Development and Validation of Stability Indicating HPTLC Method for Estimation of Salmeterol Xinafoate

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ABSTRACT
A simple, rapid validated stability indicating HPTLC method for estimation of Salmeterol xinafoate was successfully developed. This method is based on HPTLC separation followed by UV detection at 252 nm. The separation was carried out on Merck TLC aluminium sheets precoated with silica gel 60F254 using Chloroform: Methanol: Ammonia (7:3:0.5 v/v/v) as a mobile phase and scanning was done by using TLC Scanner III. Salmeterol xinafoate gave well defined and sharp peak at Rf 0.52 ± 0.05 at 252 nm. Calibration curve was linear in range 1000-3000 ng/band for Salmeterol xinafoate. Stress degradation study includes hydrolysis under different pH, oxidation, thermal and photolytic conditions. The suitability of this HPTLC method for quantitative estimation of Salmeterol xinafoate was proved by validation in accordance with requirements of ICH guidelines Q2A (R1).

Keywords: Salmeterol xinafoate, HPTLC, Stability, Forced degradation studies, Validation, ICH guideline.

INTRODUCTION
Salmeterol xinafoate belongs to Bronchodilator drugs category. Chemically it is (RS)-4-hydroxy-α-[[[6-(4-phenyloxy) hexyl] amino] methyl]-1, 3-benzenedimethanol 1-hydroxy-2-naphthoate. The chemical formula is C_{25}H_{27}NO_{4}, C_{31}H_{32}O_{3} and molar mass is 603.756g/mol. Salmeterol xinafoate is official in IP. It is freely soluble in Methanol. Literature survey reveals few analytical methods are reported for the determination of Salmeterol xinafoate viz. HPLC4, SIM RP-HPLC5, UPLC6, HPTLC7, UV spectrophotometric8 but no stability indicating HPTLC method of Salmeterol xinafoate has yet been reported. Development of SIM is based on systematic exposure of API to various stress conditions. Systematic optimization trials are required to arrive at combination of “concentration of stress reagent and duration of exposure”, to obtain degradation preferably in the 10-30% range. Typical degradative conditions involve hydrolysis under different pH conditions, photolysis, oxidation and thermal studies.

MATERIALS AND METHODS
Reagents and Chemicals
Working standard of Salmeterol xinafoate was obtained from NATCO Pharma (Hyderabad, India). Methanol (AR grade), Chloroform (AR grade), Ammonia (AR grade) Hydrochloric acid (HCl), 6% w/v Hydrogen peroxide (H_{2}O_{2}), Sodium hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. LTD. Mumbai. Preparation of Standard Stock Solution
Standard stock solution of Salmeterol xinafoate was prepared by dissolving 10 mg of drug in 10 ml of methanol get concentration of 1000 µg/ml. From the standard stock solution working standard solution of drug was prepared containing 100µg/ml of Salmeterol xinafoate using methanol.

Selection of Detection Wavelength
The λmax of Salmeterol xinafoate was determined from spectrum using UV Spectrophotometer (V-730 model) make JASCO. From the standard stock solution further dilutions were done using methanol and scanned over the range of 200 - 400 nm and the spectrum was obtained to finalise wavelength for detection.

Mobile phase optimization
The mobile phase optimized for Salmeterol xinafoate such that the Rf should be in the range of 0.2-0.8 and the band should be compact.

Chromatographic state and Instrumentation
Chromatographic separation of drug was performed on Aluminium plates precoated with silica gel 60 F_{254}, (10 cm x 10 cm with 250 µm layer thickness). Salmeterol xinafoate were applied on the plate as a band with 6 mm width using CAMAG 100 µl sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (CAMAG, Switzerland). Densitometric scanning was performed at 252 nm on CAMAG TLC scanner 3. Development of densitogram was done in twin glass chamber previously saturated with the mobile phase Chloroform: Methanol: Ammonia (7:3:0.5 v/v/v) for 15min. operated by WINCATS software (Version 1.4.3, CAMAG), slit dimensions were 5.00 x 0.45 mm and Deuterium lamp was used as a radiation source.

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Forced degradation studies
Stress testing studies were carried out on drug to provide evidence on how the quality of drug varies under the influence of variety of stress conditions like hydrolysis under different pH conditions, oxidation. Dry heat and photolytic were carried out in solid state. All studies were carried out at concentration level of 1500 ng/band and 2000 ng/band. Optimization of stress conditions was done by changing strength of reagent and duration of exposure to obtain degradation preferably in the 10-30% range.

