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Separation and determination of terbinafine and its four impurities of similar structure using simple RP-HPLC method

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Abstract

A novel reversed-phase HPLC method for the simultaneous determination of active component terbinafine, its one impurity 1-methylaminomethylnaphtalene and three degradation products, β-terbinafine, Z-terbinafine and 4-methyl-terbinafine occurring in pharmaceutical formulations after long-term stability tests, was developed and validated using propylparaben as an internal standard.

The chromatographic separation was performed on a NUCLEOSIL 100-5-CN column, mobile phase for separation of all compounds consisted of a mixture of tetrahydrofurane, acetonitrile and citrate buffer pH 4.50 (10:20:70, v/v/v). The analysis time was less than 32 min at flow-rate of 0.8 ml min^{-1}. UV detection was performed at 226 nm. The method was validated and system suitability parameters were investigated. Method robustness and short-term standard solution stability were verified. Limits of detection for terbinafine degradation products/impurity were from 0.023 to 0.098 μg ml^{-1}, limits of quantitation were from 0.078 to 0.327 μg ml^{-1}. The method was applicable for routine determination of terbinafine and all its found impurities of similar structure with sufficient selectivity, precision and accuracy.

Keywords: Terbinafine; Degradation products; HPLC; Pharmaceuticals

1. Introduction

Terbinafine (Fig. 1), chemically (*E*)-*N*-(6,6-dimethyl-2-hepten-4-inyl)-*N*-methyl-1-naphtalenemethanamine is an allylamine derivative reported to have a broad spectrum of antifungal activity. Terbinafine and its analogues have been found to be potent inhibitors of fungal squalene epoxidasis, which is an enzyme present in fungal and mammalian cell systems important in ergosterol biosynthesis [1].

Terbinafine as fungicidal agent affects dermatophytes and some yeast, it is used orally as hydrochloride for the treatment of dermatophyte infections of the skin and nails. It is also applied to the skin in the occurrence of dermatophytoses, pityriasis versicolor, and cutaneous candidiasis occurrence [2] or superficial fungal infections like seborrheic dermatitis, tinea capitis, and onychomycosis especially for its short duration therapy [3]. Terbinafine is used for treatment of dermal affections in the form of creams, gels, tablets and solutions.

Three degradation products may occur in cream formulations after several months of storage. Therefore, they should be routinely analyzed for the quality control of the formulations together with one impurity, which is a residue from manufacturing process. The impurity – 1-methylaminomethylnaphtalene – is raw material for terbinafine synthesis (MAMN, Fig. 1). β-Terbinafine is derived from the corresponding β-isomer of the raw material MAMN, 4-methyl-terbinafine is derived from the MAMN as well and Z-terbinafine is a potential decomposition product occurring after storage of the preparation. Structures of all impurities could be seen in Fig. 1.

Terbinafine, even if it is therapeutic substance used in many pharmaceutical preparations, is not official in any pharmacopoeia yet. This is why the information about monitoring its degradation products and content of active substance in one analytical run is missing. There are several

