Development and validation of high-performance liquid chromatography method for determination of miconazole, triamcinolone, methylparaben and propylparaben in cream

Z.Z. Ei\textsuperscript{1}, J. Pimthon\textsuperscript{1,2}, O. Vajragupta\textsuperscript{1,2}, J. Leanpolchareanchai\textsuperscript{3} and C.M. Phechkrajang\textsuperscript{1,2,*}

\textsuperscript{1}Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayuthaya Rd., Payathai, Rachatevi, Bangkok, 10400. Thailand
\textsuperscript{2}Excellent Center for Innovation in Drug Design and Discovery, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayuthaya Rd., Payathai, Rachatevi, Bangkok, 10400. Thailand
\textsuperscript{3}Department of Pharmacy, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayuthaya Rd., Payathai, Rachatevi, Bangkok, 10400. Thailand

Abstract

A simple, precise and accurate reversed-phase high performance liquid chromatographic method was developed for simultaneous determination of miconazole (MIC), triamcinolone (TRI), methylparaben (MEP), propylparaben (PRP) in cream using finasteride (FNT) as an internal standard. The complete separation of the compounds was achieved on a Symmetry\textsuperscript{®} C8 column (150 x 4.6 mm, 5 μm, particle size) with an isocratic elution using a mixture of 5 mM trichloroacetic acid in 0.05% phosphoric acid and acetonitrile (52 : 48 % v/v) as the mobile phase. The mobile phase flow rate was 0.9 mL/min. The UV detection wavelength was at 264 nm. All compounds were separated within 8.0 min. Analytical performance of the intended HPLC procedure was validated for system suitability, accuracy, intermediate precision, repeatability, specificity, linearity and range according to ICH guideline. The percent recoveries were 100.7-104.6 % for MIC and 97.1-99.6 % for TRI. Repeatability and intermediate precision presented as % RSD values were less than 6.1 and 1.8, respectively. The specificity results demonstrated that the determination of MIC and TRI in cream could be performed without interferences from other excipients. The regression for the calibration curve showed linear relationship with the square of correlation coefficient (r) value of MIC and TRI, 0.9988 and 0.9982, respectively, in the concentration ranges of 0.05 to 0.5 mg/mL for MIC and 0.005 to 0.05 mg/mL for TRI. This method has been applied for quantitative determination of MIC and TRI cream formulations obtained from local drugstore.

Keyword: Miconazole, Triamcinolone, Cream, HPLC

1. INTRODUCTION

Many antifungal agents such asazole, polyene and flucytosine, are used for treatment of local and systemic fungal infections\textsuperscript{1}. Azoles containing antifungal agents have broad-spectrum antifungal activities and are used for treatment of candidiasis, cryptococcosis, coccidiomycosis, blastomycosis and histoplasmosis\textsuperscript{2}. However, treatments of fungal infections are complicated with high resistant due to the narrow spectrum of activity, low tolerability and high toxicity. These problems can be solved by combination therapy of antifungal such as 5-flucytosine\textsuperscript{3-6}, amphotericin B\textsuperscript{7-10} and steroids e.g., triamcinolone acetonide\textsuperscript{11}, betamethasone\textsuperscript{12} or beclomethasone\textsuperscript{13}. The combination therapy exhibits greater potency than using monotherapy. Moreover, tolerability, safety, efficacy, broad spectrum activity and bioavailabilities are increased. The combination also decreases the number of resistant organisms and prolonged the activities of antimycotics\textsuperscript{14}. In Thailand, the topical cream combination of miconazole (MIC) and triamcinolone acetonide (TRI) is commercially available. Besides, the two active pharmaceutical ingredients, preservatives such as methylparaben (MEP) and propylparaben (PRP) are added as antimicrobial agents.

*Corresponding author: chutima.mat@mahidol.ac.th
Quantitative determination of miconazole as a single drug or in the combinations with other drugs such as sulfamethazole, metronidazole, econazole, etc can be determined by high performance liquid chromatographic (HPLC) method, spectrophotometric method, gas chromatographic method (GC), capillary electrophoresis chromatography and high-pressure thin layer chromatographic (HPTLC) method. Triamcinolone quantitative determination can be performed by UV derivative spectrophotometric and spectrodensitometric method. However, simultaneous determination of miconazole and triamcinolone in cream by HPLC method is not available. In this work, an accurate, simple and precise HPLC method was developed and fully validated for simultaneous determination of miconazole, triamcinolone, methylparaben, propylparaben in cream by using finasteride as internal standard.

2. EXPERIMENTAL

Apparatus

Chromatography was performed on a high-performance liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) consisting of a degasser DGU-12A, liquid chromatograph LC-10 AD, communications bus module CBM-10A, a UV-Visible detector SPD-10A and a data processing system (class LC-10). The analytical column was a Symmetry® C8, 150 × 3.9 mm i.d., 5µm (Waters, Ireland). Manual injection was made by using a Rheodyne model 7725 injector with a 20-µL loop.

