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

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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® 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 as azole, polyene and flucytosine, are used for treatment of local and systemic fungal infections[1]. Azoles containing antifungal agents have broad-spectrum antifungal activities and are used for treatment of candidiasis, cryptococcosis, coccidiomycosis, blastomycosis and histoplasmosis[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[3-6], amphotericine B[7-10] and steroids e.g., triamcinolone acetonide[11], betamethasone[12] or beclomethasone[13]. The combination therapy exhibits greater potency than using monotherapy. Moreover, tolerability, safety, efficacy, broad spectrum activity and bioavailabities are increased. The combination also decreases the number of resistant organisms and prolonged the activities of antimycotics[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.

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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[15], gas chromatographic method (GC)[16], capillary electrophoresis chromatography[17] and high-pressure thin layer chromatographic (HPTLC)[18] method. Triamcinolone quantitative determination can be performed by UV derivative spectro-photometric and spectrodensitometric method[19]. 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 guidances (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

linearity curve were set as the lower and upper range of the method.

Accuracy

Accuracy of the method was done by standard addition method. Three concentrations of standards mixtures were spiked into equal amount of sample. The total concentrations of target drugs were covered 50-150% concentration range in the real sample. Three replicates were performed for each concentration level. The accuracy of the method was expressed as percent recovery.

Precision

Precision of the method was performed for repeatability (intra-day precision) and intermediate precision (inter-day precision). For repeatability, three concentrations of standard spiked samples were assayed. Three determinations were performed for each concentration level on the same day. For intermediate precision, six determinations of the homogeneous sample were analyzed. The experiments were performed in two consecutive days. Percent labeled amounts of MIC and TRI in each determination were calculated. Precision was expressed as relative standard deviation (% RSD) of the repeated testing results.

Specificity

Specificity of HPLC method was demonstrated by comparing the chromatograms of standards mixture, cream sample and standards mixture spiked cream sample.

3. RESULTS AND DISCUSSION

Development of HPLC method

Development of high performance liquid chromatography (HPLC) for simultaneous determination of miconazole (MIC), triamcinolone (TRI), methylparaban (MEP), propylparaben (PRP) and an internal standard (finasteride (FNT) was performed by reversed- phase columns C8 and C18. Column C18 was selected in initial method development and prednisolone (PNS) was firstly used as an internal standard. Since, MIC has the lowest molar absorption coefficient in this group, the wavelength of the UV detector was set at the maximum absorption wavelength of MIC i.e., 264 nm. The initial mobile phase was the mixture of deionized water and acetonitrile or methanol. In these mobile phase systems, PNS, MEP, TRI and PRP were not well resolved from each others, although the ratios of mobile phase were varied. Because MIC was strongly retained in the system of acetonitrile and water and presented as a tailing peak. On the other hand, MIC peak was not observed at all ratios of methanol and water.

Further adjustment was attempted by replacing deionized water with acid solutions. The pKa of MIC is 6.77, so it may be protonated in acidic mobile phase and the interaction between MIC and C18 stationary phase may be reduced. Three acids i.e., phosphoric acid, acetic acid and trichloroacetic acid were tried. From our studied, MIC peak could be easily observed in mobile phase containing acids.

Since MIC was protonated in acidic pH and resulted in the decrease of retention time. Three hydrophobic counterions (Cl^- from KCl, CCl_3COO^- from CCl_3COOH and $B_4O_7^{2-}$ from $Na_2B_4O_7$) were tried as mobile phase modifiers to improve the separation of MIC. However, only CCl_3COO^- was affected to the retention of MIC and the effect was greater at higher concentrations of CCl_3COO^-. In fact, the effect of CCl_3COO^- on the retention behavior of MIC was probably from the acidity of CCl_3COOH that forced MIC to be presented in protonated form. In addition, CCl_3COO^- exhibited chaotropic effect to the protonated form of MIC (22-23). This was proved by the experiment results that the higher concentrations of CCl_3COO^- caused the stronger retention. We concluded that phosphoric acid and trichloroacetic acid were important to control the separation and peak shape of MIC. Therefore, they were kept in the further mobile phase systems. The best results of C18 column was obtained from the mobile phase containing methanol, acetonitrile and 5 mM trichloroacetic acid in 0.05% phosphoric acid (30/30/40, % v/v/v) (Figure 2).

