Development and Validation of Stability Indicating HPTLC Method for Simultaneous Estimation of Propranolol Hydrochloride and Flunarizine Dihydrochloride

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ABSTRACT
A simple, rapid, precise, accurate, sensitive and stability indicating high performance thin layer chromatographic method has been developed for the simultaneous estimation of Propranolol hydrochloride and Flunarizine dihydrochloride in pharmaceutical tablet formulation. The chromatographic separation was performed on silica gel 60 F254 HPTLC plates using chloroform: acetonitrile: methanol: glacial acetic acid (60:30:8:4 v/v/v/v) as a mobile phase. HPTLC separation of the two drug followed by densitometric measurement at 272 nm. The drugs were satisfactorily resolved with Rf values 0.36±0.02 and 0.70±0.02 for Propranolol hydrochloride and Flunarizine dihydrochloride, respectively. The method was found to be linear in the range of 4000-12000 ng/spot and 1000-3000 ng/spot for Propranolol hydrochloride and Flunarizine dihydrochloride, respectively. The correlation coefficient was found to be 0.993 and 0.995 for Propranolol hydrochloride and Flunarizine dihydrochloride, respectively. The LOD and LOQ were found to be 159.14 ng/spot and 482.16 ng/spot for Propranolol hydrochloride and 23.8 ng/spot and 72.3 ng/spot for Flunarizine dihydrochloride, respectively. The mean recovery was found to be 97.9-99.7% and 97.6-100.0% for Propranolol hydrochloride and Flunarizine dihydrochloride, respectively. The intra-day and inter-day precision was found to be within limit. The influences of acid, alkaline, oxidative stress, thermal stress and photolytic stress conditions on both the drugs were studied. The proposed method has adequate specificity, sensitivity and reproducibility for quality control assay of Propranolol hydrochloride and Flunarizine dihydrochloride in combined pharmaceutical tablet dosage form.

Keywords: Analytical method validation, degradation, flunarizine dihydrochloride, HPTLC, propranolol hydrochloride

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1. INTRODUCTION
Propranolol hydrochloride (PRO) is an antihypertensive agent. It is chemically (RS)-2-(4-(2-methylpropyl) phenyl) 2-Propanol, 1-[(1-methylethyl) amino]-3-(1-naphthalenyl)oxy), hydrochloride (Figure 1) [1]. Flunarizine dihydrochloride (FLU) is a calcium channel blocking agent. It is chemically 1-{bis(4-fluorophenyl)methyl}-4-(3-phenyl-2 prophenyl) piperazine (Figure 2) [2]. The combination dosage form of PRO and FLU is available in the market, and it is indicated in the treatment of hypertension and migraine [3]. Various analytical methods like potentiometry, conductometry, UV spectroscopy, HPLC, UPLC and HPTLC, LC-MS have been reported for estimation of PRO and FLU alone in their API, tablet, capsule, suspension and biological fluids [1, 2, 4-22].

Figure 1: Structure of Propranolol hydrochloride
Literature survey revealed that several analytical methods like UV spectroscopy, HPLC, and HPTLC have been reported for simultaneous estimation of PRO and FLU in their combined dosage forms [23-32]. Only one RP-HPLC stability indicating method has been reported for simultaneous estimation of PRO and FLU [33]. However, stability indicating HPTLC method has not been reported for simultaneous estimation of PRO and FLU in their combined tablet dosage form. So the aim of the present work was to develop and validate stability indicating HPTLC method for simultaneous estimation of PRO and FLU in combined tablet dosage form as per ICH guidelines [34,35].

2. MATERIALS AND METHODS

2.1 Materials

Propranolol hydrochloride was procured from Elite Pharma (Vatva, Ahmedabad, Gujarat, India) and Flunarizine dihydrochloride was procured from Sun Pharmaceuticals Ltd. (Baroda, Gujarat, India). All the chemicals used were of analytical grade (CDH chemicals, New Delhi, India). Provanol Plus 10 tablets were procured from the local market.

2.2 Instrumentation

CAMAG Linomat IV (Semiautomatic spotting device), CAMAG twin trough chamber, CAMAG TLC Scanner 3 were used. The software was CATS 4. TLC Aluminum sheet pre-coated with silica gel 60 F 254, 20×20cm² layer thickness- 0.2mm (Merck, Darmstadt, Germany) were used as the stationary phase.