Reagents

Working standard miconazole nitrate was purchased from Prostol Pharm Co.Ltd., Malta. Working standards triamcinolone acetonide, prednisolone, methylparaben and propylparaben were obtained from DMSC, Thailand. Standard finasteride was purchased from Hunan Yuxin Pharmaceutical Co, Ltd, China. Phosphoric acid and trichloroacetic acid (Analytical grade) were obtained from Sigma Chemical (S.M Chemical Co.Ltd., Bangkok, Thailand). Methanol and acetonitrile (HPLC grade) were purchased from RCI Labscan Limited (Bangkok, Thailand).

Sample preparation

About 100 mg of cream was accurately weighed into a centrifuge tube and 5 mL of methanol was added. The mixture was vortexed for 5 min, sonicated for 15 min and immersed in an ice-bath for 20 min. Then, the mixture was centrifuged at 5,000 rpm for 10 min. After that, 5 mL of 0.2% phosphoric acid and acetonitrile (1:1, v/v) was added. The sample preparation steps described above from vortex to centrifuge were repeated again. Finally, the mixture was filtered through 0.45 µm PTFE membrane before injection into HPLC instrument.

Development of HPLC method

HPLC condition was developed for acceptable resolving of miconazole, triamcinolone, methylparaben, propylparaben and an internal standard. Various mobile phase solvents and additives, columns and mobile phase flow rates were varied to achieve the optimum condition. Suitable internal standards were also tried for HPLC method development.

HPLC method validation

The developed HPLC method was validated by testing analytical performance characteristics according to USP and ICH guidelines (20-21). The testing parameters were linearity and range, accuracy, precision and specificity.

Linearity and range

Linearity of the method was performed using six concentrations of standards mixtures. The concentration ranges were 0.05-0.5 mg/mL for MIC, 0.005-0.05 mg/mL for TRI, 0.002-0.012 mg/mL for MEP and 0.0002-0.008 mg/mL for PRP. Calibration curve was separately plotted between peak area ratios of analytes and the internal standard (y-axis) and concentrations (x-axis). The square of correlation coefficient (r), y-intercept and slope of the regression line were calculated by using Microsoft® Excel 2007 program. The acceptable square of correlation coefficient (r) value is equal or greater than 0.99. The lowest and highest concentrations of
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线性曲线被设置为方法的下限和上限范围。

准确性

方法的准确性通过标准添加法来确定。将三种浓度的标准混合物分别加入等量的样品中。目标药物的总浓度覆盖了实际样品中50-150%的浓度范围。每种浓度水平进行了三次重复。方法的准确性以百分数回收率来表示。

精确性

方法的精确性通过日内重复性（日内精确性）和中间精确性（间日精确性）来测定。日内重复性中，三种浓度的标准化合物被测定。每种浓度水平分别在同一天内进行三次测定。中间精确性中，同质样品的六次测定被分析。实验在两天内进行。MIC和TRI在每种测定中的百分数标示量被计算。精确性以重复测试结果的标准偏差（% RSD）来表示。

