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Fast separation of terbinafine and eight of its metabolites by capillary electrophoresis

A rapid and simple capillary zone electrophoresis (CZE) method for the determination of terbinafine and eight of its main metabolites after incubation with rat hepatic S9 fraction was developed. The effect of the concentration and type of the running electrolyte as well as of the addition of an organic solvent were studied with emphasis on selectivity and sensitivity. All nine components obtained (unmetabolized terbinafine and up to eight of its metabolites) are baseline resolved in less than 4 minutes using a 0.05 M phosphate buffer (pH 2.2) without additives, after a solid-phase clean-up procedure of these in-vitro samples. In addition, under the conditions described, no endogenous components of the sample interfere at the detection wavelength chosen. After optimization of the separation conditions, some analytical characteristics of the developed CZE method were investigated. A limit of detection of only 0.08 μg/mL was obtained for terbinafine using a standard solution. Finally, the use of on-line CZE/diode array detection enabled to identify tentatively the presence of unmetabolized parent terbinafine and its metabolites in the mixture of nine components separated.

Key Words: Terbinafine; Drug metabolites; In-vitro analysis; Capillary zone electrophoresis; Diode array detection

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1 Introduction

Most drugs undergo extensive chemical changes known as metabolism when they reach the organism and before they are excreted. This process involves a series of complex events, which modulate the effectiveness of the administered drug in the treatment of the disease and determine its pharmacological and toxicological effects. Therefore, the subsequent isolation and structural characterization of the resulting plethora of metabolites is vitally important to understand the physical and biological effects of the parent drug [1, 2]. The enzymatic reactions involved in drug metabolism pathways are often classified into three groups: phase I, phase II, and phase III [3, 4]. Phase I metabolism consists of a variety of functionalization reactions, including oxidation, reduction, and hydrolysis, which generally produce more polar compounds that are subsequently susceptible to further modification. These reactions often result in only minor structural modifications of the parent compound. Thus, the separation of these drug metabolites by high-performance liquid chromatography (HPLC) has often been a difficult task. However, capillary electrophoresis (CE) offers a completely different selectivity process to HPLC and high separation efficiencies, which means that a small degree of selectivity can often provide acceptable resolution. These qualities with a rapid analysis and easy method development present clear advantages over the use of HPLC for the analysis of drug metabolites [4].

Terbinafine is a new powerful antifungal agent of the allylamine class that selectively inhibits fungal squalene epoxidase [5]. This drug is indicated for both oral (tablets) and topical treatment (creams) of mycoses [6], being highly effective in the treatment of dermatomycoses [7]. Terbinafine is extensively metabolized in both human beings and animals [8], mainly through *N*-demethylation and oxidation of any of the three methyl groups (see **Figure 1**).

About fifteen metabolites have been identified in human plasma [9]. However, the simultaneous determination of terbinafine and its metabolites is hindered by the large differences in hydrophobicity among the compounds. Thus, although HPLC has been used for the determination of terbinafine in tablets and creams [10], to date, only two publications report analyses of metabolites of terbinafine by HPLC. In one case, terbinafine and only its desmethyl metabolite in human plasma were determined [11], and, in the other, up to five of its main metabolites were separated

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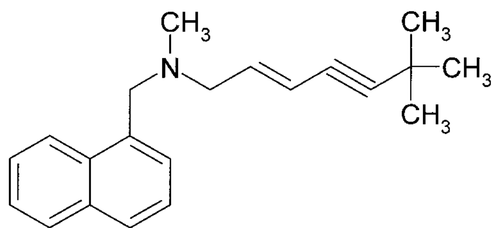

Figure 1. Structure of terbinafine.

simultaneously in human plasma and urine samples by HPLC [12]. However, no previous work has been reported in the literature for terbinafine analysis by CE.

The goal of this work is to provide a rapid and simple CE method for the determination of terbinafine and its main metabolites in rat hepatic S9 fraction incubations, after a solid-phase clean-up procedure of these in-vitro samples. The effect of the concentration and type of the running electrolyte and the addition of an organic solvent were studied with emphasis on selectivity and sensitivity. Finally, the use of on-line CE/diode array detection system was investigated to identify tentatively the presence of unmetabolized parent terbinafine and its metabolites in the mixture of nine components separated after incubation with rat liver S9 fractions.