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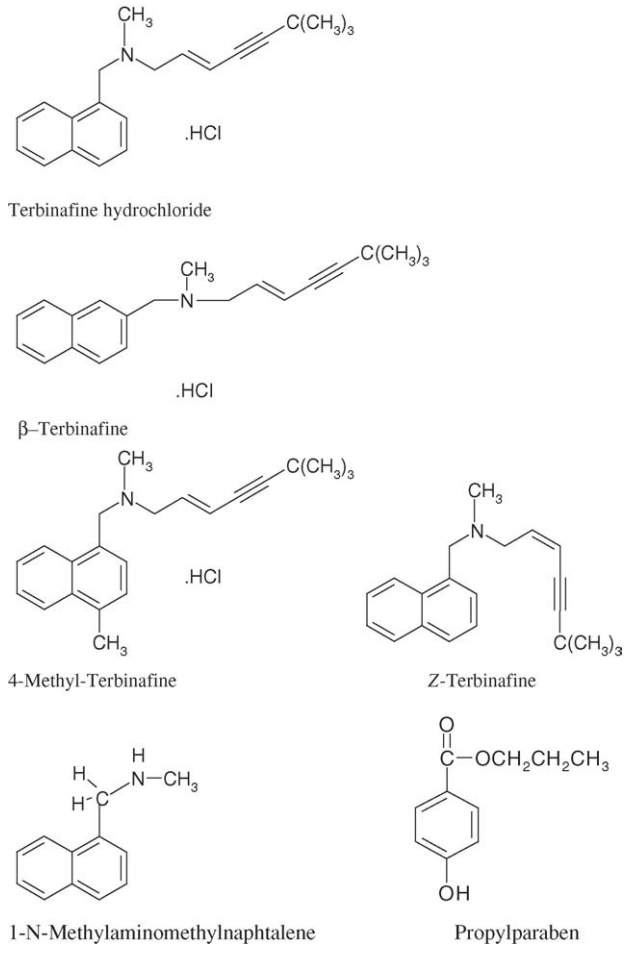

Fig. 1. Chemical structures – major component and degradation products/impurity of terbinafine with the internal standard – propylparaben.

analytical methods for terbinafine determination unfortunately none of these determines terbinafine together with its degradation products/impurities (MAMN, β-terbinafine, Z-terbinafine and 4-methyl-terbinafine).

Mostly, terbinafine has been determined using HPLC method. Recently and nowadays as well, terbinafine has been used as antimycotic in many applications, this is why it has been determined by means of HPLC in different biological matrices. The brand new methods use HPLC in connection with mass spectrometry for determination of terbinafine in human hair [4] or in human and minipig plasma [5] by means of LC–MS–MS. One method reports determination of terbinafine by HPLC–ESI–MS–MS in plasma for the purposes of bioequivalence study [6].

Simple HPLC method with UV detection was used for determination of terbinafine in plasma together with its desmethyl metabolite [7] after liquid–liquid extraction and aqueous back-extraction. This method was reported to be applicable for analysis of tissues, nails, sebum and stratum corneum as well. The same metabolite together with terbinafine and another three metabolites were determined in human plasma, milk and urine [8] by RP-HPLC, similarly as terbinafine with its five metabolites in human plasma and urine using on-line solid-phase extraction for sample clean up [9]. Terbinafine with its *N*-demethyl metabolite was determined also in skin and rat tissues using liquid–liquid extraction for isolation [10] for the purposes of tissue distribution study. A comparison of two chromatographic methods (GC and HPLC) for determination of terbinafine in cat hair was reported with higher sensitivity for HPLC [11]. Terbinafine in cat's hair and plasma was determined also in study of treatment of Microsporum canis in cats [12] and during a study of effect of ethanol and isopropyl myristate on the availability of terbinafine in human stratum corneum [13]. Only one HPLC method determines terbinafine in pharmaceutical preparation including tablets and creams [14].

Another methods used for terbinafine determination were: titrimetry in non-aqueous ambient [15], voltametry using preconcentration of terbinafine by SPE from urine [16], UV derivative spectrometry and spectrodensitometry [17] determining terbinafine hydrochloride and triamcinolone acetonide in laboratory prepared binary mixtures for a comparison of three methods based on spectrometry. Capillary electrophoresis was used for determination of terbinafine and eight of its metabolites after incubation with rat hepatic fraction [18], for determination of terbinafine and another seven antifugal compounds [19] and recently, for determination of terbinafine in pharmaceuticals (tablets, spray) and dialyzates [20].

The last method is quite new, very well developed, it is dealing even with permeation characteristics of terbinafine in gel, but it is still only terbinafine determination using internal standard descarboethoxyloratadine without its separation from degradation products. Regarding the instability of terbinafine possible in solutions, it should be taken into consideration and terbinafine should be monitored together with these compounds in one analytical run.