特异性

HPLC方法的特异性通过比较标准混合物、样品和标准混合物中标准化合物的色谱图来证明。

3. RESULTS AND DISCUSSION

开发HPLC方法

开发高性能液相色谱（HPLC）用于同时测定米康唑（MIC）、三氯生（TRI）、对羟基苯甲酸甲酯（MEP）、对羟基苯甲酸丙酯（PRP）和内标（芬那酸（FNT））的方法由反相柱（C8和C18）进行。在初始方法开发中，C18柱被选中。PNS首先用于内标。由于MIC在该组中具有最低摩尔吸收系数，因此UV检测器的波长被设置为MIC的最大吸收波长，即264 nm。初始流动相是去离子水和乙腈或甲醇的混合物。在这些流动相系统中，PNS、MEP、TRI和PRP没有很好地分离，尽管流动相的比例发生了变化。因为MIC在乙腈和水的系统中被强烈保留，表现为拖尾峰。另一方面，MIC峰在所有甲醇和水的比例中都未被观察到。进一步调整尝试使用酸性溶液。MIC的pKa是6.77，因此它可能被酸化的流动相所酸化，从而减少了MIC和C18固定相的相互作用。三种酸，即磷酸、乙酸和三氯乙酸被尝试。从我们的研究中，MIC可以在含有酸的流动相中观察到。由于MIC被酸化在酸性pH中，导致保留时间的减少。三种亲水性反离子（Cl-从KCl，CCl3COO-从CCl3COOH和B4O72-从Na2B4O7）被尝试作为流动相修饰剂来改善MIC的分离。然而，只有CCl3COO-影响了MIC的保留，且其效果随浓度的增加而增加。实际上，CCl3COO-对MIC的保留行为的影响可能来源于CCl3COOH的酸性，迫使MIC以酸化的形式存在。此外，CCl3COO-表现出强烈的亲水性，导致MIC的酸化形式被强制保留。在酸化形式下，MIC的保留行为被证明。在酸化形式下，MIC的保留行为被证明。因此，我们得出结论，磷酸和三氯乙酸酸是控制MIC的分离和峰形的重要因素。因此，它们在进一步的流动相系统中被保留。结果C18柱被选中从流动相中获得米康唑、乙腈和5 mM三氯乙酸酸的0.05%磷酸酸（30/30/40，% v/v/v）（图2）。
Figure 1. Chemical structures of the investigated compounds and internal standard, FNT.
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There are three parameters that affecting the resolution in chromatography including capacity factor (k'), selectivity factor (α) and the number of theoretical plate (N). Previously modifications of k' and α were done by adjusting mobile phase ratios as well as mobile phase solvents. Unfortunately, the acceptable chromatogram was not obtained. The final attempt was performed by replacing C18 column with C8 in order to alter the interaction strength between the analytes and stationary phase. The mixture of acetonitrile and 5 mM trichloroacetic in 0.05 % phosphoric acid (48/52; %v/v) was employed as mobile phase. An excellent chromatogram was observed from this system as shown in Figure 3. However, PNS was eluted too fast and coeluted with the system peak. Therefore, a new internal standard was searched and eventually, finasteride (FNT) displayed a suitable retention time with acceptable peak shape (Figure 4). In summary, the optimum condition for the separation of MIC, TRI, MEP and PRP was achieved using finasteride (FNT) as internal standard. The stationary phase was reversed-phase C8 and the mixture of acetonitrile and 5 mM trichloroacetic in 0.05 % phosphoric acid (48/52; %v/v) was employed as mobile phase. Isocratic elution was utilized and with the mobile phase flow rate of 0.9 mL/min. All studied compounds were well separated from each other with the total run time of less than 8.0 min.

HPLC method validation

Linearity

The calibration curve of each intended compound was linear with the square of correlation coefficient (r) greater than 0.99. Linearity curves were displayed in Figure 5.

Figure 2. A typical chromatogram of a standard mixture of MIC, TRI, MEP, PRP and PNS. Condition: column: symmetry C18; 150 x 4.6 mm i.d., 5 μm; mobile phase: MeOH / ACN / 5 mM trichloroacetic acid in 0.05 % phosphoric acid (30/30/40; %v/v/v); flow rate: 1 mL/min; UV detector: 264 nm.
Figure 3. A typical chromatogram of a standard mixture of MIC, TRI, MEP, PRP and PNS. Condition: column: symmetry C8; 150 x 4.6 mm i.d., 5 μm; mobile phase: ACN: 5 mM TCA in 0.05 % PA (48 : 52, % v/v); flow rate: 0.9 mL/min; UV detector: 264 nm.

Figure 4. A typical chromatogram of a standard mixture of MIC, TRI, MEP, PRP and FNT. Condition: column: symmetry C8; 150 x 4.6 mm i.d., 5 μm; mobile phase: ACN : 5 mM TCA in 0.05 % PA (48 : 52; % v/v); flow rate: 0.9 mL/min; UV detector: 264 nm.
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**Accuracy**

As shown in Table 1, the mean recoveries of the four compounds in this study were acceptable, the values range from 91.2-95.4% for MEP, 97.1-99.6% for TRI, 100.7-104.6% for MIC and 91.1-95.2% for PRP. These good recoveries implied that the developed HPLC method was suitable for quantitative determination of intended drugs in samples.

