Tolnaftate, discovered in Japan in 1962 by Noguchi and colleagues (1), is a thiocarbamate antifungal. It is therapeutically active against dermatophytes, *Epidermophyton*, *Microsporum*, *Trichophyton* spp., and *Malassezia furfur*. Tolnaftate is used topically in a concentration of 1% in creams, powders, solutions, aerosols, gels to treat various forms of tinea (tinea pedis, tinea cruris, tinea corporis, tinea manuum) and pityriasis versicolor. Tolnaftate is available over-the-counter and is especially valuable in the prophylaxis and treatment of tinea pedis, which affects approximately 10% of the world population (2).

Tolnaftate selectively inhibits fungal microsomal squalene epoxidase and blocks ergosterol biosynthesis in cell walls of fungi (3, 4). The accumulation of squalene and deficiency of ergosterol results in antifungal action of tolnaftate. Tolnaftate has no antibacterial activity and all tested species of *Candida* were resistant to tolnaftate (5), apparently due to its poor penetration into *Candida* cell envelope (6).

Tolnaftate is mainly restricted to dermatophyte infections. Crawford et al. (7) reviewed placebo-controlled trials of topical treatments for athlete’s foot and found that in two trials (n = 115), carried out by Fuerst et al. (8) and Tong et al. (9), 1% tolnaftate cream used for 4 weeks was significantly more effective than the placebo, producing a risk ratio of treatment failure equal to 0.3 (95% confidence interval being 0.13–0.72).

The analytical methods used for tolnaftate assay in pharmaceutical formulations mainly employ high performance liquid chromatography (HPLC) technique (10–12). Kobayashi et al. (13) studied tolnaftate permeation through the human nail plate in vitro and applied HPLC technique to assess tolnaftate permeation dependence on various donor vehicles. Patil et al. (14) used isocratic supercritical fluid chromatography to separate and deter-
mine tolnaftate and its related impurities (N-methyl-
-m-toluidine and β-naphthol-1-chlorothiocarbamate) in bulk drug. Meshram et al. (15) used high performance thin layer chromatography to determine tolnaftate in topical solution. The indirect spectro-
fluorimetric methods, based on tolnaftate alkaline 
hydrolysis (at the thiono ester bond) to β-naphthol, were developed by several authors (16, 17). Official monographs of tolnaftate offer spectrophotometric methods for tolnaftate assay in bulk drug (Eur. Ph. 
6.0: 01/2008:1158) as well as in tolnaftate creams, 
gels and topical solutions (USP 28). HPLC method is recommended to assess tolnaftate content in tol-
naftate topical powder and topical aerosol (USP 28) using expensive acetonitrile-water (2:1) composi-
tion of the mobile phase and progesterone as an internal standard.

The physicochemical properties of tolnaftate 
molecule (Fig. 1) support its penetration into super-
ficial skin layers. Low molecular weight (307.4 Da) and lipophilicity (XLogP = 5.5) of tolnaftate facil-
itate its penetration and accumulation in the stratum corneum. Hydrophilic viable skin layers form a bar-
rier for tolnaftate diffusion into blood circulation. Therefore, no systemic side effects or toxicity could be associated with tolnaftate when it is applied top-
ically in appropriate concentration.

To our knowledge, there are no recently pub-
lished data on tolnaftate penetration into skin and its accumulation in skin layers. In 1987, Szeman et al. (18) compared percutaneous absorption of tolnaf-
tate-cyclodextrin homogenized ground mixtures with tolnaftate alone and non-homogenized mix-
tures, applying them on shaved back skin of mice. Unfortunately, no other data could be found for tol-
naftate penetration into skin samples after in vitro or in vivo studies. The aim of this work, therefore, was to optimize and validate a simple, rapid, accurate and reproducible procedure for tolnaftate assay in human skin samples and to apply this procedure for in vitro tolnaftate penetration studies.

Various tolnaftate formulations available on the market contain 1% of the active ingredient. In this research 1% tolnaftate solution was applied on the human skin samples and tolnaftate amount penet-
trating into epidermis and dermis was assessed. Tolu

Materials and Methods

Chemicals and Reagents

Tolnaftate (O-naphthalen-2-yl methyl-(3-
methylphenyl)thiocarbamate, according to Eur. 
Pharm. 6.0) was a gift from pharmaceutical compa-
niny Sanitas AB (Kaunas, Lithuania). The purity of the substance was 99.7% and it was stored at ambient temperature protected from light.

