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Simultaneous determination of terbinafine (Lamisil) and five metabolites in human plasma and urine by high-performance liquid chromatography using on-line solid-phase extraction

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Abstract

The antimycotic agent terbinafine (Lamisil) and five of its main metabolites were determined simultaneously in human plasma and urine samples by an isocratic HPLC method. The compounds were separated on a phenyl column following on-line solid-phase sample clean-up with a column-switching device. Terbinafine and its metabolites were detected by monitoring the column effluent with UV light at a wavelength of 224 nm. The linear range in plasma was assessed between 0 and 2500 ng/ml for the parent drug and metabolites V, IV and I. The linear response of metabolites III and II was assessed between 0 and 1250 ng/ml. In urine, linearity was assessed between 0 and 10 000 ng/ml for metabolites V, IV, III, II and between 0 and 1000 ng/ml for the parent drug and metabolite I. Quantification limits based on a C.V. $\leq 20\%$ and a bias $\leq \pm 20\%$ ranged from 20 to 500 ng/ml depending on the compound and the matrix. Inter-day and intra-day variations were similar indicating the ruggedness of the two methods. Due to the considerable differences in hydrophobicity between the compounds, extraction efficiencies ranged from 55 to 100%. Both methods were found to be reproducible and sufficiently sensitive for the evaluation of metabolite pharmacokinetics.

1. Introduction

Terbinafine (Lamisil) [(E)-N-(6,6-dimethyl-2-hepten-4-inyl)-N-methyl-1-naphthalene methanamine hydrochloride] is a new potent antifungal agent of the allylamine class [1]. Terbinafine is extensively metabolized in both man and animals [2]. Biotransformation is mainly through N-demethylation and oxidation of the tertiary butyl group (Fig. 1). Fifteen metabolites have been identified, the most abundant in human plasma and urine being the carboxy 280-047, 280-027 (V, IV), the N-demethylated 86-621 (I) and, to a lesser degree, the alcohol 280-154, 280-022 (III, II) derivatives. The latter represent intermediates of the oxidative degradation pathway of terbinafine. In urine, the parent drug and the N-demethylated metabolite (I) are very difficult to detect even with a more sensitive method, whereas the more hydrophilic metabolites are found to be highly concentrated either in free form or as glucuronic acid conjugates. Higher concentrations especially of the alcoholic

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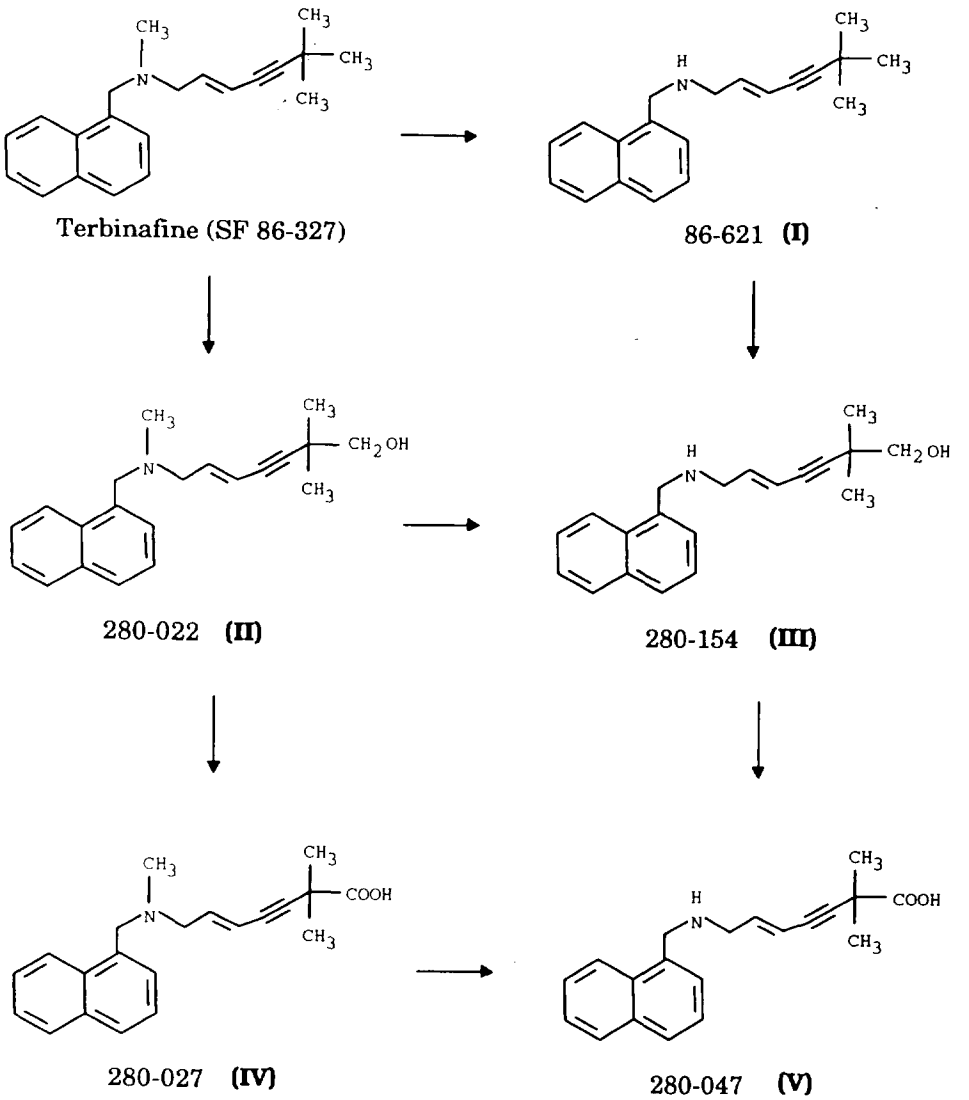