2.3 Chromatographic Conditions

Chromatography was performed on HPTLC plates coated with 0.2 mm layers of silica gel 60 F 254. The plates were prewashed with methanol and activated at 110°C for 5 min prior to chromatography. Standard and samples were applied as 5 mm bands, 10 mm apart and 10 mm from the lower edge of the plate, by means of a 100µL Hamilton (Reno, Nevada, USA) micro syringe, mounted on a Linomat IV applicator; the spraying rate was 10 sec/µL. The mobile phase consisted of chloroform: acetonitrile: methanol:glacial acetic acid (60:30:8:4, v/v/v/v). Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 10 min at 28°C±2°C. The length of chromatogram run was 6 cm. After development the plate was dried on Hot plate. Densitometric scanning at the wavelength of 272 nm was performed with a Camag TLC Scanner 3 equipped with win CATS Software Version 4.01. The slit dimensions were 4.00 mm × 0.45 mm and 100 mm/s scanning speed was employed.

2.4 Preparation of standard stock solutions

An accurately weighed PRO (400 mg) and FLU (100 mg) were transferred to 100 mL volumetric flask and dissolved and diluted to the mark with methanol to obtain a standard stock solution of PRO (S1-4000µg/mL) and FLU (S2-1000 µg/mL).

2.5 Preparation of working standard solutions

Aliquots of 1, 1.5, 2, 2.5 and 3 mL standard stock solution S1 and S2 were transferred into five different 10mL volumetric flasks and diluted up to mark with methanol (400-1200 µg/mL PRO, 100-300 µg/mL FLU).

2.6 Preparation of test sample solution

To determine the content of PRO and FLU simultaneously in conventional tablets (label claim: 40mg propranolol hydrochloride and 10 mg flunarizine dihydrochloride per tablet), twenty tablets were weighed, their mean weight determined and they were finely powdered. Then equivalent weight of the drug powder was transferred into 100 mL volumetric flask containing 5 mL methanol, sonicated for 15 min and diluted up to mark with methanol.
2.7 Analytical Method Validation
The developed method was validated as per ICH Q2 (R1) guideline for specificity, linearity and range, precision, accuracy, detection limit and quantitation limit parameters.

2.7.1 Specificity
The ability of an analytical method to unequivocally assess the analyte in the presence of other components can be demonstrated by evaluating specificity. The specificity of the HPTLC method was determined by analyzing standard drug and test sample. The spot for PRO and FLU in the sample was confirmed by comparing the Rf and spectra. The peak purity of PRO and FLU was determined by comparing the spectrum of standard drug and test sample at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

2.7.2 Linearity and range
The linearity of the developed HPTLC method was determined at the five concentration levels ranging from 4000-12000ng/spot for PRO and 1000-3000ng/spot for FLU. Working standard solutions (10 μL) were spotted on HPTLC plate to obtain a final concentration range 4000-12000ng/spot for PRO and 1000-3000ng/spot for FLU. The peak areas were recorded and calibration curve was constructed by plotting peak areas against concentration of drug (ng/spot).

2.7.3 Precision
The precision of the developed HPTLC method was verified by performing intra-day, inter-day and repeatability of sample application studies. Intra-day precision was determined by analyzing 4000, 8000, and 12000 ng/spot of PRO and 1000, 2000, 3000ng/spot of FLU for three times on the same day while inter-day precision was determined by analyzing 4000, 8000 and 12000 ng/spot of PRO and 1000, 2000 and 3000 ng/spot of FLU for three consecutive days over a period of week. Repeatability was performed by spotting 10 μL working standard solution of PRO and FLU six times on HPTLC plate. Percentage relative standard deviation (%RSD) was calculated for intra-day, inter-day and repeatability studies.

2.7.4 Limit of detection (LOD)
It is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified under the stated analytical conditions. It was calculated by using following formula.

\[
\text{LOD} = \frac{(3.3 \times \sigma)}{S}
\]

\[
\sigma = \text{Standard deviation of the Y intercept}
\]

\[
S = \text{Slope of the calibration curve}
\]

2.7.5 Limit of quantitation (LOQ)
It is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental condition. It was calculated by using following formula.

\[
\text{LOQ} = \frac{(10 \times \sigma)}{S}
\]

\[
\sigma = \text{Standard deviation of the Y intercept}
\]

\[
S = \text{Slope of the calibration curve}
\]

2.7.6 Accuracy
To examine the accuracy of the developed HPTLC method, recovery studies were carried out by standard addition method at three different concentration levels (80, 100 and 120%) in triplicate by spiking standard PRO and FLU solution in pre-analyzed tablet solution containing 400μg/mL PRO and 100μg/mL of FLU.

2.8 Force Degradation Study
Forced degradation studies an important part of the drug development process. To evaluate the stability indicating properties of the developed HPTLC method, forced degradation studies were carried out in accordance to the ICH guideline. The standard drugs were subjected to acid, base, oxidation, thermal and photo-degradation studies.