Methanol (Chromasolv®) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Ger-
many). Sodium azide (NaN₃) was obtained from POCh (Gliwice, Poland). Polyethylene glycol 400 (PEG 400) and sodium chloride (NaCl) were purchased from Carl Roth GmbH (Karlsruhe, Germany). All other reagents were of analytical grade.

Human skin was obtained when excessive skin was removed from healthy patients undergoing plas-
tic surgery in the Department of Reconstructive and Plastic Surgery, Hospital of Kaunas University of Medicine. The studies with human skin were approved by Kaunas Region Bioethical Committee. Only women skin of age 25–40 was used. The appropriately treated skin was wrapped in alumini-
um foil and stored at –20°C for not longer than 6 months before use.

Equipment

The HPLC analysis was carried out using Shimadzu Liquid Chromatograph (Shimadzu 
Corporation, Kyoto, Japan) equipped with two Shimadzu LC-10AD VP pumps, degasser DGU-
14A, auto injector SIL-10AD VP, system controller 
SCL-10A VP, UV-vis detector SPD-10A and col-
umn oven CTO-10AC VP.

The equipment for in vitro skin penetration 
experiments consisted of thermostated water bath 
(Grant GD120, Grant Instruments, (Cambridge) Ltd.), peristaltic pump (Masterflex® LS® pump 
drive (model type 7524-45) with multichannel pump 
head (model type 07535-08), Cole-Palmer 
Instrument Co., Illinois, USA) and Bronaugh-type 
flow-through diffusion cells, made from Teflon.
Extraction procedure of human skin layers was performed in the Bandelin Sonorex Digitec Ultrasonic Bath (DT 156, Bandelin electronic GmbH & Co. KG, Berlin, Germany), having ultrasonic peak output of 640 W.

**HPLC conditions**

Separation of tolnaftate from endogenous compounds, deriving from skin matrix, was accomplished on a LiChrospher®100 RP-18 Endcapped column, 125 × 4 mm, i.d., packed with 5 µm size particles (Merck KGaA, Darmstadt, Germany). A LiChrospher 100 RP-18e (5 µm) (LiChroCART 4-4) was used as a guard column.

Isocratic elution, using 70% methanol and 30% bi-distilled water as the mobile phase, resulted in tolnaftate retention time of 13.2 min and the overall running time of 15.0 min. The flow rate of the mobile phase was set to 0.8 mL/min and the injection volume was 10 µL. The column was thermostated at 40°C and tolnaftate detection was set at λ = 257 nm.

**Preparation of calibration standards**

Stock solution of tolnaftate in pure methanol was prepared at concentration of 1 mg/mL. In order to assure the dissolution of all tolnaftate particles in pure methanol, 10 min sonication at ambient temperature was applied. The prepared stock solution was stored at 4°C for up to 1 month.

Stock solution (1 mg/mL) was appropriately diluted to obtain six calibration standards at concentrations of 0.6, 1, 5, 10, 50 and 100 µg/mL. The serial dilution was performed in 10 mL volumetric flasks.

**Validation procedure**

The following criteria for validation of analytical method were considered (19–22): relationship between concentration and response, specificity, accuracy and precision, limit of quantitation (LOQ) and limit of determination (LOD), extraction recovery and stability studies.

**Relation between concentration and response:** Seven calibration standards in the concentration range of 0.1–100 µg/mL were used to define the relationship between tolnaftate concentration in pure methanol and UV-detector response (λ = 257 nm). Calibration curve was determined by least-squares linear regression analysis.

**Specificity:** Several independent sources of skin matrix were used to establish the specificity parameter. Unfrozen blank full-thickness skin was cut into small pieces of 0.64 cm² and the epidermis (n = 12) was separated from the rest of the skin (dermis) (n = 12) following heat separation procedure. Epidermis and dermis were spiked separately with 10 µL of distilled water and left for 3 h in contact. Then, 1 mL of pure methanol was added and the extraction procedure was carried out, ultrasonating the samples for 30 min.

