>
<td>3.88</td>
<td>1.33</td>
<td>4295</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PRO</td>
<td>5.07</td>
<td>1.34</td>
<td>5696</td>
<td></td>
</tr>
</tbody>
</table>
Assay

The amounts of ALP and PRO in marketed formulation were 108% and 100% respectively.

System Suitability Testing

Table 4: Results of System Suitability Parameters

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention Time (min)</th>
<th>Tailing Factor (T)</th>
<th>Theoretical Plates (N)</th>
<th>Resolution (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>3.8</td>
<td>1.31</td>
<td>4250</td>
<td>4.63</td>
</tr>
<tr>
<td>PRO</td>
<td>5.06</td>
<td>1.32</td>
<td>5517</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Forced degradation study of ALP and PRO

<table>
<thead>
<tr>
<th>Name</th>
<th>Condition</th>
<th>Time</th>
<th>% Recovery</th>
<th>% Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP Standard</td>
<td>Acid hydrolysis / 0.1N HCl/heating at 70°C</td>
<td>1 hrs</td>
<td>37.8</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td>Alkaline hydrolysis/0.1N HCL/heating at 70°C</td>
<td>24 hrs</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Oxidation/Room temp.</td>
<td>24 hrs</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Photolysis /UV Cabinet</td>
<td>24 hrs</td>
<td>98.42</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>Thermal Hot air oven/70°C</td>
<td>24 hrs</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>ALP Sample</td>
<td>Oxidation/5% H2O2</td>
<td>24 hrs</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Photolysis/ UV Cabinet</td>
<td>24 hrs</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Thermal/Hot-air oven/70°C</td>
<td>24 hrs</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>PRO Standard</td>
<td>Acid hydrolysis/ 0.1N HCl/heating at 70°C</td>
<td>24 hrs</td>
<td>98.3</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Alkaline hydrolysis/ 0.1N NAOH/heating at 70°C</td>
<td>24 hrs</td>
<td>71.84</td>
<td>28.16</td>
</tr>
<tr>
<td></td>
<td>Oxidation/ 5% H2O2 /heating at 70°C</td>
<td>24 hrs</td>
<td>73.9</td>
<td>26.1</td>
</tr>
<tr>
<td></td>
<td>Photolysis/ UV Cabinet</td>
<td>24 hrs</td>
<td>85.8</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Thermal /Hot air oven/70°C</td>
<td>24 hrs</td>
<td>95.5</td>
<td></td>
</tr>
<tr>
<td>PRO Sample</td>
<td>Oxidation/5% H2O2</td>
<td>24 hrs</td>
<td>92.3</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>Photolysis/ UV Cabinet</td>
<td>24 hrs</td>
<td>81</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Thermal/Hot-air oven/70°C</td>
<td>24 hrs</td>
<td>94.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>

CONCLUSION

Developed RP-HPLC method was simple, accurate, precise, linear, robust, economical can be applied for routine analysis of Alprazolam and Propranolol hydrochloride in tablet dosage form.

Results of stress study shows there was no other co-eluting peak with main peak and method is specific for estimation of Alprazolam and Propranolol hydrochloride in presence of their degradation products. Developed method is stability indicating method can be used for routine estimation of ALP and PRO in tablet dosage form.

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