DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF MICONAZOLE NITRATE AND CLOBETASOL PROPIONATE IN CREAM BY HPTLC METHOD

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ABSTRACT

For High Performance Thin Layer Chromatography method Identification and Determination were Performed on Pre-coated silica gel 60–F254 alumnum sheet (10×10 mm) using Toluene: Chloroform: Methanol: Ammonia (5:4:1:0.1) as Mobile phase detection done at 235 nm. The Rf values were 0.35 for Clobetasol Propionate and 0.53 for Miconazole Nitrate. Drug is extracted from cream by Soniglation, heating, cooling and Filtration. The linear response for Clobetasol Propionate and Miconazole Nitrate was observed over 20-100 ng/band (r² = 0.999) and 800-4000 ng/band (r² = 0.999) respectively. Limit of Detection and Limit of Quantification were found to be 1.21ng/band and 3.67ng/band for Clobetasol Propionate respectively and 63.88ng/band and 193.58ng/band for Miconazole Nitrate, respectively. It suitability of this HPTLC method for quantitative determination of these compounds is proved by validation in accordance with the requirements of ICH Guidelines. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form.

KEYWORDS: Miconazole Nitrate, Clobetasol Propionate, cream, HPTLC method validation.

INTRODUCTION

Miconazole Nitrate is (RS)-1-(2-(2, 4 Dichlorobenzyloyl)-2-(2,4 dichlorophenyl) ethyl)-1H-imidazole(figure:1)1, synthetic antifungal agent used to treat various fungal infection. Clobetasol propionate is [17-(2'-chlooroacetyl)-9 fluoro-11 hydroxy10,13,16 trimethyl-3-oxo 6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl] propanoate(figure:24), it has a potent glucocorticosteroid and weak mineralocorticosteroid activity. Rationale behind these combination to treat fungal infection and produce anti-inflammatory action.

There are some works dealing with the determination of Miconazole Nitrate and other ARA-II in pharmaceuticals and biological samples includes Spectrophotometric5-7, HPLC8-11, Gas Chromatography12-13, HPTLC14 Voltammetric determination15. for Clobetasol Propionate and other ARA-II in pharmaceuticals and biological samples and HPLC16-23, Spectrophotometric24 and Voltammetric determination25 methods are available.
Miconazole Nitrate Produce its anti fungal action by Lanosterol converted to Ergosterol for that required enzyme 14-α demethylase, a cytochrome P-450 enzyme these enzyme is inhibited by Miconazole Nitrate and Produce anti fungal infection \(^{(2)}\)

Clobetasol Propionate is a corticosteroid which has high glucocorticoid activity & weak minelocorticoid activity. which thought to be act by induction of phospholipase A\(_2\) inhibitory proteins, collectively called as lipocortins. \(^{(4)}\)

The aim of the present work is to develop an Accurate, Specific, and Repeatable HPTLC method for the determination of Clobetasol Propionate and Miconazole Nitrate in cream using Toluene: Chloroform: Methanol: Ammonia (5:4:1:0.1v/v/v/v) as mobile phase on silica gel 60 GF\(_{254}\) TLC plates (Merck). Quantitative estimation was accomplished by densitometric scanning with UV detector at 235 nm wavelength. The method was successfully applied to the dosage form developed.

![Figure 1](attachment:RS-1-2-(2,4 Dichlorobenzyloy)-2-(2,4 dichlorophenyl) ethyl)-1H-imidazole.png)

**Figure 1** [(RS)-1-(2-(2,4 Dichlorobenzyloy)-2-(2,4 dichlorophenyl) ethyl)-1H-imidazole]

![Figure 2](attachment:17-(2'-chloroacetyl)-9 fluoro-11 hydroxy10,13,16 trimethyl-3-oxo 6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl] propanoate

**Figure 2** [17-(2'-chloroacetyl)-9 fluoro-11 hydroxy10,13,16 trimethyl-3-oxo 6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl] propanoate
MATERIALS AND METHODS

Miconazole Nitrate was obtained as a gift samples Mahrshee Laboratories, Bharuch. Clobetasol Propionate was obtained as a gift samples Sumit Laboratories, Ahmedabad. Methanol, Chloroform, Toluene, Ammonia used was of analytical grade and obtained from RANKEM fine chemicals.