**Table 1. Accuracy and repeatability results**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration level</th>
<th>%1</th>
<th>%2</th>
<th>%3</th>
<th>Mean</th>
<th>SD</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>50%</td>
<td>94.5</td>
<td>95.2</td>
<td>96.6</td>
<td>95.4</td>
<td>1.1</td>
<td>1.1</td>
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<td></td>
<td>100%</td>
<td>95.3</td>
<td>91.5</td>
<td>95.5</td>
<td>94.1</td>
<td>2.2</td>
<td>2.4</td>
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<tr>
<td></td>
<td>150%</td>
<td>91.8</td>
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<td>93.7</td>
<td>91.2</td>
<td>2.8</td>
<td>3.1</td>
</tr>
<tr>
<td>TRI</td>
<td>50%</td>
<td>101.9</td>
<td>101.7</td>
<td>95.3</td>
<td>99.6</td>
<td>3.7</td>
<td>3.8</td>
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<tr>
<td></td>
<td>100%</td>
<td>97.2</td>
<td>99.5</td>
<td>94.6</td>
<td>97.1</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>150%</td>
<td>97.9</td>
<td>101.0</td>
<td>95.7</td>
<td>98.2</td>
<td>2.7</td>
<td>2.7</td>
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<tr>
<td>MIC</td>
<td>50%</td>
<td>102.2</td>
<td>100.9</td>
<td>98.9</td>
<td>100.7</td>
<td>1.7</td>
<td>1.6</td>
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<tr>
<td></td>
<td>100%</td>
<td>103.6</td>
<td>102.5</td>
<td>100.8</td>
<td>102.3</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>150%</td>
<td>101.0</td>
<td>107.6</td>
<td>105.3</td>
<td>104.6</td>
<td>3.4</td>
<td>3.2</td>
</tr>
<tr>
<td>PRP</td>
<td>50%</td>
<td>92.2</td>
<td>89.2</td>
<td>91.8</td>
<td>91.1</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
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<td>85.0</td>
<td>92.7</td>
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<td>5.6</td>
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<td>99.1</td>
<td>93.5</td>
<td>95.2</td>
<td>3.3</td>
<td>3.5</td>
</tr>
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</table>
**Precision**

Repeatability and intermediate precision expressed as % RSD values were less than 6.1 and 1.8 for repeatability and intermediate precision, respectively (Table 1 and 2). These results showed that the proposed HPLC method has satisfactory precision for the analysis of MIC and TRI in cream samples.

**Specificity**

Specificity of the method was studied by comparing the chromatograms of the relevant solutions and evaluation of involved chromatographic parameters. As the results showed in Figure 6, it was seen that the retention times and resolution of every peak pairs in the chromatograms of cream containing solutions were similar to chromatogram of standards mixture. Moreover, summation of peak area of each compound in chromatograms of standards mixture and cream closed to the peak area of that compound in the chromatogram of standards mixture spiked cream solution. These results indicated that the developed HPLC method was able to determine amounts of MIC, TRI and PRP in cream without interfering from any excipient.

**Quantitative determination of drugs in cream**

Eventually, the developed and fully validated HPLC method was applied to determine MIC and TRI in real samples. Four brands of cream in the market were enrolled for quantitative determination of MIC and TRI. Assay results were illustrated as percent labeled amounts (% LA) and showed in Table 3.

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### Table 2. Intermediate precision results

<table>
<thead>
<tr>
<th>Sample number</th>
<th>% Labeled amount of MIC</th>
<th>% Labeled amount of TRI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>1</td>
<td>99.7</td>
<td>98.2</td>
</tr>
<tr>
<td>2</td>
<td>98.5</td>
<td>97.3</td>
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<tr>
<td>3</td>
<td>95.3</td>
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<td>4</td>
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<td>96.6</td>
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<td>99.6</td>
<td>97.6</td>
</tr>
<tr>
<td>6</td>
<td>97.6</td>
<td>96.6</td>
</tr>
<tr>
<td>Mean</td>
<td>97.9</td>
<td>97.2</td>
</tr>
<tr>
<td>SD</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>%RSD</td>
<td>1.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

### Table 3. Percent labeled amount of MIC and TRI in commercial creams

<table>
<thead>
<tr>
<th>Brand</th>
<th>% Labeled amount (Mean ± SD, n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
</tr>
<tr>
<td>1</td>
<td>103.8 ± 2.1</td>
</tr>
<tr>
<td>2</td>
<td>101.5 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>99.4 ± 1.4</td>
</tr>
<tr>
<td>4</td>
<td>100.3 ± 1.3</td>
</tr>
</tbody>
</table>
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Figure 6. Chromatograms of (a) standard mixture solution; (b) cream solution; (c) standard mixture spiked cream solution. Condition: column: symmetry C8; 150 x 4.6 mm i.d., 5 μm; mobile phase: ACN : 5 mM TCA in 0.05 % PA (48 : 52; % v/v); flow rate: 0.9 mL/min; UV detector: 264 nm.

4. CONCLUSION
A reversed-phase high performance liquid chromatography was successfully developed for simultaneous quantitative determination of miconazole (MIC), triamcinolone (TRI), methylparaben (MEP) and propylparaben (PRP) in cream preparation using finasteride (FNT) as an internal standard. The developed HPLC method was fully validated for intended purpose. The method validation parameters were selected
according to USP and ICH guidelines for active pharmaceutical ingredient assay method. The proposed HPLC method was applied to determine MIC and TRI in the real samples. Four brands of cream in the market were analyzed for MIC and TRI contents. Results expressed in term of percent labeled amounts illustrated that the drug contents agreed with the products labels.

5. ACKNOWLEDGEMENT

The authors would like to thank the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative for financial support and Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University for research facilities.

REFERENCES