2 Materials and methods

2.1 Instrumentation

The analyses were carried out in a P/ACE 5510 CE apparatus (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector (DAD), and controlled by a Compaq Deskpro personal computer running Beckman System Gold software. Fused-silica capillary tubing (Composite Metal Services, Worcester, UK) of 27 cm total length (20 cm effective length) × 50 μm ID was used. The capillary was thermostatted and a temperature of 30°C was selected to carry out separations. Samples were injected by hydrodynamic system using N_2 pressure of 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa). For separation, a voltage of 15 kV (anode on sampling end) was applied. Electropherograms were recorded at detection wavelength of 220 nm.

The pH of the running electrolytes was measured using a Titriprocessor (mod. 670) with combined LL glass electrode from Metrohm (Herisau, Switzerland). A centrifuge, model Z231 M, from Hermle (Gosheim, Germany) was used in the preparation of in-vitro samples. A vacuum manifold (Millipore, Bedford, MA, USA) with 30-mg Oasis-HLB cartridges (Waters, Milford, MA, USA) was used for solid-phase extraction (SPE), and the eluates were evaporated to dryness under a stream of nitrogen with a Turbovap LV evaporator from Zymark (Hopkinton, MA, USA).

2.2 Chemicals

All chemicals were of analytical reagent grade and used as received. Sodium dihydrogen phosphate, phosphoric acid, and perchloric acid (70%) from Aldrich (Milwaukee, WI, USA); magnesium chloride ($MgCl_2$), formic acid, dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH), and hydrochloric acid (HCl) were supplied from Merck (Darmstadt, Germany); phosphate buffered saline (PBS) was purchased from Fluka (Buchs, Switzerland); and β-nicotinamide adenine dinucleotide phosphate (β-NADP) sodium salt, β-nicotinamide adenine dinucleotide (β-NAD), and D-glucose 6-phosphate disodium salt were from Sigma (St. Louis, MO, USA). Methanol (MeOH) and acetonitrile (AcN) were of HPLC grade from Scharlab (Barcelona, Spain). The solutions for calibration of the pH electrode (pHs 2.00, 3.00, and 4.00) were purchased from Panreac (Barcelona, Spain). Water was purified by using a Milli-Q system from Millipore. Standards of terbinafine from Novartis Pharma AG (Basel, Switzerland). Finally, rat liver S9 hepatic fractions were from ICN/Cappel (Aurora, Ohio, USA).

2.3 Incubation procedure

Incubations were carried out in screw cap vials of 24 mL. The incubation mixture contained 30 μL terbinafine (1 mg/mL in DMSO), 1 mL $MgCl_2$ (5.06 mg/mL), 1 mL PBS (0.1 M, pH 7.4), 3 mL of a nicotinamide adenine dinucleotide phosphate generating system consisting of β-NAD (60 mg), D-glucose-6-phosphate disodium salt (38 mg), and β-NADP (5 mg) all in 30 mL PBS (0.1 M, pH 7.4), and 1 mL rat hepatic S9 fraction diluted with PBS (0.1 M, pH 7.4) in a 1:5 ratio. The incubations were carried out with stirring (80 rpm) in a water bath at 37°C. At time 0 min, 30 min, and 60 min, the incubations were stopped by addition of 100 μL perchloric acid (30% in AcN) at 1 mL incubation mixture. The solutions were centrifuged at 3000 rpm for 10 min to deposit the proteins. In addition, a incubation mixture with 30 μL DMSO without terbinafine was used as blank of incubation at different times.

2.4 Solid-phase clean-up procedure

The supernatant after incubation was passed through a preconditioned 30-mg Oasis-HLB cartridge (1 mL of methanol followed by 1 mL of water) in a vacuum manifold. Then the cartridge was washed with 1 mL of methanol-water (5:95, *v/v*), and the retained compounds were eluted with 1 mL of methanol containing 2% acetic acid. The eluent was subsequently evaporated to dryness at 35°C under a stream of nitrogen. Finally, the residues were reconstituted in 250 μL of methanol/water (1:1, *v/v*), because this hydro-organic solvent increases the solubility of terbinafine (compound with low solubility in water)

and avoids evaporation when only one organic solvent is used [13].