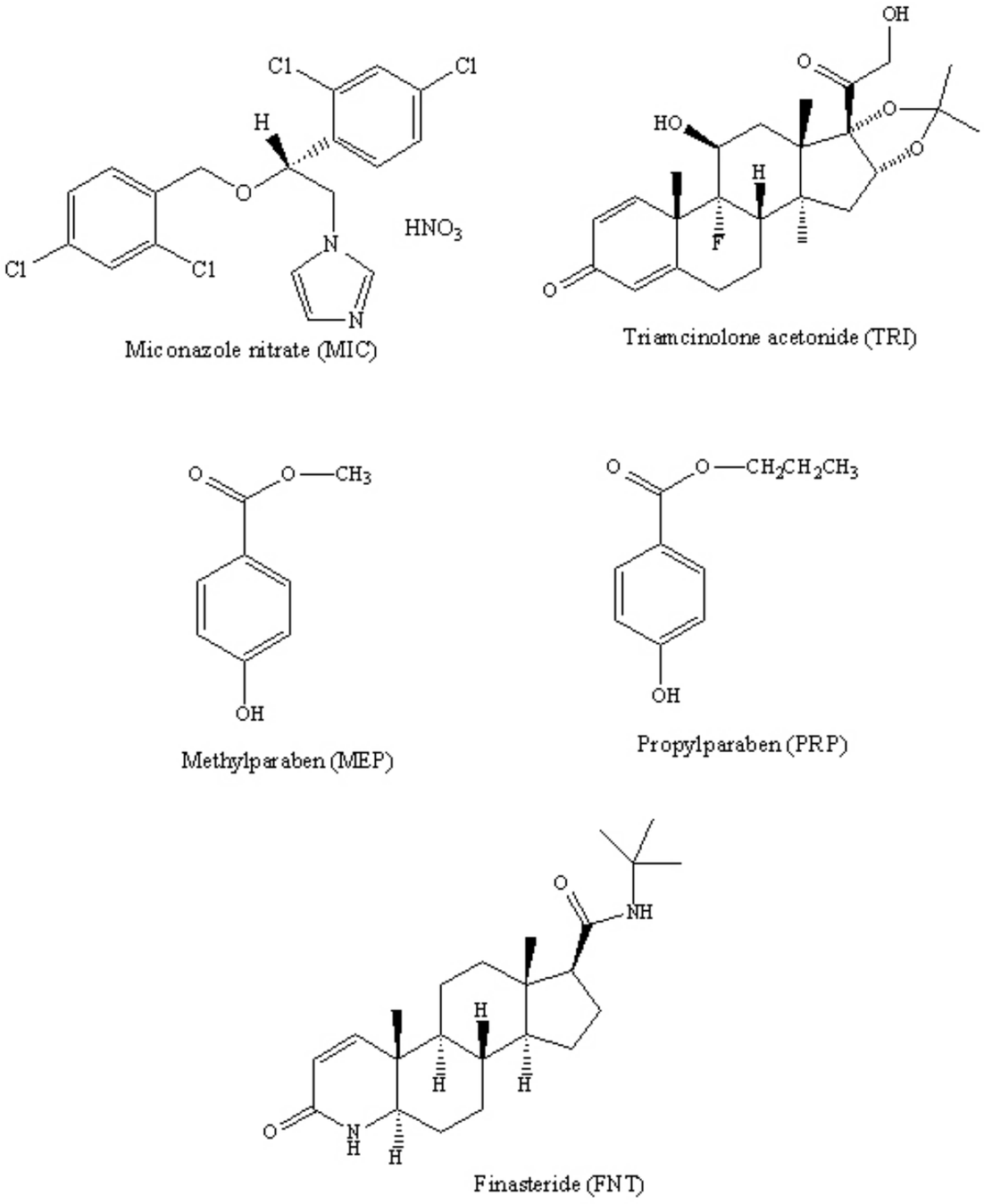

Figure 1. Chemical structures of the investigated compounds and internal standard, FNT.