Instrumentation

The HPTLC system consisted of a CAMAG Linomat 5 automatic spotting device, densitometric analysis was carried out utilizing CAMAG TLC scanner - 3, and these are supported with CAMAG WinCATS software. CAMAG twin-trough chamber (10 cm × 10 cm) was used. A 100 µl HPTLC syringe was used for application of samples. Sample application was done on precoated silica gel 60 F254. (Merck, Germany).

STANDARD SOLUTION PREPARATION

Preparation Standard solution of Clobetasol Propionate

Standard stock solutions of CLP (10µg/ml) was prepared separately by dissolving 10mg of drug in 100ml of methanol.

Preparation Standard solution of Miconazole Nitrate

Standard stock solutions of MIN (400 µg/ml) was prepared separately by dissolving 10mg of drug in 10ml of methanol, Sonigation for 5 min if required, from that taken 4 ml and volume make up to 10 ml with methanol

Preparation for Standard Solution of Clobetasol Propionate and Miconazole Nitrate in combination

Standard stock solutions of CLP (10µg/ml), and MIN (400 µg/ml) were prepared in a common volumetric flask by dissolving 10 mg and 40 mg of CLP and MIN in 100ml of methanol.

Preparation for Test Solution (From cream)

✓ Weight accurately 1 gm of the cream (contain Clobetasol propionate 0.05% and Miconazole nitrate 2.0%) dissolved in 50 ml methanol and sonigated for 15 min.

✓ Heat at 30 °C until base is dissolved, and cool at room temperature and filter the extract and make up the volume 100 ml with solvent Methanol.

✓ Final Stock solution (Containg Miconazole Nitrate 20,000µg/ml and Clobetasol Propionate 500 µg/ml)

✓ Take 0.8 mL dilute up to 10 ml(Containg Miconazole Nitrate 1600 µg/ml and Clobetasol propionate 40 µg/ml) Mobile phase from these stock 1 ml and diluted up to 10 ml(Contain Miconazole Nitrate 160µg/ml and Clobetasol Propionate 4µg/ml) with
mobile phase.

**CHROMATOGRAPHIC CONDITIONS**

Analysis was performed on 10 cm × 10 cm HPTLC silica gel 60 F254 plates. Sample and standard zones were applied to the layer as bands by means of a CAMAG. Linomat 5 automated spray - on applicator equipped with a 100 µl syringe and operated with the settings as band length 6 mm, distance from the plate side edge 15 mm, and distance from the bottom of the plate 10 mm. The chromatography developed using a mixture of Toluene: Chloroform: Methanol: Ammonia (5:4:1:0.1) as Mobile phase Pre-coated silica gel G_{60}-F_{254} aluminium sheet (10×10 mm) development under the following conditions, chamber saturation time, 30 min; temperature, 25 ± 2°C; migration distance, 80 mm. After development, the TLC plates were dried. Quantification of Miconazole Nitrate was achieved by scanning with CAMAG TLC scanner 3 (slit dimension, 5 mm × 0.45 mm; wavelength of determination, 235 nm at absorbance/reflectance mode using D2 lamp, scanning speed, 10 mm/s).

**VALIDATION OF PROPOSED METHOD**

**Calibration Plots**

A series of standard curves were prepared over a concentration range 20-100 ng/band and 800-4000 ng/band for CLP and MIN, respectively. The data of area under the peak versus drug concentration was treated by linear least square regression analysis.

**Method Validation** [9, 10, 11]

The developed method was validated as per ICH guidelines. The developed method was validated in terms of Linearity, Specificity, Recovery, Precision, Robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ).

**Specificity**

The specificity of the method was ascertained by analyzing the standard drug and sample. The spot for Miconazole Nitrate and Clobetasol Propionate in sample was confirmed by comparing the retention factor (Rf) and spectra of the spot with that of standard.

**Linearity**

Linearity of method was carried out by using standard solution of Clobetasol Propionate and Miconazole Nitrate in concentration range of 20-100 ng/band and 800-4000 ng/band. The data of area under the peak versus drug concentration was treated by linear least square regression analysis.

**Precision**

**Interday precision**
Interday variations for determination of Clobetasol Propionate and Miconazole Nitrate were carried out at three different concentration levels in triplicate - 20, 60, 80 ng/band for CLP and 800, 2400, 3200 ng/band for MIN at three different days.