The purpose of this study was to develop a new HPLC method for the simultaneous determination of five compounds in topical cream—active component terbinafine hydrochloride and its degradation products/impurities 1-methylaminomethylnaphtalene, β-terbinafine, Z-terbinafine and 4-methyl-terbinafine, using internal standard propylparaben. Thereafter, this method has been validated and successfully applied for separation, identification, quantification and stability tests of all compounds of interest in the pharmaceutical formulation—Terbinafin cream.

2. Experimental

2.1. Reagents

Reference standard compounds used in this study—standards of terbinafine hydrochloride and its degradation products and impurity were obtained from Chemagis (Bnel Brak, Izrael). Internal standard propylparaben was purchased from Sigma–Aldrich Co. (Prague, Czech Republic).

Acetonitrile for HPLC was obtained from Sigma–Aldrich Co. (Prague, Czech Republic), Tetrahydrofurane Chromasolv, for HPLC from Sigma–Aldrich Co. (Prague, Czech Republic). Citric acid was reagent grade from Penta (Prague, Czech Republic). Sodium dihydrogen phosphate anhydrous was from Kulich (Hradec Králové, Czech Republic). Phosphoric acid 85% was obtained from Merck (Darmstadt, Germany). Terbinafin cream was supplied from Herbacos–Bofarma Ltd. (Bochemie Group, Pardubice, Czech Republic). The deionised water was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Chromatographic system

Analyses were performed on Shimadzu LC-2010 C system (Shimadzu, Kyoto, Japan) with built-in UV–vis detector. The built-in autosampler was conditioned at 25 °C. Chromatographic software Class VP 5 was used for data collection and processing. The analytical column was 250 mm × 4.6 mm i.d. NUCLEOSIL 100-5-CN (Bischoff Chromatography, Leonberg, Germany) 5 μm particle size. The optimal mobile phase for separation of terbinafine and its four impurities was a mixture of tetrahydrofurane, acetonitrile and citrate buffer pH 4.50 (10:20:70, v/v/v). The finally selected and optimised conditions were as follows: injection volume 2 μl, isocratic elution at a flow-rate 0.8 ml min^{-1} at ambient temperature, detection wavelength 226 nm.

2.3. Reference standard solution preparation

Standard solutions were prepared by dissolving appropriate substance in acetonitrile. The final concentrations of the sample or reference standards were about 250 μg ml^{-1} of terbinafine, 5 μg ml^{-1} of all four impurities and 250 μg ml^{-1} of internal standard propylparaben. It is necessary to keep the solution at decreased temperature (4 °C) and in darkness.

2.4. Sample preparation

An accurately weighed portion of pharmaceutical cream corresponding to 5000 μg of terbinafine (about 0.5 g) was transferred into a 50 ml centrifuge tube and supplemented with 20.00 ml of internal standard (250 μg ml^{-1} solution of propylparaben in acetonitrile) and with 50 μl of phosphoric acid (85%). The mixture was placed into the ultrasonic bath for 15 min and then centrifuged at 2600 × *g* for 15 min. A volume of 2 μl of supernatant was injected onto the column and analyzed by HPLC.

Identification of peaks in the cream samples was based on the comparison of retention times of compounds in standard solutions.

2.5. Buffer preparation

Solution of 0.1 mol l^{-1} citric acid was prepared by dissolving of 21.01 g of citric acid monohydrate (M_r = 210.14) in 1 l of water for HPLC. Solution of 0.2 mol l^{-1} sodium dihydrogen phosphate was prepared by dissolving of 28.39 g of sodium dihydrogen phosphate anhydrous (M_r = 141.96) in 1 l of water for HPLC. Citric buffer pH 4.5 was prepared by mixing citric acid solution and sodium dihydrogen phosphate solution in the ratio 10.9:9.1. The correct value of pH was controlled by pH meter; the value was adjusted (using appropriate one of prepared solutions) when the difference higher than 0.05 pH unit occurred.

3. Results and discussion

3.1. Method development and optimization

The main criteria for development of successful HPLC method for determination of terbinafine and its degradation products and impurity in topical cream were: the method should be stability indicating, free of interference from excipients and straightforward enough for routine use in quality control laboratory.