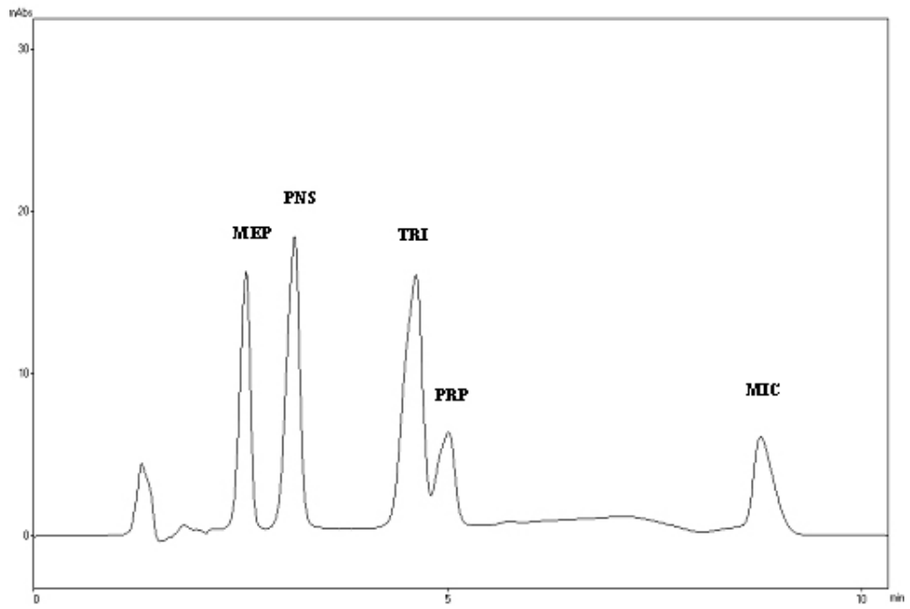

Figure 2. A typical chromatogram of a standard mixture of MIC, TRI, MEP, PRP and PNS. Condition: column: symmetry C18; 150 x 4.6 mm i.d., 5 µm; mobile phase: MeOH / ACN / 5 mM trichloroacetic acid in 0.05 % phosphoric acid (30 / 30 / 40 ; % v/v/v); flow rate: 1 mL/min; UV detector: 264 nm.

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 aceronitrile and 5mM trichloroacetic acid 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. The presence of most compounds was monitored with a UV detector at a wavelength of 264 nm. 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.

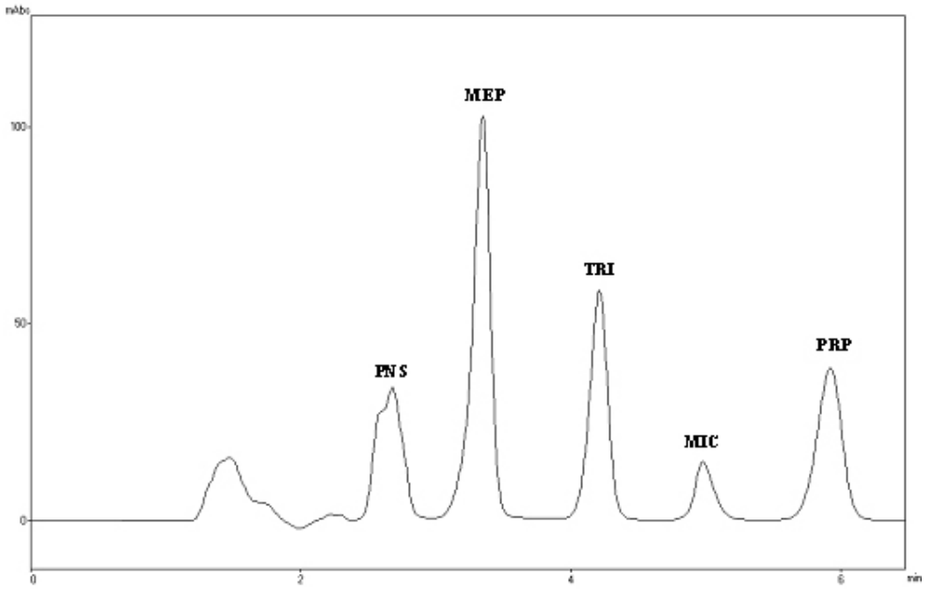

Figure 3. A typical chromatogram of a standard mixture of MIC, TRI, MEP, PRP and PNS. Condition: column: symmetry C8; 150 x 4.6 mm i.d., 5 µm; mobile phase: ACN: 5 mM TCA in 0.05 % PA (48 : 52, % v/v); flow rate: 0.9 mL/min; UV detector: 264 nm.