2.5 CE procedure

The phosphate buffers were prepared by adding appropriate aliquots of 0.5 M phosphoric acid to water and adjusting to pH 2.2 with 0.1 M NaOH. Other running electrolytes were prepared by adding appropriate aliquots of formic acid to water, measuring their pHs. The hydro-organic running electrolytes were prepared daily by mixing MeOH or AcN with appropriate aliquots of 0.1 M phosphate buffer (pH 2.2) or formic acid into water. All electrolyte solutions were filtered through 0.45 μm pore size disposable nylon filters from Millipore.

Before first use, a new capillary was preconditioned by rinsing with 1 M NaOH for 30 min, followed by a 15 min rinse with water. At the start of each day, the capillary was conditioned with the running electrolyte for 15 min, and two injections with a solution of methanol/water (1 : 1 *v/v*) were achieved to equilibrate the system. In order to increase migration time reproducibility, the capillary was rinsed between injections with the running electrolyte for 2 min. In addition, this rinsing step is needed to eliminate those components of the sample still remaining in the capillary during the analysis time. At the end of each day, the capillary was rinsed with MeOH for 2 min, 0.1 M NaOH for 2 min, 0.1 M HCl for 2 min, and water for 5 min.

3 Results and discussion

3.1 Development of CE method

The separation conditions of unmetabolized parent drug and its metabolites from the incubated terbinafine sample were achieved in two phases. First, the chemical parameters of the running electrolyte were varied, i. e. type and concentration of running electrolyte, as well as the use of an organic solvent (MeOH or AcN). Secondly, the effect of the injection volume was investigated in order to obtain the best sensitivity.

Figure 2 shows the electropherograms obtained when an incubated terbinafine sample was injected in the CE system using two running electrolytes at pHs about 2.0. According to previous results, in which fast separation of seven antifungals compounds were achieved by CZE [14], the best pH to perform the separation of several antifungal compounds was between pH 2 and 2.5. At this acidic pH, the analytes must be fully ionized, and can be separated by capillary zone electrophoresis (CZE), being the electroosmotic flow virtually eliminated [15]. It can be seen that the type of the running electrolyte, formic acid or phosphate buffer, has a major influence on the separation. In fact, it was impossible to resolve all components with formic acid at concentrations up to 0.3 M. Phosphate originated the best separation and, as could be expected, efficiency and resolution improved when increasing the running electrolyte concentration, but migration times and current intensities were also higher, then limiting its concentration at a maximum of 0.05 M (current intensities bigger than 100 μA cause bad reproducibilities in the separations, instability of the baseline, and decrease the efficiency). On the other hand, the use of organic solvents (e. g., MeOH or AcN) to modify the polarity of the running electrolyte and to improve the separation of the different metabolites was studied. However, the addition of MeOH or AcN up to 20% in the both running electrolytes was detrimental for the separation.

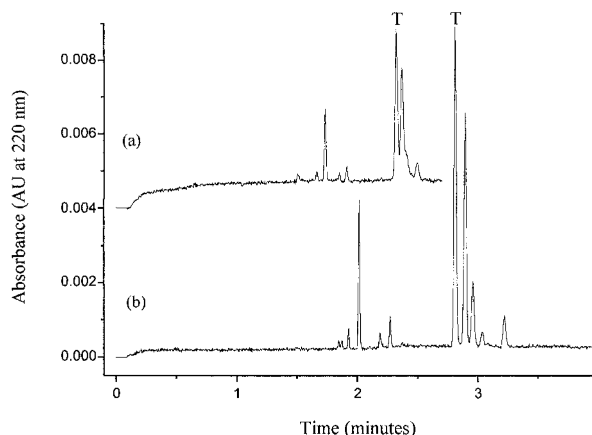

Figure 2. Electropherograms showing the effect of the type of the running electrolyte on the CZE separation: (a) 0.3 M formic acid (pH 2.1); (b) 0.05 M phosphate buffer (pH 2.2). Sample: 5 μg/mL terbinafine incubated at 60 min in rat hepatic S9 fraction after its clean-up with an off-line SPE (see Materials and Methods, Section 2.4.). Capillary, 27 cm (effective length 20 cm) × 50 μm ID. Applied voltage, 15 kV. Temperature, 30°C. Current ≈ 36 μA with (a), and ≈ 62 μA with (b). Hydrodynamic injection, 0.5 psi for 3 s. UV-detection, 220 nm. T: terbinafine. Other peaks are unknown metabolites of terbinafine.