The aim of our work was the method development for the simultaneous determination of all substances (terbinafine and its degradation products and impurity) in one step. Various types of analytical columns were tested for the convenient selectivity and separation efficiency. Firstly conventional C18 RP analytical column (Merck RP 18 (125mm × 4 mm, 5 μm) was used. Mobile phases of different compositions were tested. For a good peak shape of terbinafine, it was necessary to add buffer into the mobile phase, simple combination of methanolic, acetonitrilic and aqueous mobile phases was found not to be sufficient. Therefore, all subsequent experiments were performed using citrate buffer in pH range 2.50–8.00 either in combination with acetonitrile or methanol (20–60%). It is necessary to note, that under these conditions the separation of terbinafine isomers was just partial and the next problem was a separation of MAMN from dead retention time. The first problem was resolved with addition of tetrahydrofurane (10–20%). Using 20% methanol, 15% tetrahydrofurane, 65% citrate buffer pH 2.5 the separation of terbinafine isomers, terbinafine and methylterbinafine was very good, but the problem of early elution of MAMN still remained unresolved, even using different mobile phase compositions (low % of organic modifier).

The column was changed for X-terra RP 18 analytical column (100mm × 3.0 mm, 5 μm). Various mobile phase compositions (acetonitrile or methanol, tetrahydrofurane and citrate buffer) and different pH of aqueous part were tested. The best results were obtained using 45% of acetonitrile, 25% of tetrahydrofurane and 30% of citrate buffer pH 8.0. Unfortunately, MAMN still co-eluted with dead volume.

The acceptable results were observed using column Nucleosil CN 5 (250mm × 4.6 mm, 5 μm), probably for its higher polar proprieties and higher retention of compounds eluted early on C18. Again, different compositions of mobile phase were tested equally like pH of aqueous component of

mobile phase. Final composition of mobile phase was chosen with regards to the peak resolutions and analysis time as well. Final conditions thus were tetrahydrofurane, acetonitrile and citrate buffer pH 4.50 (10:20:70, v/v/v) at flow-rate 0.8 ml min^{-1} which was optimized with regard to the column back-pressure and analysis time as well.

The optimal detection wavelength was chosen from the UV-spectra of all analyzed compounds. UV-spectra of acetonitrilic solutions of individual impurities and terbinafine were measured. Z-terbinafine showed the highest maximum at 224 nm (the others 275 and 284 nm were much more lower), β-terbinafine at 225 nm (269 and 277 nm, respectively), 4-methyterbinafine at 228 nm (279 and 289 nm, respectively) and MAMN at 226 nm (272 nm and 281 nm, respectively). Terbinafine as an active compound absorbed at 224 nm. Detection wavelength was a compromise taking into mind that the concentrations of the impurities were very low comparing to the main active compound terbinafine—226 nm was chosen for detection.

After preliminary experiments, propylparaben (Fig. 1) was decided to be used as internal standard because of its retention time (9.60 min). Before this conclusion, many substances were tested. Unfortunately, it would be very complicated to find internal standard of similar structure as terbinafine and different form of the tested degradation products/impurity. In our opinion and experience it is not necessary to have internal standard of the same structure, when it gives reliable and precise recovery as analytes. In our laboratory, we have a lot of experience with parabens used as internal standards. Their isolation is without any problems. Moreover, chromatographic analysis of parabens is quite easy, they elute in logical order, and so the appropriate compound could be chosen. Their absorbance in UV range is sufficient and the substances are very stable (often used as preservatives). Another tested compounds were diclofenac and imidazole (very low detector response because of bad absorbance at 226 nm), paracetamol (coeluted with ballast substances from cream base, $t_r = 5.20$ min), chlorhexidin and naftochinon (were not sufficiently soluble in acetonitrile). It was necessary to add internal standard because external influences during isolation procedure and sample handling are minimized and the results are more accurate.