**Intraday precision**

Intraday precision for determination of Clobetasol Propionate and Miconazole Nitrate were carried out at three different concentration levels in triplicate - 20, 60, 80 ng/band for CLP and 800, 2400, 3200 ng/band for MIN on same day. Repeatability is also termed intra-assay or intraday precision. The reproducibility of sample application by measurement of concentration with respect area for MCN was expressed in terms of SD and % RSD for intraday and interday precision.

**Recovery**

Recovery studies were carried out by applying extraction of Drug from cream to which the known amount of Miconazole Nitrate and Clobetasol Propionate had been spiked. Recovery study was performed by spiking 80%, 100% and 120% amount of standard drug externally to the test solution of drug. The experiment was conducted in triplicate. This was conducted to check the recovery of drug at different levels.

**Robustness**

By introducing small changes in the mobile phase composition and saturation time the effects on the results were examined.

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

LOD and LOQ were calculated from equation

**Application of the Proposed Method for Analysis of CLP and MIN in Cream**

**Tenovate – M Contain**

- Clobetasol Propionate 0.05%
- Miconazole Nitrate 2.00%
- Chlorocresol (as preservative) 0.1%

w/w in non greasy base

take 1 gm of cream dissolved in 50 ml of Methanol, sonigated for 15 min, heat at 30°C until base was dissolved, then cool at room temperature, then filter until filtrate became clear, and final volume make-up up to mark 100 ml with methanol.

**RESULTS AND DISCUSSION**

**Chromatography**

In this study the quantitative HPTLC method was developed for the estimation of
Clobetasol Propionate and Miconazole Nitrate in the developed Cream. Various blends of solvent systems in varying proportions were tried as mobile phase. However, the solvent system comprising of Toluene: Chloroform: Methanol: Ammonia (5:4:1:0.1 v/v/v/v) was found to give a good separation and resolution of Clobetasol Propionate and Miconazole Nitrate without interference from the other materials. The peak area on the chromatogram was used for quantitative determination. During the development of the HPTLC method it was observed that a pre-saturation of the TLC chamber with mobile phase for at least 30 min was required to obtain a good separation with reproducible Rf values. The Rf value of Clobetasol Propionate Miconazole Nitrate was found to be 0.35 and 0.53.

Figure 1: Densitograms of mixed standard solution of CLP and MIN (40 and 1600 ng/band) using mobile phase Toluene: Chloroform: Methanol: ammonia (5:4:1:0.1)

**Linearity and Range**

Linearity in the concentration range was 20-100 ng/band and 800-4000 ng/band for CLP and MIN, respectively. (Table 1)

Correlation coefficient ($r^2$) for calibration curve of CLP and MIN was found to be 0.999 and 0.999, respectively

The regression line equation for CLP and MIN are as following,

\[ y = 15.45x + 121.7 \text{ for CLP } \quad (1) \]
\[ y = 1.138x + 238.9 \text{ for MIN } \quad (2) \]
Table 1 Calibration data for CLP and MIN *(n=6)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Concentration (ng/band)</th>
<th>Peak Area ± SD* CLP</th>
<th>Peak Area ± SD* MIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLP</td>
<td>MIN</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>800</td>
<td>575.56 ± 10.1</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>1600</td>
<td>969.2 ± 7.22</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>2400</td>
<td>1389.6 ± 11.32</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>3200</td>
<td>1764.66 ± 13.35</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>4000</td>
<td>2203.94 ± 8.71</td>
</tr>
</tbody>
</table>

Figure 2 Calibration curve for CLP

Figure 3 Calibration curve for MIN
Figure 4 overlain calibration chromatogram of CLP (20-100 ng/Band)& MIN (800-4000 ng/Band)

Precision

Intraday precision

- The data for intraday precision for combined standard solution of CLP and MIN is presented in Table 2
- The % R.S.D was found to be 0.22-0.80 % for CLP and 0.16-0.39 % for MIN.
- These %RSD value was found to be less than ± 1.0 indicated that the method is precise.

