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Determination of terbinafine in pharmaceuticals and dialyzates by capillary electrophoresis

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Abstract

A capillary electrophoresis method has been developed for the separation and determination of terbinafine (TER) in various pharmaceutically relevant matrices. Capillary zone electrophoresis (CZE) separation and UV absorbance photometric detection were carried out in a 160 mm capillary tube with a 300 μm i.d., hydrodynamically (membrane) closed. The influences of pH, carrier cation and counterion on migration parameters of TER were studied and the following conditions were selected: a 20 mmol l^{-1} glycine running buffer adjusted to pH 2.7 with acetic acid, 0.2% (w/v) methylhydroxyethylcellulose (m-HEC) as an electro-osmotic flow (EOF) suppressor, a 250 μA driving current, and 20 °C. The optimized separation conditions were convenient for the determination of TER in commercial tablets and spray and in dialyzates. Here, the dialysis was used to investigate in vitro permeation of TER through the skin from the gel. The samples of dialyzates were examined with and without simple extraction procedure and the results were compared. A permeation profile of the drug present in the gel of given composition was obtained analyzing pretreated samples. The proposed electrophoretic method was successfully validated. It was suitable for the simple, sensitive, rapid and highly reproducible assay of TER. CZE analysis was completed within 5.5 min. The detection limit of TER was 1.73 μmol l^{-1} at a 224 nm detection wavelength. The intra- and inter-laboratory precisions over the concentration range 6.0–60.0 μmol l^{-1} were between 0.32–0.69% and 1.04–1.44% including R.S.D. of migration times and peak areas, respectively. The mean absolute recoveries of drugs from samples were found to be 98.34 (tablets) and 99.47% (spray). It is suggested that there are potentialities to determine TER present in unpretreated complex samples, as CZE in a hydrodynamically closed separation system may be easily on-line combinable with purification and preconcentration CE modes (e.g., isotachophoresis, ITP).
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1. Introduction

Terbinafine (TER) [(*E*)-*N*-(6,6-dimethyl-2-hepten-4-inyl)-*N*-methyl-1-naphthalenemethanamine hydrochloride] (Fig. 1) is a new antifungal agent of the allylamine class that selectively inhibits fungal squalene epoxidase [1]. This drug is indicated for both oral and topical treatment of mycoses [2]. In the treatment of cutaneous superficial fungal infections, topical therapy is often preferred to oral drug administration. In fact, the required concentration for antimycotic activity at the skin target site may be more easily achieved after topical dosing, if good drug release and penetration are ensured [3].

TER is not yet official in any pharmacopoeia. Previously, the drug has been determined in biological fluids (plasma, urine), tissues, nail and hair by HPLC [4–9], in tablets and creams by HPLC [10] and in various dosage forms by UV-spectrophotometry, spectrodensitometry [11–13] and voltammetry [14]. Recently, CZE with off-line solid-phase extraction has been applied to in vitro metabolism studies of various antifungals (imidazole and triazole derivatives) [15]. Just one paper has been devoted to the separation of TER by CZE method so far [16]. It deals with the CZE determination

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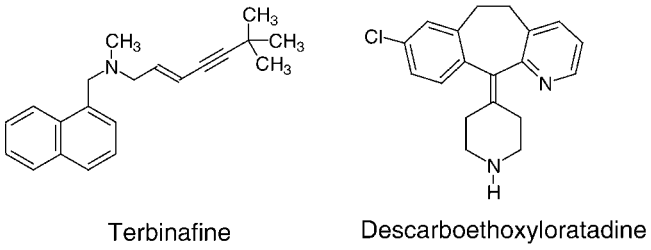

Fig. 1. Molecular structures of the compounds studied.

of TER and its metabolites after incubation with rat hepatic S9 fraction. The analytes were separated in a 50 μm i.d. capillary after a solid-phase clean-up procedure of the in vitro samples using phosphate buffer (pH 2.2). A limit of detection 2.44×10^{-7} mol l^{-1} (0.08 μg ml^{-1}) was obtained for TER using a standard solution. Intra- and inter-day reproducibility of determination (peak area) was 3.9 and 5.1%, respectively.