The final optimal composition of the mobile phase, as it is stated above, was tetrahydrofurane, acetonitrile and citrate buffer pH 4.50 (10:20:70, v/v/v). Using a 5 μm packing of the column NUCLEOSIL 100-5-CN and decreasing the flow-rate to 0.8 ml min^{-1}, we have performed the separation and analysis time for all compounds in Terbinafin cream was about 32 min (Fig. 2).

3.2. Extraction procedure

Isolation procedure was developed on a basis of methods for analysis of topical preparations routinely used in our laboratory. Usually mobile phase, acetonitrile or methanol containing internal standard were used as extraction agents.

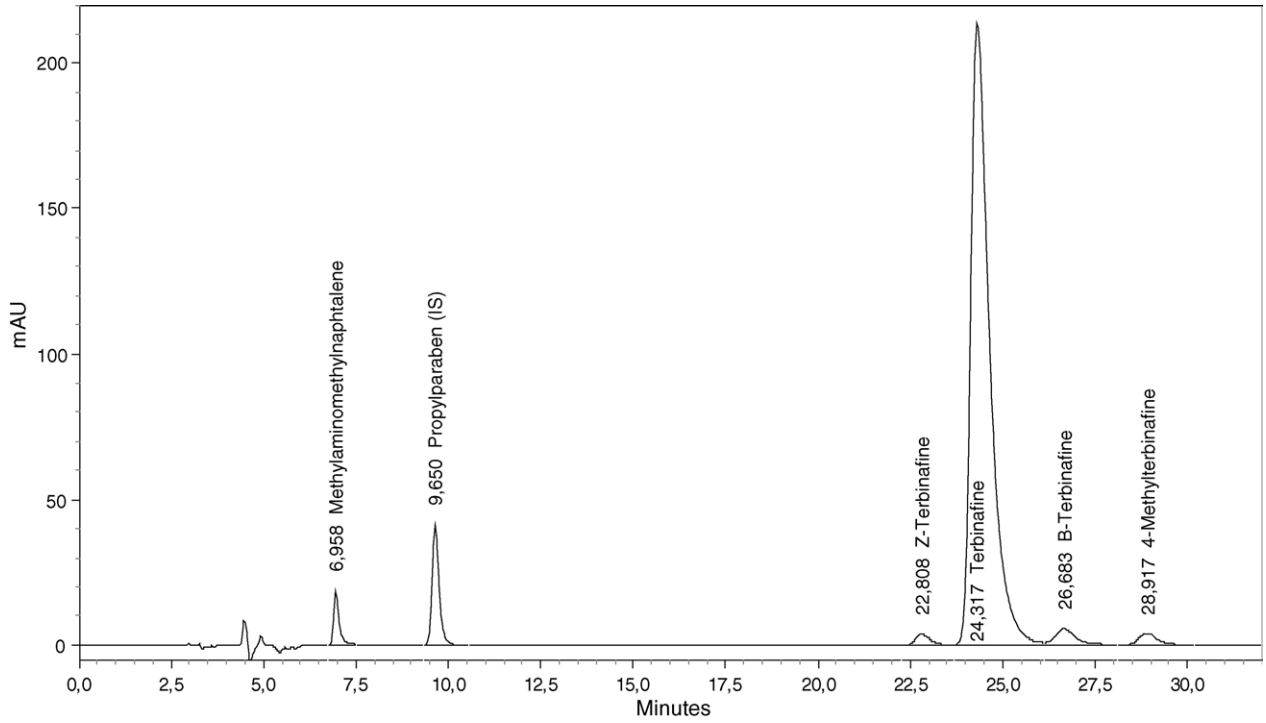

Fig. 2. Chromatogram of standard solution of terbinafine (250 μg ml^{-1}) and its degradation products – 1-*N*-methylaminomethylnaphtalene, Z-terbinafine, terbinafine, β-terbinafine and 4-methyl-terbinafine – with the internal standard (propylparaben, 250 μg ml^{-1}); UV detection at 226 nm.