Alkali hydrolytic condition
For alkali degradation study, 1 ml of (1mg/ml) stock solutions of Salmeterol xinafoate was taken into 10ml volumetric flask and 1 ml of 1N sodium hydroxide was

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structure.png}
\caption{Structure of Salmeterol xinafoate.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{uv_spectrum.png}
\caption{UV Spectrum of Salmeterol xinafoate at 252 nm.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{densitogram.png}
\caption{Densitogram of standard solution of Salmeterol xinafoate 100ng/band (Rf 0.52 ± 0.05).}
\end{figure}
### Table 1: Summary of stress degradation Salmeterol xinafoate.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Parameters</th>
<th>Condition</th>
<th>% Recovery</th>
<th>Peak purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkali hydrolysis</td>
<td>1N NaOH overnight at RT</td>
<td>75.59</td>
<td>0.999</td>
</tr>
<tr>
<td>2</td>
<td>Acid hydrolysis</td>
<td>1N HCl overnight at RT</td>
<td>80.59</td>
<td>0.999</td>
</tr>
<tr>
<td>3</td>
<td>Neutral hydrolytic</td>
<td>H2O2 overnight at RT</td>
<td>99.69</td>
<td>0.999</td>
</tr>
<tr>
<td>4</td>
<td>Oxidative stress</td>
<td>Dehydration</td>
<td>74.88</td>
<td>0.999</td>
</tr>
<tr>
<td>5</td>
<td>Dry heat degradation</td>
<td>Hot air oven at 80°C for 2 hours UV light</td>
<td>96.20</td>
<td>0.999</td>
</tr>
<tr>
<td>6</td>
<td>Photolytic degradation</td>
<td>Cool white fluro light (1.2 million lux hours)</td>
<td>95.71</td>
<td>0.999</td>
</tr>
</tbody>
</table>

### Table 2: Recovery studies of Salmeterol xinafoate at 252 nm.

<table>
<thead>
<tr>
<th>Level (%)</th>
<th>Total Conc. (ng/band)</th>
<th>Spiked Conc. (ng/band)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>1800</td>
<td>1810.59</td>
<td>100.59</td>
</tr>
<tr>
<td>100</td>
<td>2000</td>
<td>1917.94</td>
<td>98.71</td>
</tr>
<tr>
<td>120</td>
<td>2200</td>
<td>2216.52</td>
<td>100.75</td>
</tr>
</tbody>
</table>

The standard Salmeterol xinafoate were exposed to UV lamp (200 watt hours/square meter) and cool white fluorescent lamp (1.2 million lux hours). 10 mg was accurately weighed and dissolved in few ml of methanol into volumetric flask (10 ml) and made up the volume with methanol. Further diluted with methanol to attain the working standards of 100 µg/ml concentration. 15 µl and 20 µl of the resultant solution was then applied at TLC plate and densitogram was developed. Average 106.22 % and 95.71 % of Salmeterol xinafoate was recovered in UV and Fluorescence respectively with no peak of degradant.

### RESULT AND DISCUSSION

It was observed that Salmeterol xinafoate showed considerable absorbance at 252nm. Hence this wavelength was chosen for scanning the TLC plate.

#### Optimization of Densitometric Conditions

The first step in developing this stability indicating HPTLC method is to achieve the resolution of Salmeterol xinafoate with Rf in the range of 0.2 to 0.8. Various binary combinations of solvents were tried. The densitometric separation was achieved by linear ascending development in 10 cm × 10 cm twin trough glass chamber using Chloroform: Methanol: Ammonia 7:3:0.5 v/v/v as mobile phase and detection was carried out at 252 nm. The retention factor for Salmeterol xinafoate was found to be 0.52 ± 0.05. Representative Densitogram of standard solution of Salmeterol xinafoate is shown in figure 3.

#### Result of Forced Degradation Studies

After optimization of the different stress conditions, Salmeterol xinafoate was found to degrade not more than 25.22%. There was no separate peak for product of degradation observed. It was confirmed by applying 10 times higher concentration (15000ng /band for Salmeterol xinafoate) and further confirmed by multiwavelength scanning to observe if any degradation products are present. Peak purity is comparison of absorbance spectra from the start to middle (s, m) and from middle to end (m, e) of the peak to determine if they are homogenous peaks. Peak purity parameters ensured non-interference by product of degradation at Rf of Salmeterol xinafoate.