From these results, it can be stated that the best separation is obtained using a 0.05 M phosphate buffer (pH 2.2) without additives. Phosphate running buffer enables us to obtain a better sensitivity and selectivity (see Figure 2), and all nine components are baseline resolved in less than 4 min with good efficiencies (about 100 000 plates for terbinafine in only 3 min). In addition, under the described conditions, the selectivity of the developed system (sample clean-up and CZE method) enables that sample endogenous components do not interfere at the detection wavelength chosen (see **Figure 3**).

With regard to the effect of injected volume or injected plug length, which is proportional to injection time by hydrodynamic injection, an increase in the injection time up

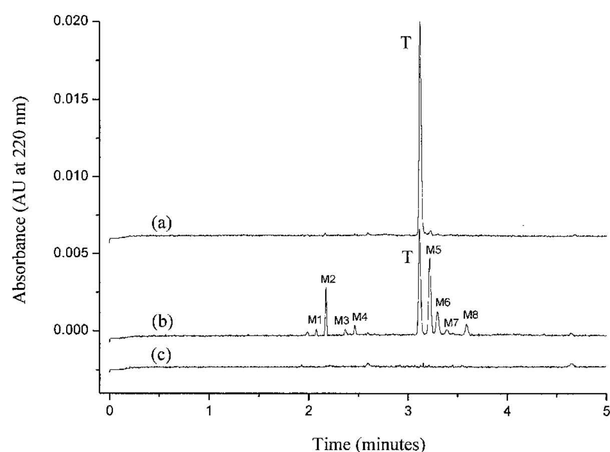

Figure 3. Electropherograms of extracts of incubations with rat hepatic S9 fraction of (a) terbinafine at 0 min, (b) terbinafine at 60 min, and (c) a blank at 60 min. Running electrolyte, 0.05 M phosphate buffer (pH 2.2). The original concentration (before incubation) of terbinafine was 5 µg/mL. Current ≈ 62 µA. Other experimental conditions as in Figure 2. T: terbinafine. Other peaks (M1–M8) are metabolites of terbinafine unknown.

to 10 s did not have a major impact on separation resolution (see **Figure 4.a**). Therefore, this large value can be chosen for a better sensitivity, but injection times larger than 10 s should be avoided unless sensitivity is needed and the loss of resolution does not preclude separation achievement.

After optimization of the separation conditions, some analytical characteristics of the developed CZE method were investigated using standard solutions of terbinafine in methanol/water (1:1, *v/v*), solvent used to reconstitute the residues after evaporated to dryness in the SPE procedure, and an injection time of 10 s at a pressure of 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa). The parameters involved were precision, linearity, limits of detection (LOD) and quantitation (LOQ), and robustness. Precision was assessed for the standard solution (5 µg/mL) on eight consecutive injections (*intra-day* reproducibility, $n = 8$) or on five different days and each day was injected in duplicate (*inter-day* reproducibility, $n = 10$). Acceptable levels of performance (measured as the relative standard deviation, RSD) were obtained in terms of *intra-day* reproducibility ($RSD_{n=8}$ from 0.3% for migration times, and from 3.9% for peak areas) and *inter-day* reproducibility ($RSD_{n=10}$ from 0.7% for migration times, and from 5.1% for peak areas). The calibration plot using peak areas showed a linear range for concentrations between 0.1 and 20 µg/mL with a squared regression coefficient (R^2) of 0.9986. The values of the LOD and LOQ were calculated from the calibration plots established with the peak heights: LOD is the concentration that equals the signal-to-noise ratio of 3, and LOQ is the concentration that equals the signal-to-noise