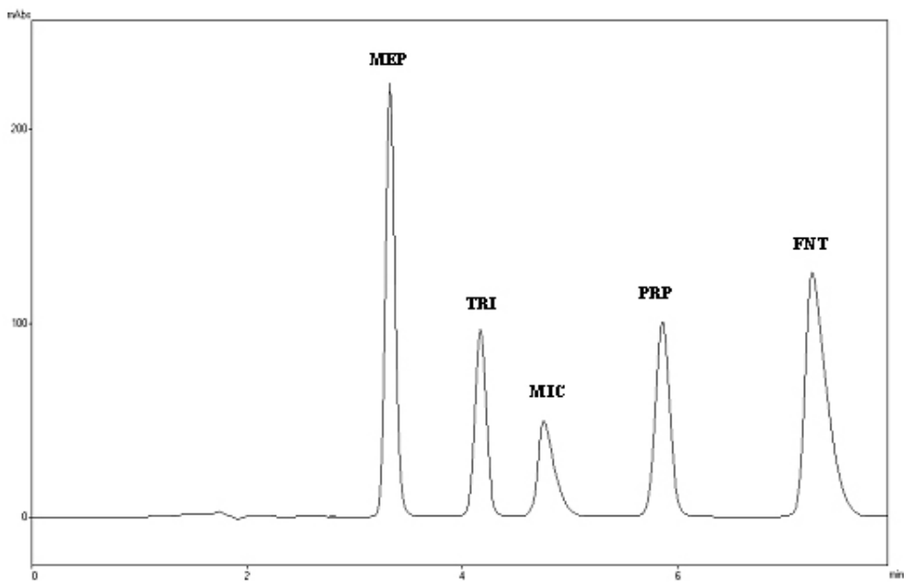

Figure 4. A typical chromatogram of a standard mixture of MIC, TRI, MEP, PRP and FNT. Condition: column: symmetry C8; 150 x 4.6 mm i.d., 5 µm; mobile phase: ACN : 5 mM TCA in 0.05 % PA (48 : 52; % v/v); flow rate: 0.9 mL/min; UV detector: 264 nm.