Fig. 1. Main biotransformation pathway of terbinafine.

intermediates (III, II) can be found in urine and in plasma following β-glucuronidase hydrolysis.

For pharmacokinetic evaluations of plasma and urine samples from volunteers or patients who had been administered terbinafine, HPLC methods have been developed for the determination of the parent drug and the N-demethylated metabolite (I) in plasma and tissues like stratum corneum, dermis–epidermis (without stratum corneum), hair, sebum and nails [3–5]. However, the simultaneous determination of terbinafine and its five main metabolites is impeded by the large differences in hydrophobicity between the compounds. Liquid–liquid extraction was tried first for sample clean-up but this procedure turned out to be rather time consuming. For routine bioanalysis of thousands of samples arising from pharmacokinetic studies, a high degree of automation is desirable and hence on-line solid-phase extraction using a column-switching device was chosen. The HPLC method described below is highly automated and

allows for the simultaneous determination of terbinafine and five metabolites in plasma or urine by isocratic reversed-phase chromatography on a stationary phenyl phase following on-line solid-phase sample preparation on a C_2-precolumn.

2. Experimental

2.1. Equipment

HPLC systems (see Fig. 2) consisted of HPLC pumps 302, 305, 307 from Gilson (Villiers Le Bel, France). Automated sample injection was performed with a Gilson 231 autosampler. Two switching-valve systems EPS 130 (Informatique and Technologie, Le Blanc-Mesnil, France) were used for the column-switching of the Perisorb RP-2 precolumns from Merck (Darmstadt, Germany) (15 × 3.2 mm I.D., 30–40 μm) enabling solid-phase extraction. The compounds were separated on a Brownlee (Roissy, France) Spheri-5 Phenyl column (220 × 4.6 mm I.D., 5 μm) tempered in a column oven (Croco-Cil from Cil, Ste. Foy La Grande, France). Both a UV detector from Knauer and a Spectroflow 783 detector from Applied Biosystems (Roissy, France) were used. Eluting peaks were recorded on Kipp and Zonen BD 41 pen-recorders (Touzart and Matignon, Vitry, France). Detector signals were transferred to the HP 1000 data acquisition system (Hewlett-Packard, Orsay, France) for peak integration and data processing via A/D interfaces.

For sample preparation, Jouan K101 (St. Herblain, France) and Sorvall RC 30 Plus (Dupont, De Nemours, France) centrifuges were used. Aliquots were transferred using automated pipets (Gilson). Polystyrene tubes for sample