Table 2 Intraday precision data for estimation of CLP and MIN *(n=3)*

<table>
<thead>
<tr>
<th>Conc. (ng/band)</th>
<th>Peak area ± SD*</th>
<th>% RSD</th>
<th>Peak area ± SD*</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLP MIN</td>
<td>CLP MIN</td>
<td></td>
<td>CLP MIN</td>
<td></td>
</tr>
<tr>
<td>20 800</td>
<td>585 ± 4.72</td>
<td>0.80</td>
<td>1151 ± 4.851</td>
<td>0.39</td>
</tr>
<tr>
<td>60 2400</td>
<td>1374 ± 5.033</td>
<td>0.36</td>
<td>2979 ± 5.033</td>
<td>0.16</td>
</tr>
<tr>
<td>80 3200</td>
<td>1750 ± 4.005</td>
<td>0.22</td>
<td>3871 ± 7.631</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Interday precision

- The data for intraday precision for combined standard solution of CLP and MIN is presented in Table 3
- The % R.S.D was found to be 0.40-0.93 % for CLP and 0.09-0.10 % for MIN.
- These %RSD value was found to be less than ± 1.0 indicated that the method is precise.
Table 3 Interday precision data for estimation of CLP and MIN *(n=3)

<table>
<thead>
<tr>
<th>Conc. (ng/band)</th>
<th>Peak area ± SD*</th>
<th>% RSD</th>
<th>Peak area ± SD*</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLP</td>
<td>MIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>589.33 ± 5.05</td>
<td>0.93</td>
<td>1147.66 ± 4.04</td>
<td>0.35</td>
</tr>
<tr>
<td>60</td>
<td>1380 ± 0.579</td>
<td>0.53</td>
<td>2979.33 ± 3.05</td>
<td>0.10</td>
</tr>
<tr>
<td>80</td>
<td>1746.33 ± 7.09</td>
<td>0.40</td>
<td>3869 ± 3.60</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Accuracy

- Accuracy of the method was determined by recovery study from Cream at three levels (80%, 100%, and 120%) of standard addition.
- The % recovery values are tabulated in Table 4 and 5.
- Percentage recovery for CLP and MIN by this method was found in the range of 100.97-102.77 % and 99.97 to 100.31 %, respectively.
- The value of %RSD within the limit indicated that the method is accurate and percentage recovery shows that there is no interference from the excipients.

Table 4 Recovery data of CLP *(n=3)

<table>
<thead>
<tr>
<th>Conc. of CLP from formulation (ng/band)</th>
<th>Amount of Std.CLP added (ng/band)</th>
<th>Total amount of CLP (ng/band)</th>
<th>Total amount of CLP found (ng/band)* Mean ± SD</th>
<th>% Recovery (n=3)</th>
<th>% RSD CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>16</td>
<td>3</td>
<td>36.35 ± 0.26</td>
<td>100.97</td>
<td>0.74</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>4</td>
<td>40.66 ± 0.32</td>
<td>101.61</td>
<td>0.79</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>4</td>
<td>45.28 ± 0.78</td>
<td>102.77</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 5 Recovery data of MIN *(n=3)

<table>
<thead>
<tr>
<th>Conc. of MIN from formulation (ng/band)</th>
<th>Amount of Std.MIN (ng/band)</th>
<th>Total amount of MIN (ng/band)</th>
<th>Total amount of MIN found (ng/band)* Mean ± SD</th>
<th>% Recovery (n=3)</th>
<th>% RSD MIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>640</td>
<td>1440</td>
<td>1439.6 ± 7.61</td>
<td>99.97</td>
<td>0.52</td>
</tr>
<tr>
<td>800</td>
<td>800</td>
<td>1600</td>
<td>1603.63 ± 7.25</td>
<td>100.22</td>
<td>0.45</td>
</tr>
<tr>
<td>800</td>
<td>960</td>
<td>1760</td>
<td>1765.45 ± 11.41</td>
<td>100.31</td>
<td>0.64</td>
</tr>
</tbody>
</table>
Limit of Detection and Limit of Quantitation

- The LOD for CLP and MIN was conformed to be 1.21 ng/band and 63.88 ng/band respectively.
- The LOQ for CLP and MIN was conformed to be 3.67 ng/band and 193.58 ng/band respectively.

Robustness and Ruggedness

- The obtained Ruggedness and Robustness results are presented in Table 6
- The % R.S.D was found to be 0.34-0.87% for CLP and 0.39-0.52 % for MIN.
- These %RSD value was found to be less than ± 1.0 indicated that the method is precise.
- No significant changes in the Peak area were observed, proving that the developed method is rugged and robust.