Firstly, the extraction by mobile phase was tested. The recovery was very low (<80%); therefore, buffer pH was changed from 4.50 to 2.50. Terbinafine and its impurities are basic amines, therefore it was supposed to improve their solubility by pH decrease. This theory was confirmed; recovery was increased over 95% as it was recommended by the validation rules. The problem in this case was sample cleaning-up. Using ultrasonic bath the cream was dissolved for unclear "milk" which was impossible neither to remove by centrifugation nor by filtration (obstruction of filters occurred). In addition, terbinafine was adsorbed on the used filters and even after their washing, it contaminated subsequent samples.

Extraction using pure acetonitrile gave recovery of about 95% (near the validation requirements), but the results were not precise enough. Recovery was increased more by acidification by phosphoric acid 85% (50 μl) and this way the method was also sufficiently precise. The extraction was performed by ultrasonication for 15 min and then centrifugation at 2600 × *g* for 15 min.

The procedure as described above gives recovery from 96.32 to 102.34%. Chromatogram at Fig. 3 is an illustration of separation of all tested compounds after isolation from pharmaceutical preparation. Only MAMN as an impurity from manufacturing process was observed while the degradation products were not present due to only short period of storage of preparation during stability studies. Their concentrations were below the limit of detection or they were not present at all. There are no other substances, which may coelute with tested analytes as was verified during method validation-selectivity testing.

3.3. Analytical parameters and validation

The aim of method validation was to demonstrate the method suitability for its intended purpose as stated in ICH guidelines Q2A and Q2B [21,22]. The optimized method was validated by a standard procedure to evaluate adequate validation characteristics (accuracy, precision, linearity, selectivity, sensitivity-LOD, LOQ, robustness and stability).

Accuracy (% of recovery, % of R.S.D.) was investigated using placebo samples spiked with standard solution. Comparison of real sample concentration and determined concentration was calculated with the results from 97.07 to 100.29% for recovery, 0.92–1.77% for R.S.D., respectively. Precision (% of R.S.D.) was investigated using sample preparation procedure for six real samples with the results from 1.31 to 4.81% R.S.D. Selectivity was verified by injection of standard solution, placebo of pharmaceutical preparation and pharmaceutical preparation treated according to sample preparation procedure. No interferences were observed as it could be seen from Fig. 3.

Linearity (described by equation and corresponding correlation coefficient) was determined using six calibration levels for all compounds (at 50, 75, 100, 115, 135, 150% levels). The concentrations of calibration solutions of degradation products/impurity were from 0.20 to 5 μg ml^{-1}. The method of linear regression was used for data evaluation. Peak area