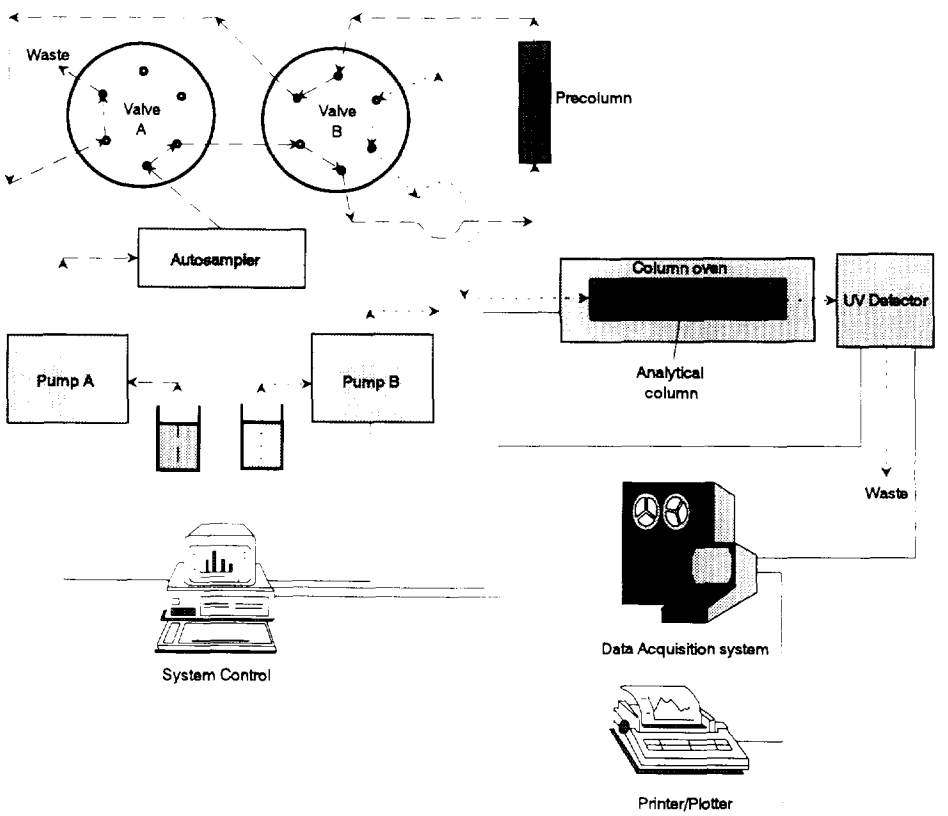

Fig. 2. HPLC system set-up for on-line sample preparation by a column-switching device.

handling and 2-ml capped glass vials for HPLC analysis were used.

2.2. Chemicals

KH_2PO_4, Normapur, and orthophosphoric acid, Normapur, were from Prolabo (Paris, France). Milli Q water (Millipore, St. Quentin-en-Yvelines, France) was used throughout. Methanol, UV-grade and acetonitrile, UV-grade were from Carlo Erba Farmitalia (Val de Reuil, France). Triethylamine was from Fluka (Buchs, Switzerland) and β-glucuronidase (bovine liver, 1.7 g, ~1 000 000 IU) was from Sigma (St. Louis, MO, USA). All other chemicals were of the highest purity commercially available. Terbinafine hydrochloride, metabolite V free base, metabolite IV free base, metabolite III free base, metabolite II free base and metabolite I hydrochloride as well as the internal standard IW 85-190 hydrochloride (I.S.) were obtained ≥98% pure from Sandoz Research Institute (Vienna, Austria).

2.3. Solutions

All solutions were prepared at ambient temperature and stored at 4°C or −20°C if not stated otherwise. The stock solution for the preparation of spiked samples was prepared by dissolving each of the solid powders of terbinafine and the metabolites in methanol to a final concentration of 1 mg/ml for each compound. The stock solution was further diluted by addition of water in order to prepare the whole range of concentrations for the spiked plasma or urine samples. These were aliquoted and stored at −20°C pending analysis.

Washing solution for the pre-column and mobile phase for the isocratic elution of the compounds on the HPLC column were prepared as follows: the aqueous washing phase A consisted of 20 m*M* KH_2PO_4 and 0.25‰ triethylamine. The pH was adjusted to 3.8 by the addition of 1 *M* H_3PO_4. Phase B consisted of 20 m*M* KH_2PO_4, 0.125‰ triethylamine in water, brought to pH 3.8 by the addition of 1 *M* H_3PO_4. Phase B and acetonitrile were mixed

Table 1
Sample pretreatment for solid-phase extraction of human plasma and urine samples

