SIMULTANEOUS ESTIMATION OF CHLORHEXIDINE DIGLUCONATE AND MICONAZOLE NITRATE BY RP-HPLC

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ABSTRACT
A new RP-HPLC method was developed and validated for the simultaneous determination of chlorhexidine digluconate and miconazole nitrate in a shampoo formulation. The chromatographic separation was achieved by gradient mode with 0.3% Orthophosphoric acid and Acetonitrile, as mobile phase and using RP C-18e (25.0 cm × 4.6 mm i.d, 5µ particle size) column as stationary phase, with a flow rate of 1.0 ml/min at 35°C. The quantification of the analytes was monitored by UV detection at a wavelength of 225 nm.

The optimized method was subjected to validation for specificity, accuracy, precision, linearity range, ruggedness and robustness. The calibration graphs were linear in the concentration range of 0.00010 – 0.00015 mg/ml for chlorhexidine digluconate and 0.00012 – 0.00018 mg/ml for miconazole nitrate. The results were evaluated as per the International Conference on Harmonization (ICH) guidelines. The precision of the method in terms of RSD is not more than 2% for both the analytes. The method is sensitive, specific, fast and accurate with minimum sample preparation.

KEYWORDS: Chlorhexidine digluconate; Miconazole nitrate; HPLC method; Shampoo.

INTRODUCTION
Chlorhexidine digluconate, 1,6-Bis(N5-[p-chlorophenyl]-N1-biguainido)hexane;1,1’-Hexamethylenes (5-[p-chlorophenyl]biguanide) is an antibacterial agent used in disinfectants, cosmetics and pharmaceutical products like preservative in eye drops and antiseptic mouthwashes.[1] Miconazole nitrate (RS)-1-(2-(2,4-Dichlorobenzyloxy)-2-(2,4-dichlorophenyl) ethyl)-1H-imidazole is an antifungal agent commonly applied topically to the skin or to mucous membranes to cure fungal infections.[2] A combination of both is used to treat fungal infections of the skin affecting dogs and cats.[3]

Several methods have been published for determination of Chlorhexidine digluconate and Miconazole nitrate separately using High Performance Liquid Chromatography (HPLC), colorimetry, Capillary Electrophoresis, High Performance Thin Layer Chromatography (HPTLC),[4,5,6] Gas Chromatography[7] and Spectrophotometry.[8] Since both the compounds are highly polar, it is challenging to achieve desired resolution between the peaks during the simultaneous estimation.

It was realized that there is no simple and convenient analytical method for simultaneous estimation in our shampoo formulation containing both the components Chlorhexidine digluconate and miconazole nitrate for regular quality control checks. In the present study, a simple, rapid, and accurate method for simultaneous quantitative analysis of Chlorhexidine digluconate 20% solution and miconazole nitrate in a shampoo formulation used for pets particularly dogs and cats was developed using High Performance Liquid Chromatography. The method was validated with respect to linearity, precision, accuracy and robustness as per the International Conference on Harmonization (ICH) guidelines.[9]

MATERIALS AND METHODS
Chemicals and reagents
Chlorhexidine digluconate and Miconazole nitrate standards from sigma were used. Acetonitrile (HPLC grade), Orthophosphoric acid HPLC grade and Deionized water was purified using Milli Q water system. All other reagents and solvents used in study were of analytical grade.

Equipment
A Shimadzu HPLC system equipped with a quaternary pump, auto sampler, photo diode array detector was used. The system was connected with the help of LC
solution software in a computer system for data collection and processing.

Chromatographic conditions
Column: RP C-18e (25.0 cm × 4.6 mm), 5 µm
Mobile phase: A - 0.3% of orthophosphoric acid and B - Acetonitrile (ACN)
Gradient Program: Gradient programme is mentioned in Table 1
Flow rate: 1.00 ml.min⁻¹
Wavelength: 225 nm
Inj. Vol: 10 µl
Oven Temp: 350°C
Run time: 20 minutes

The mobile phase was filtered using 0.45 µm nylon membrane filter and was degassed by sonication prior to use.

Table 1: Gradient Programme.

<table>
<thead>
<tr>
<th>Time (in min)</th>
<th>Mobile Phase A (in %)</th>
<th>Mobile Phase B (in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>15</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>Stop</td>
<td></td>
</tr>
</tbody>
</table>

Standard and Sample Preparation
Standard Preparation
Stock solution: 25 mg of Chlorhexidine digluconate standard solution and 30 mg of Miconazole nitrate was accurately weighed into a 100 ml volumetric flask and dissolved in methanol. The solution was sonicated for 2 minutes and then made up to the mark with methanol.

Working dilution: From the above stock solution, 5 ml was withdrawn into a 10 ml volumetric flask and made up to the mark with water - methanol mixture (1:1). The solution was filtered using 0.45 µm syringe filter prior to injecting into HPLC.