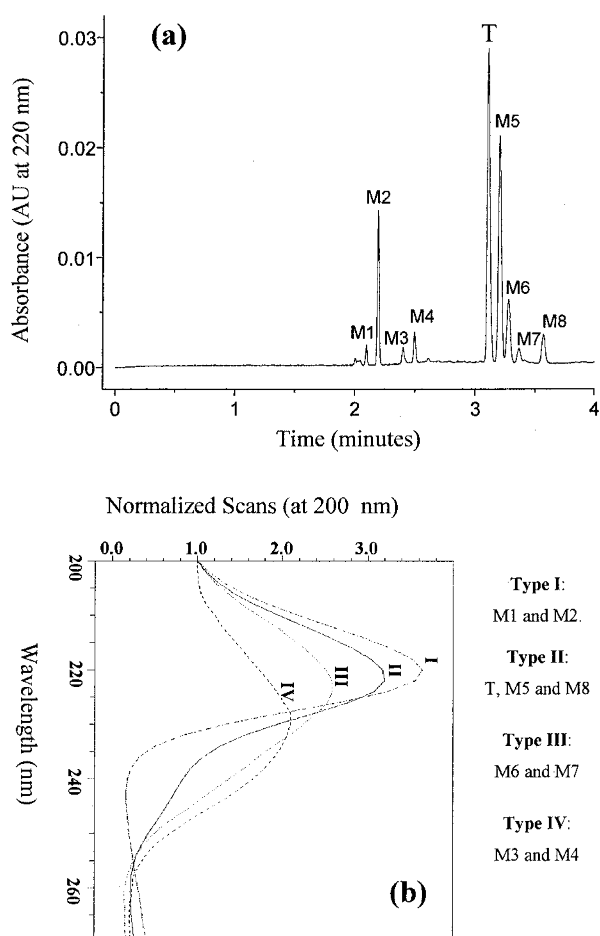

Figure 4. (a) Electropherograms using an injection of 0.5 psi for 10 s of an incubated terbinafine sample (5 µg/mL) at 60 min in rat hepatic S9 fraction after its clean-up with the off-line SPE; other experimental conditions as in Figure 3. (b) Different types of UV-spectra (200–270 nm) acquired by CZE/DAD of the components separated in the electropherogram shows in (a). T: terbinafine. Other peaks (M1–M8) are unknown metabolites of terbinafine.

ratio of 10. The noise was estimated as the largest deviation of detector signal from baseline measured in a section of about 5 min in the absence of analyte (1.0×10^{-4} AU at 220 nm and 0.05 M phosphate running buffer). The LOD was 0.08 µg/mL and the LOQ was 0.28 µg/mL. Finally, the robustness of the method was measured as the RSD for inter-day calibration slopes using new phosphate running buffer every day ($n = 5$). A value from 2.5% was obtained using peak areas to establish the calibration plots.

3.2 CZE-DAD

In the analysis of drug metabolites, samples with unknown compounds, any structural information is important. Thus, the UV spectrum of an analyte obtained with a DAD is particularly useful in the case of drug metabolism studies, in

which the UV spectrum of parent drug is usually only slightly modified in functionality due to its biotransformation. Therefore, the use of on-line CZE/DAD can be utilized for the screening of complex mixtures and tentatively identifying metabolites based on their CZE migration time and UV spectrum. This approach was used in this work to investigate the mixture of nine components derived from an in-vitro hepatic incubation, which were baseline resolved as already shown in Figure 4.a providing the acquisition of their UV spectrum.

First, it was observed that the major component (peak T) had a UV spectrum identical to a terbinafine standard (Pearson's correlation coefficient between spectra, R = 0.99993). Therefore, comparison of migration times (Figure 3.a and Figure 3.b) and UV spectra allow tentative identification of the major component of the mixture as unmetabolized parent terbinafine. Second, comparison of the UV spectrum of all components in the electropherogram of Figure 4.a revealed four types of spectra with only slightly differences (see **Figure 4.b**) due to the small structural modifications when the parent drug is subjected to biotransformation on phase I metabolism. All of them have a maximum between 218 nm and 228 nm. Type I at 218 nm corresponds to peaks M1 and M2 with $R = 0.9994$. Type II at 220 nm corresponds to peak T (unmetabolized terbinafine), M5 and M8 with $R = 0.9998$ between T and M5, and $R > 0.998$ between T and M8 or M5 and M8. Type III at 222 nm corresponds to peaks M6 and M7 with $R = 0.986$, and type IV at 228 nm corresponds to peaks M3 and M4 with $R = 0.985$. Although the latter values of R are lower than 0.99, which could indicate a loose match of the spectra, visually one can conclude that the UV spectra of the peak M6 and M7 or M3 and M4 do not exhibit important differences (**Figure 5**). It is possible that the small values of R between the spectra of type III and IV are due to the small peaks obtained for M3 and M7.