Plasma	Urine
-thaw all samples at ambient temperature	-thaw all samples at ambient temperature
-mix for 10 s on a vortex-mixer	-mix for 10 s on a vortex-mixer
-centrifuge for 5 min at 1000 *g*	
-transfer 0.75 ml into polysterene hemodialysis tubes	-transfer 0.5 ml into polysterene hemodialysis tubes
-add 50 μl internal standard solution (20 μg/ml final conc.) and 25 μl H_3PO_4 85%	-add 50 μl internal standard solution (20 μg/ml final conc.)
-add 0.75 ml of an ethanol–2-propanol mixture (75:25, v/v)	
-mix for 10 s on a vortex-mixer	-add 0.2 ml β-glucuronidase solution
-keep chilled on crushed ice for 30 min	-leave samples for 18 h at 37°C
	-add 0.3 ml of 0.01 *M* phosphate buffer pH 5
	-mix for 10 s on a vortex-mixer
-centrifuge for 15 min at 1000 *g*	-centrifuge for 18 min at 2500 *g*
-transfer 0.4 ml of the supernatant into 2-ml glass vials	-transfer 1 ml into 2-ml glass vials
-add 0.4 ml of 0.01 *M* phosphate buffer pH 5 and vortex-mix for 10 s	
-inject 250 μl onto the precolumn	-inject 100 or 250 μl onto the precolumn

(45:55, v/v) to prepare the mobile phase for the separation of the compunds on the analytical column.

The β-glucuronidase solution for the hydrolysis of the metabolite conjugates in urine (and occasionally in plasma) samples was prepared by dissolving one vial ($\approx 10^6$ IU) in 10 ml of a 0.01 *M* phosphate buffer pH 5. This solution was stored frozen at −20°C until used.

2.4. Chromatography

Prior to injection onto the precolumn, the samples were pretreated as listed in Table 1. Terbinafine and metabolites were adsorbed to the stationary C_2-phase of the precolumn. After washing with phase A, the compounds were transferred with mobile phase by back-flush desorption to the analytical column where they were separated from other matrix components by reversed-phase chromatography. Details of the isocratic separation are summarized in Table 2.

2.5. Data acquisition and statistical analysis

The UV detector signals were transferred via A/D interfaces to the HP 1000 data acquisition system. Peak areas were integrated and concentrations were calculated from linear regression curves of the peak area vs. concentration response of the calibration standards [6] using RS/1, a table calculation and graphics software from BBN Software Products (Cambridge, MA, USA) running on a VAX computer (Digital Equipment, Maynard, MA, USA). Intra- and

Table 2
Chromatographic conditions for the determination of terbinafine and five metabolites in human plasma and urine

Time (min)	Flow-rate (ml/min)	Eluent	Operation
0–4	1.0	A	Adsorption of terbinafine and metabolites to the precolumn
4–5	1.0	A	Back-flush washing
5–15	1.0	B–acetonitrile 45:55 (v/v)	Back-flush transfer of the analytes to the analytical column
15.1–45	0.1	A	Reconditioning of the precolumn
	1.0	B–acetonitrile 45:55 (v/v)	Separation of the analytes on the analytical column
Column temp.			30–35°C
Detection wavelength			224 nm
Total run-time			45 min
Retention times (plasma and urine)	metabolite V:		11.5–12.5 min
	metabolite IV:		13.5–14.5 min
	metabolite III:		15.5–16.5 min
	metabolite II:		17.0–18.0 min
	metabolite I:		28.5–30.5 min
	terbinafine		34.0–36.0 min

inter-day variability as well as the bias and relative standard deviations were calculated using RS/1 procedures.

3. Results and discussion

3.1. Extraction

Plasma or urine samples were injected onto the precolumn (Fig. 2) for solid-phase extraction following a short manual pretreatment (see Table 1). Only in plasma samples, proteins were precipitated prior to the submission of the supernatant to solid-phase extraction (see Table 1 for details). An ethyl phase (RP-2) proved to be most appropriate for the adsorption of the analytes. Acidification of plasma and urine was mandatory for good extraction efficiencies especially for the carboxy metabolites (V and IV). When compared to reference solutions of the same concentration but without any sample preparation, the extraction recoveries were found to be 55 to 100% depending on the analyte. The more hydrophobic compounds, e.g. parent drug, metabolite I and the internal standard (I.S.), were extracted with higher efficiency.

3.2. Chromatographic separation

Separation of the early eluting metabolites (V, IV, III and II) from interfering substances was the most cumbersome part of the method development. A phenyl phase turned out to be best suited with regard to selectivity. It should be noted that some column batches from the manufacturer (Brownlee) differed in their selectivity, giving rise to peak interference of a co-extracted substance from urine samples particularly with metabolite I. A slight variation in the composition of the mobile phase (phase B:acetonitrile) was shown to improve peak separation. Temperature did not have a major impact on separation efficiency but was chosen between 30 and 35°C for a better temperature control. Although there are considerable differences in lipophilicity between e.g. the carboxylated metabolites (V, IV) and the parent drug, isocratic separation gave a better reproducibility than gradient elution. The concomitantly long retention times of 30 to 40 min for the highly lipophilic compounds (metabolite I and terbinafine) led to a decreased sensitivity for these compounds. This was tolerated because the methods were developed above all for the quantification of the more hydrophilic metabolites (II,III,IV,V).