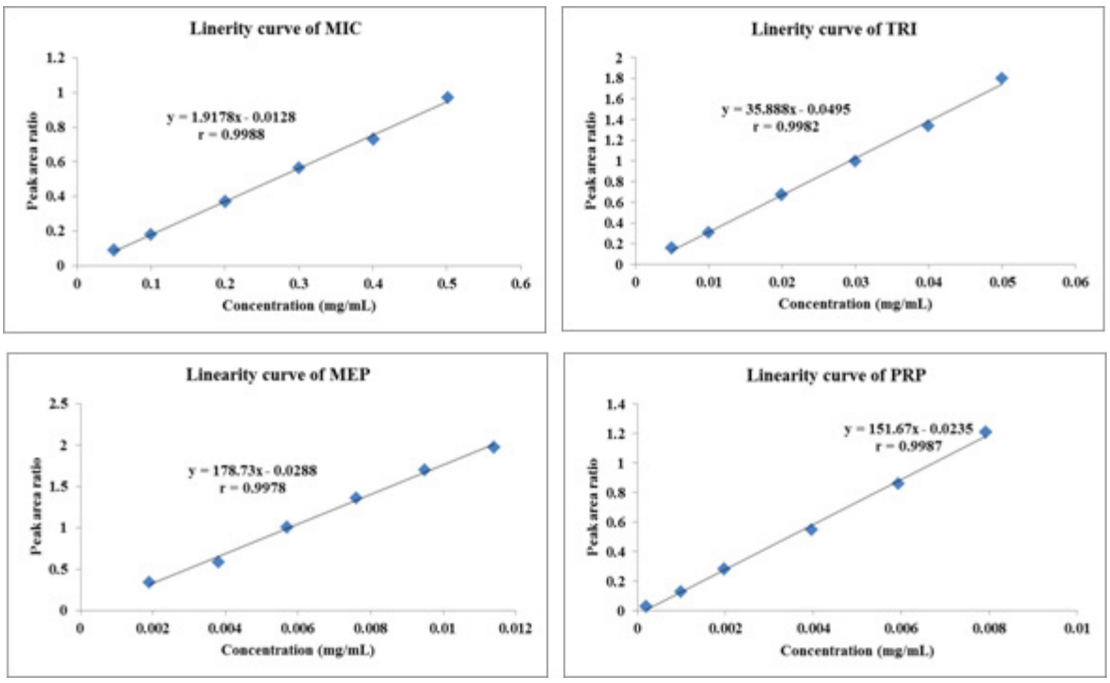

Figure 5. Calibration curves of (A) MIC, (B) TRI, (C) MEP and (D) PRP.

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

Compound	Concentration level	% Recovery					
		#1	#2	#3	Mean	SD	%RSD
MEP	50%	94.5	95.2	96.6	95.4	1.1	1.1
	100%	95.3	91.5	95.5	94.1	2.2	2.4
	150%	91.8	88.2	93.7	91.2	2.8	3.1
TRI	50%	101.9	101.7	95.3	99.6	3.7	3.8
	100%	97.2	99.5	94.6	97.1	2.5	2.5
	150%	97.9	101.0	95.7	98.2	2.7	2.7
MIC	50%	102.2	100.9	98.9	100.7	1.7	1.6
	100%	103.6	102.5	100.8	102.3	1.4	1.4
	150%	101.0	107.6	105.3	104.6	3.4	3.2
PRP	50%	92.2	89.2	91.8	91.1	1.6	1.8
	100%	95.9	85.0	92.7	91.2	5.6	6.1
	150%	93.1	99.1	93.5	95.2	3.3	3.5

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.

Table 2. Intermediate precision results

Sample number	% Labeled amount of MIC		% Labeled amount of TRI	
	Day 1	Day 2	Day 1	Day 2
1	99.7	98.2	106.5	105.9
2	98.5	97.3	105.8	105.8
3	95.3	97.0	106.4	105.2
4	96.5	96.6	107.6	106.6
5	99.6	97.6	106.3	106.0
6	97.6	96.6	107.9	105.7
Mean	97.9	97.2	106.7	105.9
SD	1.8	0.6	0.8	0.4
%RSD	1.8	0.6	0.8	0.4

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

Brand	% Labeled amount (Mean ± SD, n = 3)	
	MIC	TRI
1	103.8 ± 2.1	104.7 ± 2.1
2	101.5 ± 1.1	101.3 ± 1.0
3	99.4 ± 1.4	114.2 ± 1.3
4	100.3 ± 1.3	117.7 ± 1.5