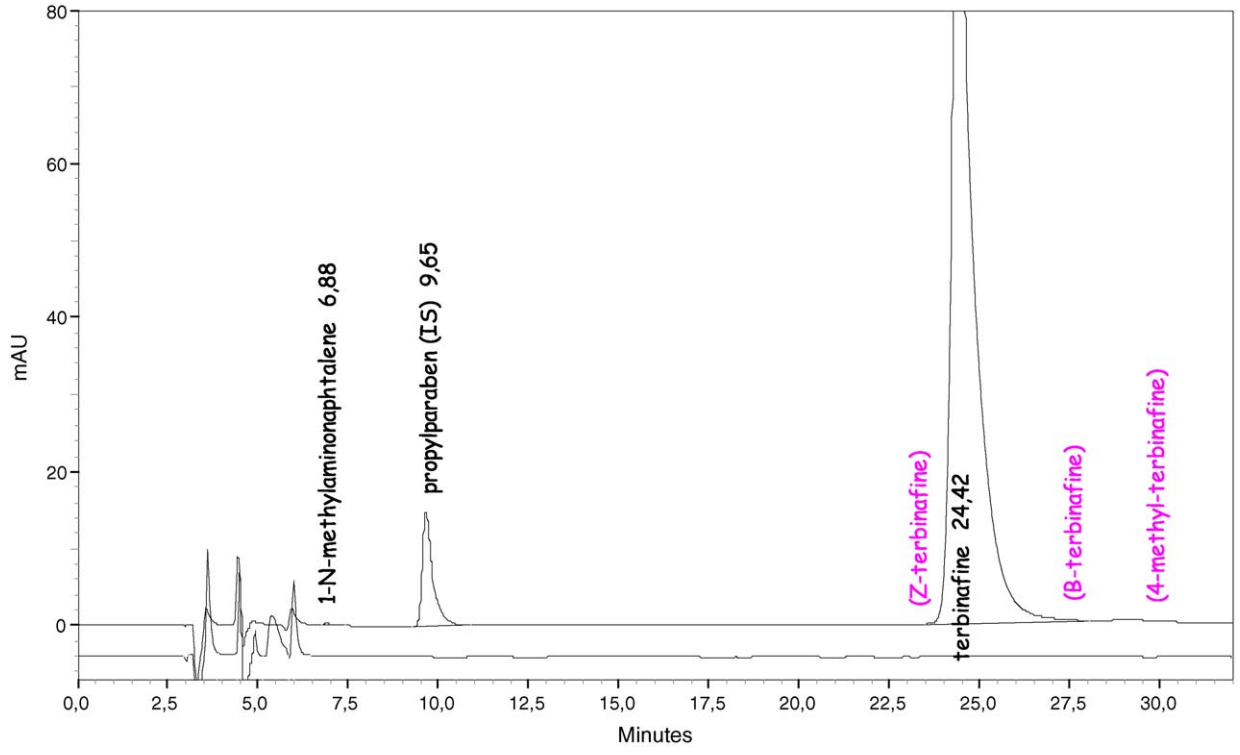

Fig. 3. Chromatogram of placebo of Terbinafin cream and pharmaceutical formulation Terbinafin cream.

Table 1
Method validation results for individual compound

	MAMN	Z-terbinafine	Terbinafine	β-terbinafin	4-methylterbinafine	Limits
SST						
Theoretical plates[a]	12394	16249	12309	16274	15245	$N > 2000$
Asymmetry[a]	1.37	1.40	1.50	1.27	1.48	$T < 1.5$
Resolution[a]	9.33	25.22	1.89	2.73	2.53	$R_{ij} > 1.5$
Repeatability-t_r[b]	0.00	0.02	0.26	0.03	0.01	R.S.D. < 1%
Repeatability-A[b]	0.28	0.73	0.26	0.79	0.92	R.S.D. < 1%
Validation						
Intra-day precision[c] (% R.S.D.)	3.66	1.31	2.71	4.02	4.81	R.S.D. < 5%
Linearity[d] (correlation coefficient)	0.99986	0.99973	0.99976	0.99983	0.99925	R > 0.9990
Linearity[d] (equation)	$y = 0.654 \times x + 0.0054$	$y = 0.4041 \times x - 0.0002$	$y = 0.5529 \times x + 0.16$	$y = 0.518 \times x - 0.0101$	$y = 0.599 \times x - 0.0018$	–
Accuracy[c] (% R.S.D.)	1.35	0.92	1.41	1.45	1.77	R.S.D. < 5%
Accuracy[c] (% recovery)	98.58	99.27	97.07	100.29	99.71	$100 \pm 5\%$
Selectivity	No interference	No interference	No interference	No interference	No interference	No interference
LOD ($\mu g\,ml^{-1}$)	0.023	0.058	–	0.098	0.083	–
LOQ ($\mu g\,ml^{-1}$)	0.078	0.193	–	0.327	0.278	–
Stability—ambient [%][e]	14.33	308.59	37.08	43.17	60.04	1%
Stability—4 °C (%)[e]	1.88	0.83	0.83	5.08	0.62	1%

[a] Made in six replicates.
[b] Made in six replicates.
[c] Six samples injected three times each.
[d] At 50, 75, 100, 115, 135, 150% levels, three replicates.
[e] (%) express change in concentration during four days of storage in comparison with freshly prepared solution.

ratios of standard compounds and internal standard were plotted against theoretical concentrations of standards. Linearity was described by equation and as well, correlation coefficient was determined.