By now, no attention has been paid to use CE in hydrodynamically closed separation system for the analysis of TER. Although used in practice rather rarely, this CE technology, minimizing impacts of non-separative transport processes on the migration velocities of the separated constituents, has inherent analytical advantages in situations when highly reproducible migration velocities are desired (e.g., peak area based quantitation of the analytes). Moreover, the use of capillary of a larger i.d. was preferred as it should enable to enhance some performance parameters, such as sample loadability, separation capacity, and sensitivity (see Ref. [17] and references given therein). The aim of the present work was, (i) to study analytical capabilities of such CE mode, as an alternative to the other separation techniques (HPLC, open CE mode), for the analysis of TER and (ii) to apply this capillary electromigration technique for the determination of TER in pharmaceuticals as well as for the monitoring in vitro permeation process of TER (terbinafine enriched gel-skin-saline ternary system).

2. Experimental

2.1. Instrumentation

2.1.1. Capillary electrophoresis

A CS Isotachophoretic Analyzer EA 101 (Villa-Labeco, Spišská Nová Ves, Slovak Republic) was used in a single-column configuration of the separation unit. The separation unit consisted of the following modules: (i) a CZE injection valve with a 100 ml internal sample loop; (ii) a column provided with a 300 μm i.d. (650 μm o.d.) capillary tube made of fluorinated ethylene-propylene copolymer (FEP) of 210 mm total length (160 mm to the photometric detector); (iii) a counter-electrode compartment with a hydrodynamically (membrane) closed connecting channel to the separation compartment.

The CZE column was provided with an on-column conductivity detector (Villa-Labeco) and with a LCD 2083 on-column photometric detector with variable wavelengths, 190–600 nm (Ecom, Praha, Czech Republic). In this work the photometric detector was set at 224 nm detection wavelength. The signals from the detectors were led to a PC via a Unilab data acquisition unit (Villa-Labeco). ITP Pro32 Win software (version 1.0) obtained from KasComp (Bratislava, Slovak Republic) was used for data acquisition and processing.

A CS Isotachophoretic Analyzer ZKI 01-1 (Villa-Labeco) and a LCD 2083 on-column photometric detector (Ecom), with the same parameters of both as those about mentioned, were used to assess reproducibility of optimized method.

Prior to the use, the capillary was not particularly treated to suppress an electro-osmotic flow (EOF). A dynamic coating of the capillary wall by means of a 0.2% methylhydroxyethylcellulose (m-HEC 30000; Serva, Heidelberg, Germany) in background electrolyte solutions served for this purpose [18]. CZE analyses were carried out in cationic regime of the separation with direct injections of the samples. The experiments were performed in constant current mode [17]. The driving current applied ranged from 120 to 250 μA and the temperature was 20 °C.

2.1.2. Dialysis

The dialysis experiments were performed by Prof. V. Koprda (Slovak Tech. Univ., Fac. Chem. Technol., Bratislava, Slovakia). The experimental arrangement consisted of Franz-type vertical diffusion cell [19] (Fig. 2) completed with shed snake skin as an in vitro diffusion membrane [20,21] (active area 0.8 cm^2). The internal volume of the donor and acceptor compartment was 1.0 and 7.3 ml, respectively. The acceptor compartment was furnished by a stirring with defined speed. The device was kept at a constant temperature during the experiment.

2.2. Chemicals and samples

The carrier electrolyte solution was prepared from chemicals obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland) in water demineralized by a Rowapure-Ultrapure water purification system (Premier, Phoenix, Arizona, USA). All chemicals used were of analytical grade or additionally purified by the usual methods. The solutions of the electrolytes were filtered before use through disposable

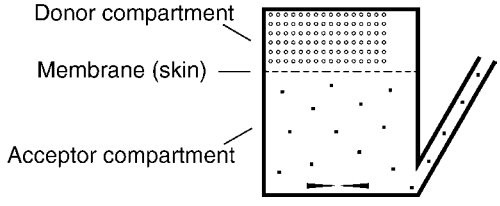

Fig. 2. Franz vertical diffusion cell for in vitro permeation experiment.

membrane filters (a 1.2 μm pore size) purchased from Sigma (St. Louis, MO, USA).