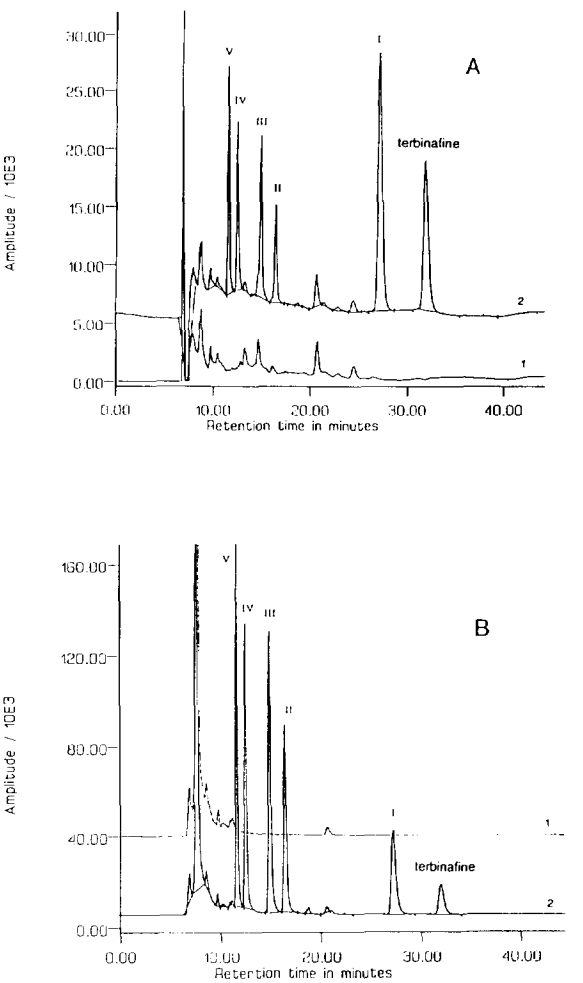

Fig. 3. Typical chromatograms of the determination of terbinafine and five metabolites in human plasma (A) and urine (B) by isocratic reversed-phase HPLC. Trace 1 corresponds to a plasma or urine blank. Trace 2 represents a sample spiked with 1000 ng/ml plasma (A) or 2000 ng/ml urine (B) of terbinafine and metabolites I to V.

3.3. Method selectivity

The selectivity of the method was shown for each of the compounds by the lack of peak interferences form co-eluted matrix substances (Fig. 3A,B). Only in urine, an un-identified peak interfered with metabolite I leading to a decreased sensitivity for this compound (see chromatogram of Fig. 3B).

3.4. Stability of the compounds in plasma or urine

Spiked plasma and urine samples with low, mid and high concentrations of each compound were subjected to repeated freeze–thaw cycles during four weeks. Determination of the concentration of each compound by HPLC did not indicate any relevant concentration decrease irrespective of the compound investigated (data not shown).

3.5. Method performance

The simultaneous determination by HPLC of terbinafine and its five main metabolites in human plasma and urine samples was validated by calculating performance data for several batches analyzed on different days and by different analysts. The data are shown in Tables 3 and 4 for plasma and urine, respectively. All determinations were performed at least in triplicate but in general more than tenfold. Inter-day variability in plasma expressed as the relative standard deviation (C.V.%) ranged from 2.9 to 50.7% depending on the compound and the concentration. In urine, the C.V. ranged from 1.3 to 45.4%. The bias (=[(measured conc. − nominal conc.)/nominal conc.] · 100) of all compounds determined in plasma and urine did not indicate any trend towards a systematic under- or over-estimation and ranged from −21.1 to 24.3%. Only for metabolite I and terbinafine a

Table 3
Validation of the HPLC method. Inter-day variability of the determinations of terbinafine and five metabolites in human plasma