Limits of detection (LOD) and limits of quantitation (LOQ), as a measure of method sensitivity, were provided for degradation products and impurity calculated by means of the method of signal-to-noise ratio. These limits are parameters of quantitative assays of low level compounds in the sample and they are used especially for the determination of impurities as in our case. Thus, limits of detection for terbinafine degradation products/impurity were in a range 0.023–0.098 $\mu g\,ml^{-1}$, limits of quantitation were in a range 0.078–0.327 $\mu g\,ml^{-1}$. The details could be seen in Table 1.

System suitability parameters were measured so as to verify the system performance. All important characteristics including repeatability, peak resolution, theoretical plate number and peak asymmetry were measured and calculated using standard solution injection in six replicates. The results of method validation and system suitability test in comparison with the required limits could be seen in Table 1.

The robustness as a measure of method capacity to remain unaffected by small, but deliberate, variations in method parameters was studied testing influences of small changes in mobile phase composition (±10%). It was investigated, that mobile phase composition had very strong influence on peak retention time and separation, especially for critical separation of terbinafine and β-terbinafine. Individual components of mobile phase has to be present in the ratio tetrahydrofurane, acetonitrile and citrate buffer pH 4.50 (10:20:70, v/v/v) as it was investigated during method optimization, especially a content of tetrahydrofurane and citrate buffer is necessary to keep as precise as possible.

Stability of standard solutions was tested by storage at 4 °C and at ambient temperature (about 20 °C) and in darkness in parallel. Concentrations of freshly prepared solution of all standards were measured and then the concentrations were observed in four days. Changes in concentrations were compared to the first day concentrations. The decrease should be less than 1% to considered solution to be stable. Terbinafine solutions are very susceptible to degradation, as it could be seen from Table 1. Standard solutions in acetonitrile have to be stored at decreased temperature (4 °C). All compounds are stable only up to 48 h. Later the stability of β-terbinafine is not sufficient. In addition, storage in darkness is recommended.

3.4. Analysis of impurities

For determination of the actual amount of degradation products, it is necessary to keep in mind that they occur in very low concentration levels in comparison to the Terbinafine hydrochloride (about 100–1000 times less). Generally, this is the reason why methods for determination of an active pharmaceutical compound and degradation products are different. In our case, we have finally found sufficient separation of all compounds and therefore we could include the whole procedure into one step.

The chromatogram in Fig. 2 was obtained using the described HPLC method with the standard solution of terbinafine and all four impurities. All compounds presented in the solution – terbinafine, impurities and internal standard – are clearly separated. The chromatogram in Fig. 3 shows the analysis of the placebo of Terbinafin cream and Terbinafin cream pharmaceutical preparation. There were not any peaks of interfering substances in the retention times of impurities.

The results of accelerated stability tests (formulation stored 6 months in original packing at a temperature of 40 ± 2°C and relative humidity of 60% ± 5%) were 99.6 ± 8.2% of the labelled amount of terbinafine. The amount of the degradation product 1-*N*-methylaminomethylnaphtalene was found to be 0.253% of the amount of the active compound terbinafine, the other degradation products were not present.

4. Conclusion

A novel HPLC method for simultaneous determination of terbinafine, its three degradation products and one impurity of very similar structure was developed. Propylparaben was used as internal standard. Demanding separation was carried out using NUCLEOSIL 100-5-CN analytical column and mobile phase consisting of tetrahydrofurane, acetonitrile, citrate buffer pH 4.5 (10:20:70, v/v/v). Detection of analytes and their quantitation was performed using UV–vis detector at set up at 226 nm.

The total analysis time was less than 32 min, extraction procedure was optimized. Method has been validated the results obtained were selective, precise and accurate. This method was successfully applied for the identification, quantitative analysis, homogeneity tests and stability tests of all compounds in a topical cream—Terbinafin cream.

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