Table 6 Robustness and Ruggedness data of CLP and MIN *(n=3)*

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Factor</th>
<th>Drug</th>
<th>Level</th>
<th>Peak area ± SD</th>
<th>%RSD</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Change in the Saturation time</td>
<td>CLP (60 ng/band)</td>
<td>20 min</td>
<td>1398.34 ± 0.74</td>
<td>0.74</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 min</td>
<td>1438 ± 13</td>
<td>0.87</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIN (2400 ng/band)</td>
<td>20 min</td>
<td>2913.33 ± 15.27</td>
<td>0.52</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 min</td>
<td>3063.6 ± 15.35</td>
<td>0.48</td>
<td>0.57</td>
</tr>
<tr>
<td>2.</td>
<td>Change in the Ratio of mobile phase</td>
<td>CLP (60 ng/band)</td>
<td>9.8</td>
<td>1448.33 ± 7.63</td>
<td>0.52</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.2</td>
<td>1450 ± 5</td>
<td>0.34</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIN (2400 ng/band)</td>
<td>9.8</td>
<td>2964.33 ± 0.47</td>
<td>0.47</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.2</td>
<td>3003.66 ± 0.39</td>
<td>0.39</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Application of the Proposed Method for Analysis of CLP and MIN in Cream (TENOVATE-M)

- Chromatogram of the Test solution containing 60ng/band of CLP and 2400ng/band of MIN was recorded and peak areas were noted for estimation of CLP and MIN, respectively.
- The concentration of CLP and MIN in Cream was determined against the standard CLP and MIN.

- The results from the analysis of Cream containing CLP (60 ng/band) and MIN (2400 ng/band) in combination are presented in Table 7.2.3.10.

- The percent assay shows that there is no interference from excipients and the proposed method can successfully applied to analysis of commercial formulation containing CLP and MIN. The % assay values are tabulated in Table 7.2.3.10.

Table 7: Analysis data of commercial formulation *(n=3)

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Formulation (Cream) (ng/band)</th>
<th>Peak area CLP</th>
<th>% Assay CLP±RSD*</th>
<th>Peak area MIN</th>
<th>% Assay MIN ±SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>1373</td>
<td>99.47 ±</td>
<td>2950</td>
<td>99.04 ± 0.017</td>
</tr>
<tr>
<td>2</td>
<td>2400</td>
<td>1375</td>
<td>0.56</td>
<td>2943</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1369</td>
<td></td>
<td>2955</td>
<td></td>
</tr>
</tbody>
</table>

Table 8: Summary of validation parameters

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>RP-HPTLC Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clobetasol Propionate</td>
</tr>
<tr>
<td>Concentration range (ng/band)</td>
<td>20-100</td>
</tr>
<tr>
<td>Regression equation</td>
<td>y = 15.45x + 121.7</td>
</tr>
<tr>
<td>Correlation Coefficient (r²)</td>
<td>0.999</td>
</tr>
<tr>
<td>Accuracy (% Recovery) (n=3)</td>
<td>101.80%</td>
</tr>
<tr>
<td>Intra-day Precision (%RSD) (n=3)</td>
<td>0.22-0.80</td>
</tr>
<tr>
<td>Inter-day precision (% RSD) (n=3)</td>
<td>0.40-0.93</td>
</tr>
<tr>
<td>LOD (ng/spot)</td>
<td>1.21</td>
</tr>
<tr>
<td>LOQ (ng/spot)</td>
<td>3.67</td>
</tr>
<tr>
<td>Ruggedness and Robustness (%)</td>
<td>0.34-0.87</td>
</tr>
<tr>
<td>% Assay</td>
<td>99.47</td>
</tr>
</tbody>
</table>
CONCLUSION

Indicating method was developed on HPTLC for Clobetasol Propionate and Miconazole Nitrate in cream formulation, for the first time in order to analyze more samples in less time. The proposed method is easy to perform, precise, accurate, rapid and reasonably specific and rugged. The whole procedure may be extended to pharmaceutical preparations and other applications on the same drugs for routine screening without any interference from the excipients. The presented method is an affirmation of both the effectiveness and ecological quality of modern instrumental TLC. As a result of the timely combination of a traditional method and spectrometry, computer-aided technologies, and qualitative as well as quantitative modern planar chromatography are rapidly gaining acceptance throughout the laboratories.

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