Compound	Spiked concentration (ng/ml)	Calculated concentration (mean ± S.D.) (ng/ml)	*n*	Inter-day C.V. (%)	Bias (%)
Metabolite V	100	99 ± 16	20	16.3	−1.0
	750	740 ± 98	20	13.3	−1.3
	2500	2568 ± 174	20	6.8	2.7
Metabolite IV	100	99 ± 13	20	13.5	−0.9
	750	784 ± 71	20	9.1	4.5
	2500	2541 ± 187	20	7.4	1.6
Metabolite III	50	52 ± 9	16	17.2	4.0
	500	498 ± 27	17	5.5	−0.4
	1250	1287 ± 79	11	6.2	3.0
Metabolite II	50	58 ± 9	16	15.4	15.0
	500	498 ± 27	17	5.4	−0.4
	1250	1232 ± 64	11	5.2	−1.4
Metabolite I	20	16 ± 8	20	50.7	−21.1
	500	505 ± 30	20	5.9	1.0
	2500	2479 ± 72	20	2.9	−0.8
Terbinafine	20	20 ± 4	20	18.5	−2.3
	500	498 ± 24	20	4.8	−0.4
	2500	2475 ± 92	20	3.7	1.0

Table 4
Validation of the HPLC method. Inter-day variability of the determination of terbinafine and five metabolites in human urine

Compound	Spiked concentration (ng/ml)	Calculated concentration (mean ± S.D.) (ng/ml)	*n*	Inter-day C.V. (%)	Bias (%)
Metabolite V	100	93 ± 13	11	13.5	−7.3
	2500	2493 ± 50	12	2.0	−0.3
	10 000	9849 ± 132	12	1.3	−1.5
Metabolite IV	100	100 ± 5	7	12.6	0.0
	2500	2592 ± 268	8	10.3	3.7
	10 000	10 077 ± 848	8	8.4	0.8
Metabolite III	100	98 ± 13	12	13.5	−2.2
	2500	2455 ± 160	12	6.5	−1.8
	10 000	10 120 ± 707	12	7.0	1.2
Metabolite II	100	102 ± 22	12	21.7	1.6
	2500	2458 ± 176	12	7.2	−1.7
	10 000	10 027 ± 721	12	7.2	0.3
Metabolite I	100	124 ± 56	12	45.4	24.3
	250	247 ± 74	12	29.9	−1.2
	1000	1072 ± 144	12	13.4	7.2
Terbinafine	100	99 ± 30	12	29.9	−1.1
	250	257 ± 34	12	13.3	2.6
	1000	980 ± 104	12	10.6	−2.0

high variability (>29%) was found for the low (both) and mid (I) concentrations. In the case of metabolite I, this is due to the peak interference form a non-identified matrix component. The linear range of the determinations in plasma was assessed from 0 to 2500 ng/ml for terbinafine, metabolite I, IV and V and from 0 to 1250 ng/ml for metabolites II and III. In urine, linearity was shown from 0 to 1000 ng/ml for terbinafine and metabolite I, and from 0 to 10 000 ng/ml for the metabolites V, IV, III and II. The limits of quantification, based on a C.V. ≤20% and a bias ≤ ±20%, ranged between 20 and 500 ng/ml.

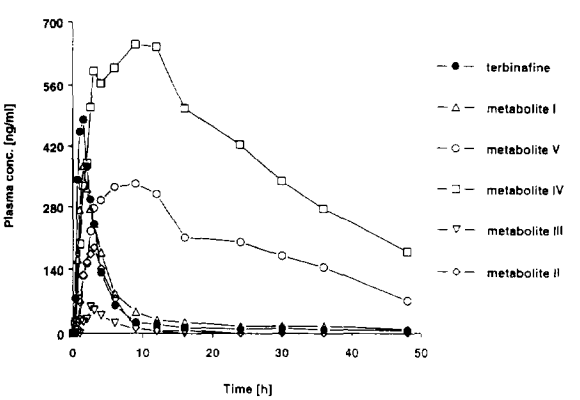

Fig. 4. Mean time–concentration profiles of terbinafine and five metabolites determined in plasma samples of a human pharmacokinetic study. The subjects had been administered 125 mg terbinafine per day orally.

4. Conclusions

The HPLC method described above allows for the simultaneous determination of terbinafine and five of its metabolites in human plasma and urine samples. The method has been developed above all to determine the more hydrophilic carboxy (V, IV) and hydroxy (III, II) metabolites. The sensitivity was found to be sufficiently high to evaluate the pharmacokinetic profiles of these compounds (Fig. 4). Several human phar-

macokinetic studies have been successfully analyzed applying the HPLC method described here indicating its reliability and reproducibility which is at least partially due to the high degree of automation.

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