2.8.1 Acid degradation
Aliquot 2 mL standard stock solution of PRO (4000μg/mL) and FLU (1000μg/mL) were transferred to 10 mL of volumetric flask and diluted up to the mark with 5 N HCl. This solution was refluxed for 3 hours and neutralized using 5 N NaOH. This solution (10 μL) was spotted on TLC plate along with standard solution and analysis was performed as described under chromatographic conditions.

2.8.2 Alkali degradation
Aliquot 2 mL standard stock solution of PRO (4000μg/mL) and FLU (1000μg/mL) were transferred to 10 mL of volumetric flask and diluted up to the mark with 1 N NaOH. This
solution was refluxed for 3 hours and neutralized using 1 N HCl. This solution (10 μL) was spotted on TLC plate along with standard solution and analysis was performed as described under chromatographic condition.

2.8.3 Oxidative degradation
Aliquot 2 mL standard stock solution of PRO (4000μg/mL) and FLU (1000μg/mL) were transferred to 10 mL of volumetric flask and diluted up to the mark with 6% H₂O₂. This solution was refluxed for 30 minutes. This solution (10 μL) was spotted on TLC plate along with standard solution and analysis was performed as described under chromatographic condition.

2.8.4 Thermal degradation
An accurately weighed PRO (400 mg) and FLU (100mg) were mixed in porcelain dish and exposed to a temperature of 80°C for 24 hours in hot air oven. After 24 hours, PRO (8 mg) and FLU (2 mg) were transferred to 10 mL volumetric flask. Dissolve it and diluted up to the mark with methanol. This solution (10μL) was spotted on TLC plate and analysis was performed as described under chromatographic condition.

2.8.5 Photolytic degradation
An accurately weighed PRO (400 mg) and FLU (100mg) were mixed in porcelain dish and exposed to UV light for 24 hours. After 24 hours, PRO (8 mg) and FLU (2 mg) were transferred to 10 mL volumetric flask. Dissolve it and diluted up to the mark with methanol. This solution (10μL) was spotted on TLC plate and analysis was performed as described under chromatographic condition.

3. RESULT AND DISCUSSION
3.1 Chromatographic development
The goal of the present study was to develop a rapid, precise, accurate and cost effective stability indicating HPTLC method for the simultaneous estimation of PRO and FLU in combined pharmaceutical tablet formulation. The TLC procedure was optimized for simultaneous determination of PRO and FLU. The mobile phase chloroform: acetonitrile: methanol: glacial acetic acid (60:30:8:4,v/v/v/v) resulted in good resolution, sharp and symmetrical peaks at R₀.36±0.02 for PRO and 0.70±0.02 for FLU (Figure 3).

3.2 Analytical Method Validation
The developed HPTLC method was validated with respect to specificity, linearity and range, accuracy, precision, LOD and LOQ parameters as per ICH guideline.

![Figure 3: Chromatogram of PRO (Rf = 0.36±0.02) and FLU (Rf = 0.7±0.02)](image)

3.2.1 Specificity
The mobile phase was designed to resolve both the drugs very efficiently. The R₁ values of PRO and FLU were found to be 0.36±0.02 and 0.70±0.02, respectively. The peak purity of PRO was tested by comparing the standard and sample spectrum of PRO at the peak start (S), peak apex (M) and at the peak end (E) positions. Correlation between standard and sample spectrum of PRO was found to be r(S, M)=0.999 and r(M, E)=0.997. Same procedure was followed for FLU. Correlation between standard and sample spectrum of FLU was found to be r(S, M) = 0.999 and r(M, E) =0.999. A good correlation was obtained between the standard and sample spectra of PRO and FLU indicate specificity of the proposed HPTLC method.

3.2.2 Linearity and Range
The method was linear in the concentration range from 4000-12000ng/spot and 400-1200ng/spot for PRO and FLU, respectively. The calibration curve was constructed by plotting concentration drug (X) versus the mean peak area of drug (Y). The correlation coefficient was found to be 0.993 and 0.995 for PRO and FLU, respectively. The regression equation was found as $y=10.483x + 7744.6$ and $y=2.04x -1588.1$ for PRO and FLU, respectively. Where, $y$ is the peak area and $x$ is the concentration.
The results showed excellent correlation between the peak area and the concentration of drug in the range tested (Table 1).