**Accuracy and precision:** The accuracy and precision of the assay were established within-day (n = 3) and between-day (n = 3) using 4 control samples at concentrations of 0.6 (LOQ), 1, 10 and 100 µg/mL. For within-day accuracy and precision, analysis of above mentioned concentrations in triplicate was performed the same day. For between-day accuracy and precision, control samples were analyzed once a day, following three days. Accuracy was calculated as follows: [mean found concentration / nominal concentration] × 100, and precision was measured in terms of imprecision and expressed as the percent relative standard deviation (RSD).

**Limit of quantitation (LOQ) and limit of determination (LOD):** The LOD was established as the response that corresponds to a signal-to-noise ratio of 3. The LOQ was the lowest amount of tolnaftate in pure methanol that can be quantitatively determined with adequate precision and accuracy. The LOQ and LOD were determined by analyzing tolnaftate solutions at concentrations ranging from 0.1 µg/mL to 0.75 µg/mL.

**Extraction recovery:** Tolnaftate absolute recovery from epidermis and dermis was evaluated according to the procedures described in the literature (23–30). The optimal volume of extraction solvent (having influence on the sensitivity of tolnaftate determination) as well as extraction mode and duration of the procedure were selected after preliminary testing their influence on the highest and fastest tolnaftate recovery from skin samples. Blank skin sections of 0.64 cm² were exposed to heat (60°C) for epidermis separation from the rest of the skin (dermis). Then blank epidermis and dermis were spiked separately with 10 µL of methanolic tolnaftate solutions at concentrations of 1000 µg/mL and 100 µg/mL. The amounts of tolnaftate added were 10 and 1 µg, respectively. The spiked epidermis and dermis were allowed to rest for 3 h at ambient temperature in order to achieve drug penetration. After adding 1 mL of pure methanol (extraction solvent), the spiked samples were extracted following bath sonication for 30 min and then analyzed using HPLC technique. The areas under the peaks of extracted samples were compared with unextracted standards, representing 100% recovery.
Stability study: Stability of tolnaftate stock solution, kept at +4°C, was verified. Appropriately diluted stock solution was injected in triplicate immediately after preparation and after 1 month of storage. Short term stability of tolnaftate in epidermis and dermis extracts was proven over 24 h at room temperature (20°C). Long term stability of tolnaftate, keeping extracts from spiked epidermis and dermis at 4°C and −20°C, was also verified. For short term and long term stability studies three replicates of spiked (with 10 µL of methanolic tolnaftate solution at concentration of 100 µg/mL) and then extracted epidermis and dermis were prepared and kept under appropriate conditions. The assessment of tolnaftate stability in epidermis and dermis extracts was achieved by comparing the responses of freshly (reference value) prepared samples and samples after storage.

In vitro skin penetration experiments

Bronaugh-type flow-through diffusion cells, having an effective diffusional area of 0.64 cm² and the receptor volume of 0.13 mL, were used in in vitro skin penetration experiments. Seven cells, mounted with human skin, were operated in one run. The diffusion cells were placed on the heating block in order to maintain 37°C.

Before the experiments, the full-thickness human skin was thawed and the residues of subcutaneous fatty tissue were removed. The skin samples were appropriately trimmed off to fit diffusion cells and then the equilibration period of 12 h, during which the circulation of 0.9% NaCl solution with 0.005% NaN₃ was maintained, was performed. After the equilibration period, the infinite dose of 1% (w/w) tolnaftate solution in PEG 400 (approximately 200 mg of solution) was applied on the stratum corneum side of skin surface. The cells were occluded to avoid compositional changes. The NaCl solution (0.9%) with 0.005% NaN₃ (4.0 mL) was used as a receiver fluid and it was pumped at a rate of 0.6 mL/min by the peristaltic pump. After 4, 8 and 24 h, the system was rinsed with 0.5 mL of the fresh receiver fluid and then the acceptor medium was entirely replaced by the fresh portion (4.0 mL).

After 24 h, the donor solution was removed and the outer residuals of skin samples were trimmed off, leaving the central circles with area of 0.64 cm².