No peak for degradation product was observed for Salmeterol xinafoate. During either of the stress conditions like hydrolysis, oxidation, dry heat and photolysis. Results of the stress degradation studies are presented in Table 1.
For confirmation of any degradation product is present or not 10 times spotting is done on TLC plate. The overlain spectra of peak obtained at Rf 0.7 is similar for the tracks standard and stress degradation samples, thus indicating that this is not a degradation product developed on

Figure 4: Densitogram of Salmeterol xinafoate after acid and alkaline hydrolysis track 1: methanol, track 2: standard 1500 ng/band, track 3, 4: acid hydrolysis sample (1500 and 15000 ng/band), 5, 6: alkaline hydrolysis sample (1500 and 15000 ng/band).

Figure 4.1: I- Overlay spectrum of Salmeterol xinafoate peak at Rf 0.5 on track 2, 3 & 4 respectively, II- Overlay spectrum of peak obtained at Rf 0.7 on track 2, 3 & 4.

Figure 4.2: Overlay spectrum of peak obtained at Rf 0.5 & 0.6 on track 2 & 4 respectively.
Figure 4.3: III- Overlay spectrum of Salmeterol xinafoate peak at Rf 0.5 on track 2, 5 & 6 respectively, IV- Overlay spectrum of peak obtained at Rf 0.7 on track 2, 5 & 6 respectively.

Figure 4.4: Overlay spectrum of peak obtained at Rf 0.5 & 0.6 on track 2 & 6 respectively.

Figure 5: Densitogram of Salmeterol xinafoate after oxidative hydrolysis and thermal degradation track 1: methanol, track 2: standard 1500ng/band, track 3,4: oxidative hydrolysis sample (1500 and 15000ng/band), 5,6: thermal hydrolysis sample (1500 and 15000ng/band).
Figure 5.1: Overlay spectrum of Salmeterol xinafoate peak at Rf 0.5 on track 2, 3 & 4 respectively, Overlay spectrum of peak obtained at Rf 0.8 on track 2, 3 & 4 respectively.

Figure 5.2: Overlay spectrum of Salmeterol xinafoate peak at Rf 0.5 on track 2, 5 & 6 respectively, Overlay spectrum of peak obtained at Rf 0.8 on track 2, 5 & 6 respectively.

Figure 6: Densitogram of Salmeterol xinafoate after neutral hydrolysis and thermal degradation track 1: methanol, track 2: standard 1500ng/band, track 3,4: oxidative hydrolysis sample (1500 and 15000ng/band), 5, 6: thermal hydrolysis sample (1500 and 15000ng/band).
Figure 6.1: I- Overlay spectrum of Salmeterol xinafoate peak at Rf 0.5 on track 2, 3 & 4 respectively, II- Overlay spectrum of peak obtained at Rf 0.7 on track 2, 3 & 4 respectively.

Figure 7: Densitogram of Salmeterol xinafoate after UV and fluro sample
Track 1: methanol, track 2: standard 1500ng/band, track 3-4: uv sample (1500 and 15000ng/band), 5-6: fluro sample (1500 and 15000ng/band).

Figure 7.1: I- Overlay spectrum of Salmeterol xinafoate peak at Rf 0.5 on track 2, 3 & 4 respectively, II- Overlay spectrum of peak obtained at Rf 0.7 on track 2, 3 & 4 respectively.
exposure to stress conditions.

**Validation of the method**

The method was validated for various parameters in accordance with ICH guidelines.9-10.

**Specificity**

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 0.999, indicating the non-interference of any other peak of degradation product or impurity.

**Linearity**

The calibration curve was obtained in the range of 1000-3000ng/band for Salmeterol xinafoate by applying different volumes on TLC of stock solution (100μg/ml) and peak areas were recorded. Standard calibration graph was plotted of peak area Vs amount applied. The equation of the calibration curve found for Salmeterol xinafoate was 
\[ y = 3.2255x + 2820.1 \]

The coefficient of correlation (r²) was found to be 0.9953 for Salmeterol xinafoate shown in Figure 5.