### Table 1: Linearity study of PRO and FLU

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (ng/spot)</th>
<th>Peak area (n=6)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>4000</td>
<td>11593.7±53.12</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>6000</td>
<td>14331.8±82.62</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>8000</td>
<td>16364.6±74.42</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>18244.25±105.73</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>12000</td>
<td>20120.4±112.47</td>
<td>0.56</td>
</tr>
<tr>
<td>FLU</td>
<td>1000</td>
<td>3865.75±10.27</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>5403.6±24.32</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>6608.2±29.09</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>7651.2±30.52</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>8852.05±24.71</td>
<td>0.28</td>
</tr>
</tbody>
</table>

3.2.3 Precision
The %RSD for intra-day precision was found to be 0.65-0.90% and 0.18-0.38% for PRO and FLU, respectively. The % RSD for inter-day precision was found to be 0.20-0.24% and 0.19-0.66% for PRO and FLU, respectively. The %RSD for repeatability was found to be 0.33% and 0.33% for PRO and FLU, respectively. The % RSD for intra-day, inter-day and repeatability precision was found to be less than 2% indicating good precision of the developed HPTLC method. The results of precision were shown in (Table 2).

### Table 2: Precision study for PRO and FRO

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (ng/spot)</th>
<th>Intra-day %RSD (n=3)</th>
<th>Inter-day %RSD (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>4000</td>
<td>0.66</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>8000</td>
<td>0.65</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>12000</td>
<td>0.90</td>
<td>0.20</td>
</tr>
<tr>
<td>FLU</td>
<td>1000</td>
<td>0.18</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>0.28</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>0.38</td>
<td>0.19</td>
</tr>
</tbody>
</table>

3.2.4 Limit of detection (LOD)
The LOD value for PRO and FLU were found to be 159.14 ng/spot and 23.8 ng/spot, respectively.

3.2.5 Limit of quantitation (LOQ)
The LOQ value for PRO and FLU were found to be 482.16 ng/spot and 72.3 ng/spot, respectively.

3.2.6 Accuracy
Accuracy of the proposed HPTLC method was ascertained by recovery studies and the results are expressed as % recovery. The mean recovery was found to be 97.9-99.7% and 97.0-100.0% for PRO and FLU, respectively indicating the accuracy of proposed HPTLC method (Table 3).

### Table 3: Recovery study of PRO and FLU

<table>
<thead>
<tr>
<th>Drug</th>
<th>Initial amount (µg/mL)</th>
<th>Amount added (µg/mL)</th>
<th>% Recovery (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>400</td>
<td>-</td>
<td>99.3±0.53</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>200</td>
<td>97.9±0.81</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>800</td>
<td>99.0±0.88</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>1000</td>
<td>99.7±0.92</td>
</tr>
<tr>
<td>FLU</td>
<td>100</td>
<td>-</td>
<td>98.0±0.65</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50</td>
<td>97.6±0.57</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>97.0±0.50</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>150</td>
<td>100±0.82</td>
</tr>
</tbody>
</table>

3.4 Analysis of marketed formulation
The proposed method was successfully applied to the analysis of marketed tablet formulation and the results obtained are given in (Table 4). The average drug content was found to be 99.3±0.53 and 98.0±0.89 for PRO and FLU, respectively.

### Table 4: Analysis of marketed formulation

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Amount Claimed (mg)</th>
<th>% Assay Mean ±SD (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol HCl</td>
<td>40 mg</td>
<td>99.3±0.53</td>
</tr>
<tr>
<td>Flunarizinedihydrochloride</td>
<td>10 mg</td>
<td>98.0±0.89</td>
</tr>
</tbody>
</table>

3.5.1 Acid degradation study
One degradation product of FLU was observed when it was subjected to 5NHCl for 3 hours reflux condition while PRO was found to be stable (Figure 4).

**Figure 4: Acid Degradation**
(1 Degradation product of FLU 2. PRO 3. FLU)
3.5.2 Alkali degradation
Both PRO and FLU was found to be stable when they were subjected to 1N NaOH for 3 hours reflux condition (Figure 5).

3.5.3 Oxidative degradation
Both PRO and FLU were degraded when they were subjected to 6% H₂O₂ for 30 minutes reflux condition (Figure 6).

3.5.4 Thermal degradation
Both PRO and FLU was found to be stable when they were exposed to 80°C for 24 hours in hot air oven (Figure 7).

3.5.5 Photolytic degradation
Both PRO and FLU was found to be stable when they were exposed to UV light for 24 hours. (Figure 8).

4. CONCLUSION
Based on the results obtained, it is found that the developed HPTLC method is accurate, precise, reproducible, sensitive, specific and stability indicating. It can become effective analytical tool for routine quality control of PRO and FLU in combined tablet dosage form. The method can be used for determination of the degradation kinetics of PRO and FLU.

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