In order to determine tolnaftate content in epidermis and dermis, the epidermis was peeled back from the dermis after a very short heating (60°C) on the hot surface (heat-separation method). The obtained epidermis and dermis were separately extracted following extraction procedure as described above.

The samples of receptor fluid and methanolic extracts of epidermis and dermis were analyzed using HPLC.

RESULTS AND DISCUSSION

Optimization of HPLC conditions

Several different columns and mobile phase compositions were tested. Pathfinder® AP Silica 100 3.5 µm RP (150 × 4.6) and Pathfinder® EP Silica 100 3.5 µm RP (150 × 4.6) columns from Shimadzu were not effective for tolnaftate analysis as very wide and asymmetrical (tailing factor > 1.5) tolnaftate peak was observed. This was due to weak basic properties of tolnaftate substance, which tended to attach to free silanol groups of stationary phases. Endcapped LiChrospher®100 RP-18 column (125 × 4 mm, 5 µm) from Merck performed very well and was chosen for tolnaftate assay.

The preliminary mobile phase composition for HPLC was chosen with the reference to published data. Optimal composition of mobile phase was selected after testing methanol : 25 mM KH₂PO₄ (80:20, v/v) (11, 12) and methanol (60–85%) : water (13). Total time of analysis, tolnaftate retention time, economical aspects of methanol consumption and simplicity of mobile phase preparation were taken into account. Methanol : water (70:30, v/v) were chosen as the best mobile phase composition for tolnaftate elution.

Chromatographic system precision and its performance

The performance of the chromatographic system (repeatability of response, efficiency of the column, etc.) was evaluated.

In order to check the precision of the system the tolnaftate standard solution at concentration of 5 µg/mL was injected 10 times in succession. The RSD of tolnaftate retention time and peak area was calculated. The repeatability of tolnaftate retention time (tᵣ = 13.265 ± 0.015, RSD = 0.114) and peak area (RSD = 1.016) was very good, confirming the suitability of the system.

The value of capacity factor k’ for tolnaftate retention in the stationary phase was 8.09 ± 0.24. The tolnaftate peak was symmetrical (the symmetry factor being 0.99 ± 0.06) and narrow (w = 1.3 ± 0.43 min). The number of theoretical plates, expressing the efficiency of the column, was approximately 1700.

The efficiency of separation of tolnaftate from endogenous compounds deriving from epidermis
Assay of tolnaftate in human skin samples after in vitro penetration studies using high performance... 331

and dermis matrices, could be demonstrated by the representative chromatograms of extracts from spiked epidermis and dermis. Adequate separation of peaks is presented in Figure 2.

Assessment of validation criteria

The optimized HPLC method was used to validate tolnaftate assay for linearity, specificity, accuracy, precision, limit of quantitation, limit of determination, drug extraction recovery and stability in skin extracts.

Relationship between concentration and response: Least-squares linear regression analysis revealed the linearity in the concentration range of 0.6–100 µg/mL. The regression line equation obtained had a mean slope of 25.66 ± 0.46 (n = 5), a
mean intercept of $-1.79 \pm 1.12$ (n = 5) and coefficient of determination equal to $R^2 = 0.9998$ (RSD = 0.022, n = 5).

**Specificity:** The specificity of the analytical method was demonstrated by chromatograms of blank epidermis and dermis. The retention times of endogenous compounds were compared with tolnafate retention time ($t_R = 13.2$ min). Representative chromatograms showing no interference of endogenous compounds for the determination of tolnafate are presented in Figure 3.

**Accuracy and precision:** The results for within-day and between-day accuracy and precision of the analytical procedure are presented in Table 1. The obtained values for both within-day and between-day accuracy and precision were within the recommended limits: within-day and between-day recovery values ranged from 98.2 to 104.2% and from 98.7 to 101.4%, respectively. RSD values for within-day and between-day imprecision were = 3.8%.

**Limit of quantitation (LOQ) and limit of determination (LOD):** The LOD was established to be 0.4 µg/mL (the response corresponds to a signal-to-noise ratio of 3) and the LOQ was found to be 0.6 µg/mL (conformity with acceptable limits of accuracy and precision was achieved).