Preparation of sample: 1.4 g of the shampoo was accurately weighed into a calibrated 100 ml volumetric flask and added 75 ml of methanol and sonicated for 2 minutes. Finally the volume was made up to mark with methanol. 5 ml of this solution was diluted up to 10 ml with water – methanol mixture (1:1). The solution was filtered using 0.45 µm syringe filter prior to injecting into HPLC.

RESULTS AND DISCUSSION
Method development
The system suitability test was carried out by preparing a freshly prepared standard solution of chlorhexidine digluconate solution and miconazole nitrate to check various parameters. The system suitability results are tabulated in Table 2. The chromatogram of the standard is shown in Figure 1.

Table 2: System suitability (N=6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Retention time (RSD %)</th>
<th>Theoretical plate</th>
<th>Tailing factor</th>
<th>Calibration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine digluconate</td>
<td>1.49</td>
<td>26661</td>
<td>1.96</td>
<td>0.0001 mg/ml – 0.00015 mg/ml</td>
</tr>
<tr>
<td>Miconazole nitrate</td>
<td>0.62</td>
<td>35606</td>
<td>2.01</td>
<td>0.00012 mg/ml – 0.00018 mg/ml</td>
</tr>
</tbody>
</table>

N: Number of repetitions.

Figure 1 - Chromatogram of the standard solution of Chlorhexidine digluconate and Miconazole nitrate

Method validation
Specificity: The placebo excluding both chlorhexidine digluconate and miconazole nitrate was prepared and injected. No peaks were observed in the placebo chromatograms corresponding to chlorhexidine digluconate and miconazole nitrate (Fig 2).

Figure 2 - Chromatogram of the placebo solution

Linearity and range: The linearity of the method was observed within the expected concentration range demonstrating its suitability for analysis. The correlation coefficient (R²) was found to be 0.9968 and 0.9935 for chlorhexidine digluconate and miconazole nitrate respectively. The calibration curves for linearity data is shown in Figure 3 and Figure 4.
Figure 3 – Calibration curve for Chlorhexidine digluconate.

Figure 4 – Calibration curve for Miconazole nitrate.

**Accuracy:** The accuracy of the methods was determined by addition of the active ingredients in different concentrations to the placebo matrix. The accuracy was evaluated by the recovery of chlorhexidine and miconazole at three different levels like 80, 100 & 120 % of the label claim. The mean recovery was calculated as shown in Table 3.

<table>
<thead>
<tr>
<th>Level</th>
<th>Recovery % (± SD) (N=3)</th>
<th>80 %</th>
<th>100 %</th>
<th>120 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine gluconate</td>
<td>100.73 ± 0.32</td>
<td>100.08 ± 0.53</td>
<td>99.29 ± 0.53</td>
<td></td>
</tr>
<tr>
<td>Miconazole nitrate</td>
<td>100.44 ± 0.53</td>
<td>99.41 ± 0.65</td>
<td>101.72 ± 0.22</td>
<td></td>
</tr>
</tbody>
</table>

**Intermediate precision:** Intermediate precision was performed by 2 analysts on three replicates of each sample at different points of time. The RSD was found to be < 2%.

**Reproducibility:** The ruggedness study was carried out for miconazole nitrate and chlorhexidine digluconate on the Shampoo base formulation at 100% level of the final test concentration with different analytical equipment’s on different days. The RSD percentage values showed that the results of the assay of the two active ingredients are within a suitable range of < 2%.

**Robustness:** The robustness of analytical method was studied by analyzing the samples with slight variation in the concentration of the buffer (0.29 % and 0.31%). The RSD was found to be < 2%.

The statistical evaluation of the two active ingredients for repeatability, Intermediate precision, reproducibility and robustness are tabulated in Table 4.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chlorhexidine digluconate</th>
<th>Miconazole nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSD, % (Repeatability) (N=6)</td>
<td>1.70</td>
<td>1.29</td>
</tr>
<tr>
<td>RSD, % (IntermediatePrecision)</td>
<td>1.00</td>
<td>1.12</td>
</tr>
<tr>
<td>RSD, % (Reproducibility) (N=3)</td>
<td>0.39</td>
<td>1.30</td>
</tr>
<tr>
<td>RSD, % (Robustness) Condition -1</td>
<td>0.50</td>
<td>1.84</td>
</tr>
<tr>
<td>RSD, % (Robustness) Condition -2</td>
<td>1.20</td>
<td>1.18</td>
</tr>
</tbody>
</table>

**CONCLUSION**

The proposed method is simple, sensitive, precise and accurate. There are no additional extraction or separation procedures to extract the actives from the formulation matrix thereby decreasing the error in quantitation. Also the method is convenient for routine use and is less time consuming. Therefore this method can be applied for routine quality control analysis of a combination of Chlorhexidine digluconate and Miconazole nitrate in the shampoo formulation.

**ACKNOWLEDGEMENT**

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**REFERENCES**

2. Indian Pharmacopeia, Miconazole nitrate Monograph, 2014; 2: 2226-2227.