TER and descarboethoxyloratadine (DES) were obtained as working standards from Zentiva (Hlohovec, Slovakia) and Schering-Plough (Heist-op-den-Berg, Belgium), respectively. Analyzed tablets Lamisil®, being claimed to contain 250 mg of the drug, and Lamisil spray®, 1% (w/v), were obtained commercially. Substance of TER, used for the in vitro experiment, was obtained from Zentiva as a powder of pharmaceutical grade (assigned purity 99.4%).

The gel sample (with or without TER), used for the dialysis, was kindly provided by Prof. K. Ducková (Department of Galenic Chemistry, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia). A preparation and composition of the gel as well as a role of paticular compounds included in it are presented in [22].

2.3. Procedures for sample and standard solution preparations

2.3.1. Standard solutions

Pure standard stock solutions of TER and DES (serving as internal standard) were prepared in concentrated acetic acid and stored at 4 °C. Working solutions were made by appropriate dilutions in demineralized water or in saline solution, as required.

2.3.2. Tablets

A total of 10 tablets (Lamisil) were weighed and finely powdered. A portion of the powder equivalent to 10 mg TER was weighed accurately, transferred to a 10 ml volumetric flask and suspended in 8 ml of concentrated acetic acid. An appropriate amount of the internal standard was added. The flask was placed in ultrasonic water bath for 10 min before completion to volume with the same acid. The mixture was centrifuged (5000 rpm) for 10 min. The resulting solution was transferred into a proper flask and stored in a freezer at 4 °C. It was properly diluted with demineralized water prior to the analysis and filtered [a 1.2 μm pore size (Sigma)] before the injection into the CE equipment.

2.3.3. Commercial solution

The drug sample (Lamisil spray) containing TER as the active ingredient at a 1% (w/w) concentration with the internal standard was appropriately diluted with demineralized water prior to the analyses and directly injected.

2.3.4. Dialyzates

Permeation of TER from gel across the shed snake skin was studied in vitro using the vertical diffusion cell (Fig. 2). The donor compartment was filled up with gel while the acceptor compartment with phosphate buffered saline (0.9% NaCl, pH 7.4). Gel employed contained the substance of TER at a 1% (w/w) concentration. The solution was stirred at 200 rpm and the temperature was kept at 32 °C during the experiment. A yield of the permeation was monitored in various periodes of the permeation process. TER was determined in aliquots (TER enriched solutions) sampled at 1, 3, 5, 6, 7, 9 and 24 h after starting the experiment. Both, unpretreated and pretreated samples were examined in our study. A sample preparation was based on a liquid–liquid extraction procedure.

The solution (7 ml) from acceptor compartment and an appropriate amount of the internal standard were transferred to a proper vessel, alkalinized by 1 mol l^{-1} NaOH (1 ml) and mixed with chloroform (3 ml × 5 ml). The mixture was vigorously shaken for 5 min. The aqueous layers were discarded while the organic portions were consecutively transferred to a 10 ml tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in $CH_3OH{\cdot}HCl$ (2 ml) in order to make the hydrochlorides up, and evaporated to dryness. Finally, the residue was reconstituted in demineralized water (500 μl), vortexed briefly and centrifuged for 5 min at 10 000 rpm. The resulting solution was directly injected into the CE equipment.

The unpretreated samples were filtered before use through disposable membrane filters (a 1.2 μm pore size) and injected directly into the CE equipment.

3. Results and discussion

3.1. Optimization of the CZE method

CZE in a hydrodynamically closed separation system with suppressed EOF was used to analyze TER. The principal operating parameters (type and concentration of carrier cation and counterion, pH) were optimized with respect to the buffer capacity, analysis time and the separation efficiency. Electrolyte systems (ESs) used are set in Table 1.