**Extraction recovery:** Mean absolute extraction recoveries of tolnafate from epidermis and dermis (independent of spiked amount of tolnafate) were satisfactory and reaching 90% (93.2% for epidermis and 89.3% for dermis). Table 2 presents the mean absolute extraction recoveries (ERs) of tolnafate from epidermis and dermis calculated separately for each amount of tolnafate spiked on epidermis or dermis. The results obtained are indicative of efficient quantitative extraction procedure. RSD values for within-day and between-day precision were ≤ 4.5%, showing that extraction procedure was precise and reproducible.

**Stability study:** Stock solution of tolnafate, kept in refrigerator at 4°C for 1 month, did not show any changes compared to freshly prepared stock solution. Short term stability study of tolnafate in epidermis and dermis extracts during 24 h at ambient temperature showed no sign of significant change in accordance to the reference samples. Long term stability of tolnafate in epidermis and dermis extracts, stored at 4°C and −20°C and tested after 1 month period, showed neither degradation nor significant losses. The results of long term stability, including accuracy, precision and tolnafate mean absolute extraction recovery from epidermis and dermis, are presented in Table 3. Tlnaftate was considered stable over all storage conditions tested as tolnafate losses were not higher than 6% and no additional or interfering peaks were observed.

**Application of the method to quantify tolnafate in skin layers after in vitro skin penetration experiments**

The validated reliable method was applied for tolnafate determination in skin layers after in vitro skin penetration studies. Tlnaftate is a hydrophobic substance, used against dermatophytes affecting *stratum corneum* and viable epidermis. Therefore, tolnafate permeation into the receptor fluid is undesirable and its high amounts in hydrophilic dermis are unacceptable. During our experiment, no drug was detected in the receptor fluid and only small amounts reached the hydrophilic dermis.

### Table 2. Mean extraction recoveries of tolnafate from epidermis and dermis.

<table>
<thead>
<tr>
<th>Spiked amount of tolnafate (µg)</th>
<th>Extraction recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epidermis</td>
</tr>
<tr>
<td>1 (n = 6)</td>
<td>93.4 (RSD = 3.7%)</td>
</tr>
<tr>
<td>10 (n = 3)</td>
<td>93.0 (RSD = 4.5%)</td>
</tr>
</tbody>
</table>

*Relative standard deviation for between-day precision. Relative standard deviation for within-day precision.

### Table 3. Long-term stability study of tolnafate in epidermis and dermis extracts (n = 3).

<table>
<thead>
<tr>
<th>1 month storage at:</th>
<th>Extract of:</th>
<th>Accuracy (%)</th>
<th>Precision (%)</th>
<th>ER (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 °C</td>
<td>Epidermis</td>
<td>98.4</td>
<td>3.2</td>
<td>91.0</td>
</tr>
<tr>
<td></td>
<td>Dermis</td>
<td>101.2</td>
<td>4.4</td>
<td>93.6</td>
</tr>
<tr>
<td>−20 °C</td>
<td>Epidermis</td>
<td>100.3</td>
<td>1.6</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td>Dermis</td>
<td>98.8</td>
<td>4.7</td>
<td>91.1</td>
</tr>
</tbody>
</table>
Tolnaftate amounts penetrating into the epidermis and dermis (µg/cm²) from 1% (w/w) tolnaftate solution in PEG 400 after 24 h of topical application are illustrated in Figure 4. The average amount of tolnaftate penetrating into the epidermis and dermis was 2.60 ± 0.28 µg/cm² and 0.92 ± 0.12 µg/cm², respectively (n = 7).

CONCLUSIONS

The optimized HPLC method for tolnaftate assay in human skin samples was validated and applied for tolnaftate determination in human epidermis and dermis after in vitro penetration studies. The very precise, accurate, sensitive and specific analytical method, simple and rapid tolnaftate extraction procedure from epidermis and dermis with reproducible extraction recoveries could be applied for biopharmaceutical evaluation of tolnaftate preparations and studies of pharmacokinetics in human skin after in vitro penetration studies.

ACKNOWLEDGMENT

The authors are thankful to the Research Foundation of Kaunas University of Medicine for supporting transdermal penetration research project.

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Received: 23. 11. 2009