**Assay**

Assay was performed on marketed formulation. Assay was determined by extrapolation of peak area from linearity equation which was found to be 104.47% for Salmeterol xinafoate.

**Accuracy**

To check accuracy of the method, recovery studies were carried out by adding standard drug to assay at three different levels 80, 100 and 120 %. The drug concentrations were calculated from respective linearity equation.

**Table 3: Robustness study.**

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Parameters</th>
<th>Robust Condition</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Time from spotting to development</td>
<td>Immediate</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After 60 min.</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After 120 min.</td>
<td>1.10</td>
</tr>
<tr>
<td>2</td>
<td>Time from development to scanning</td>
<td>0 min.</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After 60 min.</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After 120 min.</td>
<td>1.55</td>
</tr>
<tr>
<td>3</td>
<td>Mobile phase ratio variation</td>
<td>Chloroform</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>: Methanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>: Ammonia (6.8:3.2:0.5)</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>: Methanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>: Ammonia (7:3:0.5)</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloroform</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>: Methanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>: Ammonia (7.2:8:0.7)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4: Summary of validation study.**

[Figure 7: Densitogram of linearity of Salmeterol xinafoate (Rf 0.57 ± 0.5) 200-1000ng/band.]

[Figure 7.2: III- Overlay spectrum of Salmeterol xinafoate peak at Rf 0.5 on track 2, 5 & 6 respectively. IV- Overlay spectrum of peak obtained at Rf 0.7 on track 2, 5 & 6 respectively.]
<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Validation Parameters</th>
<th>Salmeterol xinafoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linearity Equation $Y = 3.2255x + 2820.1$ ($r^2 = 0.9953$)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Precision (% RSD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intraday</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Interday</td>
<td>0.83</td>
</tr>
<tr>
<td>3</td>
<td>Accuracy % Recovery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>100.59</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>98.71</td>
</tr>
<tr>
<td></td>
<td>120%</td>
<td>100.75</td>
</tr>
<tr>
<td>4</td>
<td>Limit of Detection 92.99 ng/band</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Specificity</td>
<td>Specific</td>
</tr>
<tr>
<td>7</td>
<td>Robustness</td>
<td>Robust</td>
</tr>
<tr>
<td>8</td>
<td>Solution stability</td>
<td>Stable</td>
</tr>
</tbody>
</table>

The results of the recovery studies indicated that the method is accurate for estimation of drug in the blend. The results obtained are shown in Table 2.

**Precision**

The precision of the system was demonstrated by intra-day and inter-day studies. In the intra-day study 3 replicates of 3 standard concentrations (1000, 1500 and 2000ng/band for Salmeterol xinafoate) were analysed in a day and percentage RSD was calculated. For the inter-day study, 3 replicates of 3 different concentrations were analysed and percentage RSD was calculated. For intra-day system precision 0.95, 0.52 and 0.97 % for Salmeterol xinafoate. For inter-day RSD was found to be 0.47, 0.83 and 1.03 % for Salmeterol xinafoate.

**Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

LOD and LOQ were calculated as $3 \sigma/S$ and $10 \sigma/S$, respectively;

Where $\sigma$ is the standard deviation of the lowest concentration response and $S$ is the slope of the calibration plot. The LOD and LOQ were found to be.

LOD of Salmeterol xinafoate = 92.99 ng/band
LOQ of Salmeterol xinafoate = 281.81 ng/band

**Robustness**

Robustness of the method was determined by carrying out the analysis under conditions during which chamber saturation time were altered, Time was also changed from spotting to development and development to scanning and the effects on the peak area was noted.

**Solution stability**

Standard stock solution of Salmeterol xinafoate was found to be stable for 72 hrs if stored at RT.

**CONCLUSION**

Literature survey revealed that few HPLC, simple HPTLC and UV method have been reported for estimation of Salmeterol xinafoate. The present study was aimed to develop stability indicating method for Salmeterol xinafoate that may be used to monitor stability of Salmeterol xinafoate. Validation of stability indicating method for Salmeterol xinafoate using HPTLC confirms that the developed method is precise, specific, and accurate. The present study of stability indicating method for Salmeterol xinafoate may be used to monitor stability of Salmeterol xinafoate.

**ACKNOWLEDGEMENT**

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**REFERENCES**