In our investigation slowly migrating organic cations were suitable as the carriers in terms of a low conductivity of the buffer (elimination of thermal dispersion due to a Joule heat) and a good match of the effective mobilities of the migrants (elimination of electrodispersion). On the other hand, an inorganic buffer like phosphate, commonly used in 25–75 μm i.d. capillaries and applied recently also for CZE analysis of TER [16], was unsuitable to use in the wide-bore (300 μm)

Table 1
Electrolyte systems

Parameter	ES 1	ES 2	ES 3
Solvent	Water	Water	Water
Carrier cation	ε-ACA	Gly	Gly
Concentration (mmol l^{-1})	20	20	20–25
Counter ion 1	AcH	HCl	AcH
pH	4.5	2.6	2.6–3.2
EOF suppressor	m-HEC	m-HEC	m-HEC
Concentration (%, w/v)	0.2	0.2	0.2
Voltage/120 μA (kV)	6.7	1.6	2.6–7.7

ε-ACA: ε-aminocaproic acid; Gly: glycine; AcH: acetic acid; m-HEC: methylhydroxyethylcellulose.

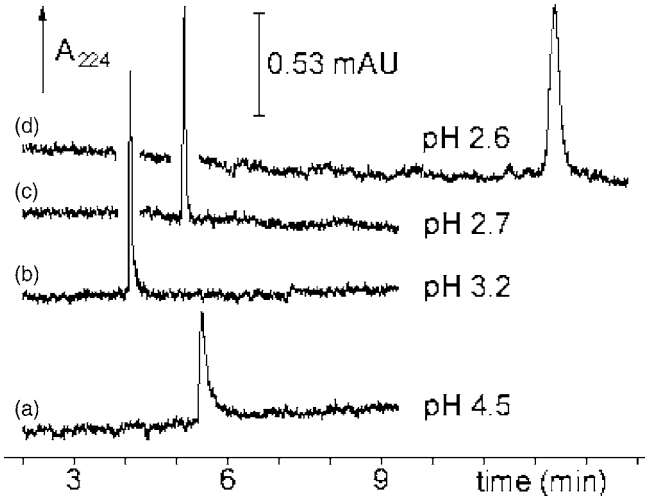

Fig. 3. Effect of CZE separating conditions on migration parameters of TER. The separations were carried out in various ESs (Table1); (a) ES 1, (b) ES 3, 25 mmol l^{-1} glycine, pH 3.2, (c) ES 3, 20 mmol l^{-1} glycine, pH 2.7, (d) ES 2. Concentration of TER in the analyzed standard sample was 30 μmol l^{-1}. The driving current was stabilized at (a and b) 120 μA, and (c and d) 250 μA, the corresponding driving voltages were 6.7 and 7.7 kV, and 5.6 and 2.8 kV, respectively. The detection wavelength was 224 nm.

capillary from reasons discussed elsewhere [23]. A combination of carrier ion with weak acid as a counterion was favored to the strong acid for providing better results in term of electric field (driving force) generated during the separation, see voltages of the buffer systems in Table 1.

An influence of pH on CZE analyses of TER was studied in pH range 2.6–4.5. The obtained results suggested a full protonation of TER at pH 3.2 (Fig. 3). Obviously, the migration time of TER rose due to a bit lower electric field in the separation system as pH decreased (for the voltage see the legend to Fig. 3). On the other hand, a shape (symmetry) of the peak was improved, probably due to a lower adsorption of the analyte in the separation compartment. With regard to a sufficient protonation of TER, reasonable ionic strength/conductivity of the buffer, analysis time, and proper shape of the peak, pH 2.7 (ES 3) was chosen as an optimal value.

The controlled current and corresponding voltage were optimized with respect to the separation efficiency (influenced mainly by diffusion and thermal dispersion). It was demonstrated (ES 3, pH 2.7) that an increase of the current (120–250 μA) markedly enhanced the efficiency of separations due to shortening analysis time. The thermal dispersion was low enough (up to a 250 μA driving current) and did not influence the separation efficiency significantly.

A 20 mmol l^{-1} glycine running electrolyte adjusted to pH 2.7 with acetic acid (ES 3) and a 250 μA stabilized driving current were chosen for further experiments as optimal.

Wavelengths between 210 and 260 nm were assessed for maximum response. Use of 224 nm gave the highest response for TER and it was suitable also for the detection of the internal standard.

3.2. Validation

After optimization of the separation conditions, some analytical characteristics of the developed CZE method were investigated using standard solutions (validation samples), as given in the Experimental section. The parameters involved were sensitivity, linearity, precision (intra- and inter-laboratory) and accuracy (recovery).