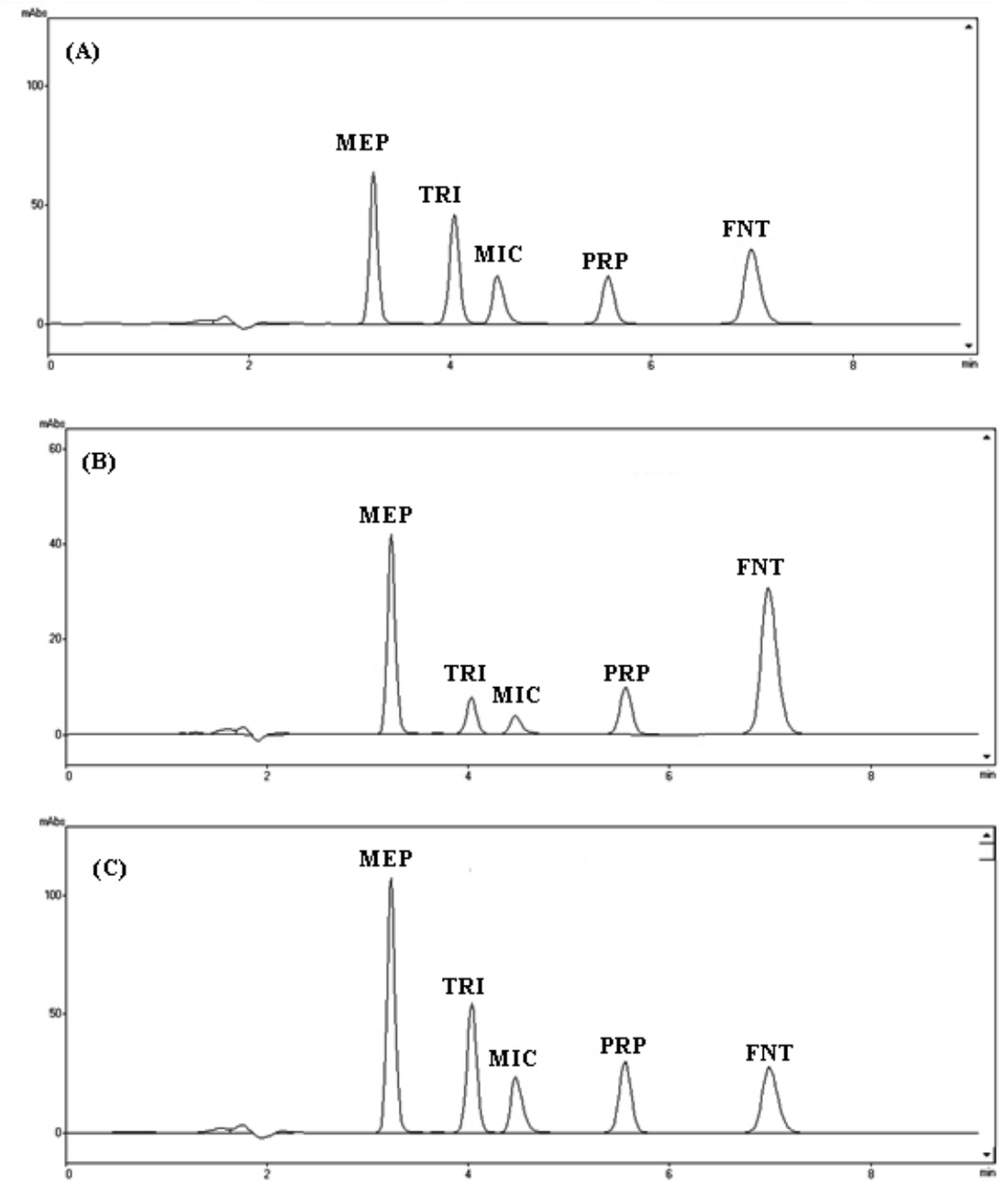

Figure 6. Chromatograms of (a) standard mixture solution; (b) cream solution; (c) standard mixture spiked cream solution. Condition: column: symmetry C8; 150 x 4.6 mm i.d., 5 μm; mobile phase: ACN : 5 mM TCA in 0.05 % PA (48 : 52; % v/v); flow rate: 0.9 mL/min; UV detector: 264 nm.

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.