Finally, the similarity between the UV spectra of the peaks T and M5 together with the nearness between their migration times, could lead to uncertainty in the identification of peaks T or M5 as unmetabolized terbinafine. However, electropherograms obtained after incubations at 30 and 60 min showed a decrease in the size of peak T and an increase in the size of the peak M5 when increasing the incubation time (data not shown). These results enabled us to confirm that peak T corresponded to unmetabolized terbinafine while peak M5 corresponded to one of its metabolites. In fact, migration times for peak T and terbinafine standard were the nearest and the R value for the comparison of the spectra of peak T and the terbinafine standard was higher than the R value corresponding to the comparison of the spectra of peak M5 and the terbinafine standard. These results confirm the CZE/DAD features for monitoring drug metabolism.

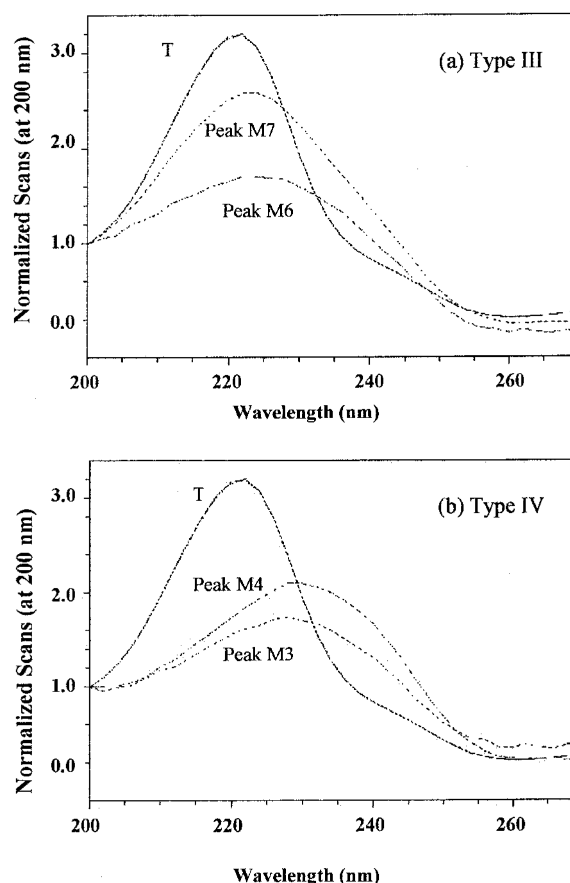

Figure 5. UV-spectra (200–270 nm) acquired by CZE/DAD of the terbinafine and four components obtained in the electropherogram shows in Figure 4.a: (a) type III spectra, and (b) type IV spectra.

4 Concluding remarks

The type of the running electrolyte, formic acid or phosphate buffer, had a major influence on the separation of all components originated from an incubated terbinafine sample (unmetabolized parent drug and its metabolites) after its clean-up with off-line SPE. On the other hand, the use of MeOH or AcN as additives in the running electrolyte was detrimental for the separation. The best separation was obtained using a 0.05 M phosphate running buffer (pH 2.2) without additives, all nine components are baseline resolved in less than 4 min. Under the described conditions, the selectivity of the developed system (sample clean-up and CZE method) means that sample endogenous components do not interfere at the detection wavelength chosen.

This is the first separation with so many metabolites of terbinafine resolved. The result shows that CZE is a promising alternative to HPLC for separations of metabolites. In this particular case, the separation of metabolite mixtures

of terbinafine previously reported by HPLC requires a longer analysis time (more than 30 min for a separation of five metabolites [12]) than those achieved in this work (only 4 min for eight metabolites). Thus, the proposed method is rapid, offers the possibility of improving detection of metabolites, and it is sensitive enough to be used in stability studies within Drug Discovery programs.

Finally, the use of CZE with DAD enabled the identification of unmetabolized parent terbinafine in the mixture of nine components separated after incubation with rat liver S9 fractions. In addition, the comparison of the UV spectrum of all components baseline separated showed four types of spectra with only slightly differences, all of them having a maximum between 218 nm and 228 nm, due to the small structural modifications when the parent drug is subjected to biotransformation on phase I metabolism. Further investigations should be focused towards the coupling of CZE with mass spectrometry (MS), taken into account the enormous separation performance of CZE-MS in structural elucidation studies of metabolites.

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