The limit of detection (estimated as 3σ) of TER was 1.73 μmol l^{-1} when a 224 nm detection wavelength used while the limit of quantification (estimated as 10σ) was 5.77 μmol l^{-1}. The concentration of the analyte in validation sample, corresponding to the limit of quantification, was determined with acceptable precision (R.S.D. = 0.78%, n = 7) and accuracy (R.S.D. = 98.76%, n = 7) under the stated conditions.

The linearity of detector response (peak area) for TER was assessed over the range 6.0–60.0 μmol l^{-1}. This represents an interval suitable for evaluation of TER in various commercial preparations and dialyzates (pretreated samples) used in our study. The straight line equation was $y = (0.3855 \pm 0.5261) + (22.97 \pm 0.48)x$ and corresponding determination coefficient $R^2 = 0.9992$. Use of the internal standard (DES) slightly improved the determination coefficient (∼0.02%) reducing scatter of points due to random error.

The method was validated by evaluation of run-to-run and day-to-day precision (intra-laboratory precision). In the range of 6.0–60.0 μmol l^{-1} the percent R.S.Ds on the basis of migration time and peak area ratios for five replicate injections were found to be between 0.32–0.65% and 0.56–1.04%, respectively. The inter-day precision was evaluated by comparing the linear regressions of the five standard plots prepared on five different days, over a one-week period. The average determination coefficient was $R^2 = 0.9990$ and the R.S.D. of the slope of the five lines was 0.61%. Analysis of variance of the data indicated no significant difference in slopes of the five calibration curves ($P < 0.01$). Inter-laboratory precision (reproducibility) corresponded to conditions where test results were obtained with the same method in different laboratories with different operators using different equipment (see Experimental). Reproducibility was assessed for the standard solution (30 μmol l^{-1}) on five consecutive injections. Acceptable levels of performance (R.S.D.) were obtained for migration time (0.69%) and peak area (1.44%). These results clearly indicated that CZE separations in a hydrodynamically closed separation system provided highly precise migration data.

3.3. Application

A validated method was applied for the determination of TER in commercial tablets and spray (both Lamisil) and in dialyzate samples descending from the in vitro permeation experiment.

Results from the determinations of TER in pharmaceuticals are in Table 2. The contents of TER obtained by the

Table 2
Determination of TER in pharmaceutical preparations

Parameter	Tablets			Solution		
	1	2	3	1	2	3
Average content[a] (mg per tablet or g)	249.10	250.42	247.40	10.02	9.97	10.03
Purity (%)	99.64	100.17	98.96	100.20	99.70	100.30
R.S.D.[b] (%)	0.69	0.65	0.77	0.59	0.63	0.57

The separations of TER in three different batches of two commercial preparations of Lamisil were carried out in the optimized ES 3; TER in each sample injected was at a 30 μmol l^{-1} declared concentration and corresponding peak area was compared with that of standard sample obtained at the same concentration.

[a] For calculations, relative migration data were used (DES served as an internal standard in the sample preparation); the peak areas were corrected by migration times (an actual peak area of the analyte was divided by its migration time).

[b] The R.S.D. values of the peak areas are means of seven determinations.

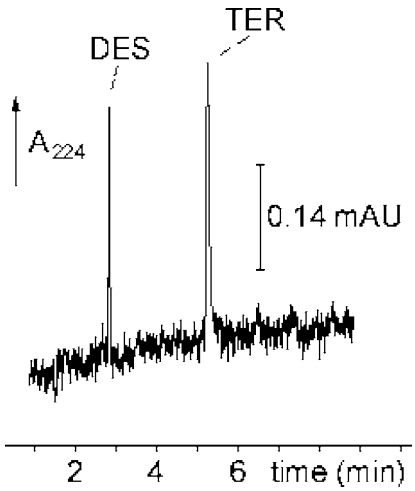

Fig. 4. Electropherogram from the determination of TER in pharmaceutical tablets (Lamisil). The separation was carried out in optimized ES 3. DES was used as an internal standard in the sample preparation (Section 2). TER and DES were separated at 9 and 36 μmol l^{-1} concentrations, respectively. Other experimental conditions as in Fig. 3c.

proposed method were in a good agreement with those declared. The absolute differences between determinated and declared values of TER in different batches of the preparations ranged from 0.17 to 1.04% (tablets) and from 0.20 to 0.30% (spray). The mean coefficient of variation was 0.70 and 0.60% for tablets and spray, respectively. The recovery test and precision data for commercial tablets and spray are shown in Table 3. The mean absolute recoveries, determined by adding known amounts of TER reference substance (2.0, 4.0, 6.0, 8.0, 10.0 μmol l^{-1}) to the samples at the beginning of the process, were found to be 98.34 (tablets) and 99.47% (spray). No detection interference was occurred separating the pharmaceuticals at a 224 nm detection wavelength (Fig. 4).