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REFERENCES

1. Aderiye BI, Oluwole OA. Disruption of fungi cell membranes by polyenes, azoles, allylamines, amino acids and peptides. Int Sci Res J. 2015;1(7):108-116.
2. Abu-Elteen KH, Hamad M. Antifungal agents for use in human therapy. In: Kavanagh K, editor. Fungi Biology and Applications. England: John Wiley and Sons Ltd; 2005. p. 191-217.
3. Barchiesl F, Gallo D, Caselli F, Francesco LFD, Arzeni D, Giacometti A, et al. In-vitro interactions of itraconazole with flucytosine against clinical isolates of *Cryptococcus neoformans*. J Antimicrob Chemother. 1999; 44:65-70.
4. Barchiesi F, Schimizzi AM, Najvar LK, Bocanegra R, Caselli F, Cesare SD, et al. Interactions of posaconazole and flucytosine against *Cryptococcus neoformans*. Antimicrob Agents Chemother. 2001;45(5):1355–1359.
5. Girmenia C, Venditti M, Martino P. Fluconazole in combination with flucytosine in the treatment of fluconazole-resistant Candida infections. Diagn Microbiol Infect Dis. 2003;46:227-231.
6. Scheven M, Jünemann K, Schramm H, Hühn W. Successful treatment of *Candida albicans* sepsis with a combination of flucytosine and fluconazole. Mycoses. 1992;35:315-316.
7. Barchiesi F, Schimizzi AM, Caselli F, Novelli A, Fallani S, Giannini D, et al. Interactions between triazoles and amphotericin B against *Cryptococcus neoformans*. Antimicrob Agents Chemother. 2000;44(9):2435–2441.
8. Lewis RE, Lund BC, Klepser ME, Ernst EJ, Pfaller MA. Assessment of antifungal activities of fluconazole and amphotericin B administered alone and in combination against *Candida Albicans* by using a dynamic in vitro mycotic infection model. Antimicrob Agents Chemother. 1998;42(6):1382–1386.
9. Sanati H, Ramos CF, Bayer AS, Ghannoum MA. Combination therapy with amphotericin B and fluconazole against invasive candidiasis in neutropenic-mouse and infective-endocarditis rabbit models. Antimicrob Agents Chemother. 1997;41(6): 1345–1348.
10. Najvar LK, Cacciapuoti A, Hernandez S, Halpern J, Bocanegra R, Gurnani M, et al. Activity of posaconazole combined with amphotericin B against *Aspergillus Favus* infection in mice: Comparative studies in two laboratories. Antimicrob Agents Chemother. 2004;48(3):758–764.
11. Baker MM, Belal TS, Mahrous MS, Ahmed HM, Daabees HG. A validated stability-indicating HPLC-DAD method for simultaneous determination of econazole nitrate, triamcinolone acetonide, benzoic acid and butylated hydroxyanisole in cream dosage form. Anal Methods. 2016;8:2185-2200.
12. Manassra A, Khamis M, El-Dakiky M, Abdel-Qader Z, Al-Rimawi F. Simultaneous HPLC analysis of betamethasone and clotrimazole in cream formulations. Pharm Anal Acta. 2010;1(2):1-3.
13. Dhudashia KR, Patel AV, Patel CN. Development and validation of a reversedphase HPLC method for simultaneous estimation of clotrimazole and beclomethasone dipropionate in lotion and cream dosage form. Chronicles of Young Scientists 2013; 4(2):102-107.
14. Mukherjee PK, Sheehan DJ, Hitchcock CA, Ghannoum MA. Combination treatment of invasive fungal infections. Clin Microbiol Rev. 2005;18(1):163–194.

15. Alizadeh N, Ansari R, Rezaee NK. Extractive spectrophotometric determination of miconazole nitrate in pure and pharmaceutical preparation. J Pharm Health Sci. 2012; 1(4):45-52.
16. Ashour S, Kattan N. Simultaneous determination of miconazole nitrate and metronidazole in different pharmaceutical dosage forms by gas chromatography and flame ionization detector (GC-FID). Int J Biomed Sci. 2010;6(1):13-18.
17. Hermawan D, Yatim IM, Rahim KA, Sanagi MM, Ibrahim WAW, Aboul-Enein HY. Comparison of HPLC and MEEKC for miconazole nitrate determination in pharmaceutical formulation. Chromatographia. 2013;76:1527–1536.
18. Meshram DB, Bagade SB, Tajne MR. Simultaneous determination of metronidazole and miconazole nitrate in gel by HPTLC. Pak J Pharm Sci. 2009;22(3):323-328.
19. El-Saharty YS, Hassan NY, Metwally FH. Simultaneous determination of terbinafine HCL and triamcinolone acetonide by UV derivative spectrophotometry and spectrodensitometry. J Pharm Biomed Anal. 2002; 28:569–580.
20. The United States Pharmacopeia 39- The National Formulary 34, United States Pharmacopeial Convention, Rockville, MD, 2016.p.459-470.
21. Validation of Analytical Procedures: Text and Methodology (Q2(R1)), ICH Harmonised Tripartite Guideline,finalized on Nov.2005.
22. Kazakevich Y, Lobrutto R, editors. HPLC for Pharmaceutical Scientists. Canada: A John Wiley and Sons, Inc; 2007. p. 139-240.
23. Phechkrajang CM. Chaotropic effect in reversed-phase HPLC : A review. MUJPS 2010;37(1-2):1-7.