The gel of a particular composition [22] was used for the in vitro experiment. Some of the additives/drug enhancers included in it were intended to control in vitro permeation ability of TER, however, an optimization of gel composition will be a subject of another work. A lipophilic membrane seemed to be suitable to use in this study with respect to lipophilic character of the antifungal monitored. The optimized CZE method was used for the analyses of TER enriched dialyzate samples. The analyses of unpretreated samples were complicated by a high concentration of ionic compounds (inorganic ions from saline solution). A shape of the peak was influenced by a high ionic strength in the sample plug (the

Table 3
Recovery of drug from samples with known concentrations

Product	Amount of standard (μmol l^{-1})		
	Added	Found	Recovery[a] (%)
Tablets	2.00	1.95	97.50
	4.00	3.93	98.25
	6.00	5.89	98.17
	8.00	7.87	98.38
	10.00	9.94	99.40
Solution	2.00	1.98	99.00
	4.00	3.95	98.75
	6.00	5.98	99.67
	8.00	8.01	100.13
	10.00	9.98	99.80

The separations were carried out in the optimized ES 3.

[a] The R.S.D. values of the corrected peak areas are means of seven determinations.

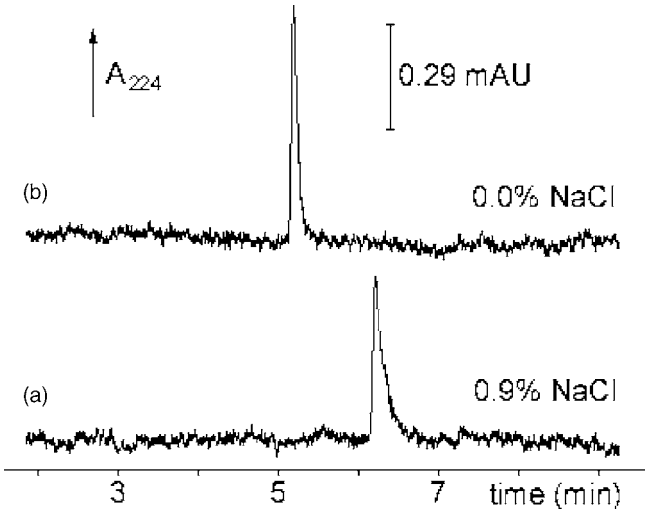

Fig. 5. Influence of ionic compound (NaCl) dissolved in the sample on migration characteristics of TER. The separations of TER, dissolved in (a) saline (0.9% w/v NaCl), (b) demineralized water, were carried out in optimized ES 3. Concentration of TER in the analyzed standard sample was 16 μmol l^{-1}. Other experimental conditions as in Fig. 3c.

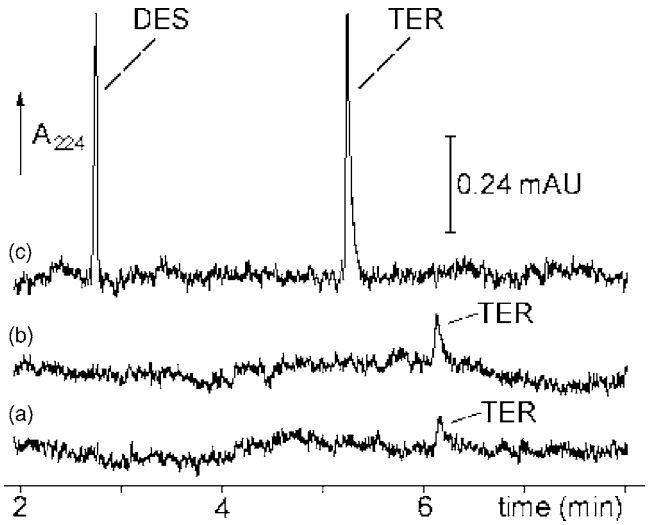

Fig. 6. Electropherograms from the determinations of TER in dialyzates (Section 2). The separations were carried out in optimized ES 3; (a) unpretreated sample, 6 h dialysis, (b) unpretreated sample, 24 h dialysis, (c) sample after extraction procedure (Section 2). The concentration of TER in the preconcentrated sample (a) was 17 μmol l^{-1}. Other experimental conditions as in Fig. 3c.

distortion as a result of electromigration and/or thermal dispersion; see Fig. 5). The increased migration time indicates that in the separations of high salinity samples transient ITP was effective with Na^+ acting as the leading cation in the initial phase of the CZE separation (see, e.g., [24]). Moreover, it was not possible to monitor the permeation process shorter than 6 h because of a low concentration of the analyte in the sample (Fig. 6). Here, the sample preparation based on a liquid–liquid extraction procedure was effective to overcome the above problems [compare electropherograms (a), (b), and (c) in Fig. 6]. Now the sensitivity was found to be sufficiently high to evaluate the in vitro permeation profile of TER (Fig. 7). The dependence obtained illustrated possibilities of lipophilic antifungal to penetrate through the lipophilic membrane from less to more polar substance.

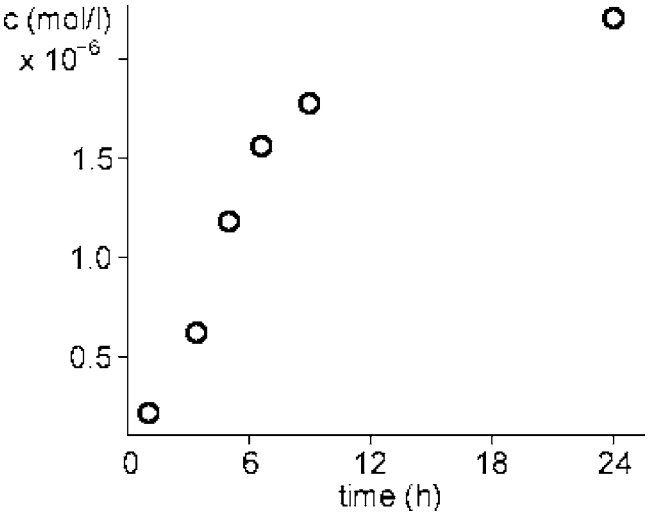

Fig. 7. Effect of the dialysis time (1, 3, 5, 7, 9 and 24 h) on the concentration of TER in dialyzate. The pretreated samples (Section 2) were analyzed using optimized ES 3. The mean concentrations were obtained from five consecutive determinations. The corrected peak areas were used in the calculations. Other experimental conditions as in Fig. 3c.

4. Conclusion

This work outlined a significant potential of CZE working in a hydrodynamically (membrane) closed separation system for providing higly effective analyses of antifungals. TER represents a molecule possessing majority of hydrophobic moieties. Here, CZE and aqueous separating conditions should be an alternative to HPLC, commonly used for TER and similar antifungals, avoiding organic solvents in the separations. In the contrary to the hydrodynamically open CE system, the capillary of a larger i.d. (300 μm) employed provided favorable conditions in term of a sample loadability and separation capacity. These parameters should be advantageous when unpretreated complex samples analyzed using on-line coupled CE methods [25,26].

The proposed CZE method was successfully applied for the determination of TER in commercial pharmaceutical formulations, including tablets and spray. Moreover, it was useful to monitor an in vitro permeation process of TER on the skin. It should enable to examine various gel additives and drug enhancers in order to find the optimal composition of a potential pharmaceutical intended to the topical treatment of mycoses. A high reproducibility, separation efficiency, short analysis time and a low cost are advantages of the method. It could be further improved accomplishing the sample pretreatment on-line to the analysis (ITP-CZE) avoiding an external sample manipulation.

Successful validation was achieved including suitable assessments of sensitivity, linearity, precision and recovery. It is concluded that the reported operating conditions are suitable for the routine assay of TER in pharmaceutically relevant samples.

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